Application of Biotechnology in Dairy and Food Processing

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1.0. INTRODUCTION

Milk occupies a pride of place as the most coveted food in the Indian diet and fulfills almost all the basic nutritional requirements of our diet. For bulk of the Indian population particularly belonging to vegetarian section, milk and its products constitute the major affordable source of animal proteins for growth and health. Because of special status of milk in our nutrition, milk production in the country has always received top priority. As a result of white revolution through operation flood program, India is now a major player in the world market with the output touching 86 million tons mainly contributed by 134 million cows and 125 million buffaloes. Almost half of the milk produced in the country is consumed as fluid milk in the urban areas and the remaining processed at organized and unorganized sectors. In the Indian context of poverty and malnutrition, milk has a special role to play for its many nutritional advantages as well as providing supplementary income to some 70 million farmers in over 5,00,000 remote villages. Dramatic increase in milk production has also resulted into tremendous expansion of India’s modern dairy sector. The organized dairy sector is presently handling some 20 million lpd in over 400 dairy plants. With this, the challenges confronting the dairy industry have also increased considerably since they have to cope up with the expectations of the consumers in the domestic as well as world market in terms of producing high quality dairy products.

Notwithstanding such a dramatic boost in milk production, the per capita availability of milk in the country is only 220 ml which is far below the minimal requirement of 280 ml. This is precisely due to population which is growing at the rate of 1.8 per cent. Hence, the projected figures for the milk requirements for 2030 are staggering 130-152 MT, which is going to be a herculean task to achieve. If India has to achieve this target and to sustain its position in the world market, it would require huge efforts to fill the gap. It is, in this context, Biotechnology can play a very effective and forceful role in enhancing the food and nutritional security in the country. Recent developments in Biotechnology have opened up new and exciting possibilities in dairying for enabling the availability of milk and milk products within the reach of poor and to cater to the needs of large sections of our population. Dairy Industry in particular can immensely benefit through Biotechnological interventions which can not only improve the overall quality and safety of processed dairy foods but also enhance their commercial value for local consumption and exports. Since, the major obligation of dairy industry is to provide high quality nutritious, clean, wholesome, healthful and inexpensive dairy foods to the consumers, it has become inevitable to integrate Biotechnological interventions at different stages of milk production and processing. In this presentation, the status of present and future
biotechnological interventions in some emerging priority areas catering to the needs of the dairy industry and the consumers will be highlighted.

2.0. POTENTIAL APPLICATIONS OF BIOTECHNOLOGY IN DAIRY SECTOR

Biotechnology has already made significant contributions in dairy industry. Some of the potential applications and target areas where Biotechnology has already made its impact along with future prospects are given below:

2.1. Dairy Production

- Recombinant Bovine/Buffalo Somatotropin - rbst
- Recombinant Vaccines
- Rumen manipulation
- DNA finger printing and RFLP
- PCR based diagnostics (Animal Health care)
- Embryo Transfer Technology
- Animal Cloning
- Gene Pharming and Transgenics

2.2. Dairy Processing

- Designing milk through genetic engineering
- Genetically Modified Organisms (GMOs)
  - Starter Cultures
  - Genetically modified foods
- Food grade Biopreservatives
- Recombinant dairy enzymes/proteins
- Accelerated cheese ripening
- Probiotics
- Functional foods and nutraceuticals
- Dairy waste management and pollution control
- Gene probes and PCR based pathogen detection

Out of these envisaged areas, some have already been realized while others are still in the developmental stages and will take some time before reaching the end users. Some of these promising areas and their potential application in dairy industry will be highlighted in this presentation.

2.2.1. Genetically Engineered milk - Humanization of Bovine Milk

Breast milk is nature's perfect food for human infants providing them all aspects of nutrition and protection against infections. However, a considerable number of infants are fed formulae based on bovine/buffalo milk. The composition of these infant formulations can be improved if the proteins contained therein more closely resemble those of human milk. It is now possible to add human lactoferrin or lysozyme to bovine milk by genetic engineering to produce new functional foods. The shelf life of such products is also expected to be very high due to anti microbial activity of these proteins.

The goal of the dairy industry has been to create an efficient, healthy cow/buffalo that can serve all the needs of the industry. Genetic engineering offers the opportunity for a
paradigm shift, a reshaping of the industry from the producers to the processing plants. Dairy producers have the opportunity to choose to produce high protein milk, milk destined for cheese manufacture that has accelerated curd clotting time, milk containing neutraceuticals, orally administered biologicals that provide health benefits or a replacement for infant formulae. Such a scenario would be a radical change for the dairy industry. There is now little doubt that the products of genetic engineering will become a part of the dairy industry in the new millennium.

2.2.2. Genetic Manipulation of Starter Cultures

Good quality starter cultures (Lactic Acid Bacteria, LAB) are the pre-requisite for preparing good quality fermented foods. The commercial value of the fermented foods is, therefore, mainly dependent upon the performance of the starter cultures. One of the most notable features of lactic starter cultures is that they generally harbour large complements of plasmids. It is very significant that many commercially important traits of these bacteria are in fact plasmid encoded. These include lactose and citrate utilization, phage resistance, proteinase production and bacteriocin production/immunity. The location of such important genetic information of considerable industrial value on the plasmids could be detrimental to the host as these extra chromosomal elements may be lost from the cell population on extended cell culture especially in absence of any selective pressure to ensure their continued maintenance. However, it has proved to be a blessing in disguise to the researchers since the plasmid encoded genes are more accessible for manipulation and more easily analyzed than the genes located on the chromosomes.

Recent advances in gene transfer and cloning technologies provided numerous opportunities for the application of genetic approaches to strain development programme appropriate for their application in dairy industry. In addition, significant advances have already been made in the analysis of lactococcal and lactobacillus gene expression. Genetic manipulation of these bacterial has now been developed to the point where it is now possible to clone and express homologous and heterologous genes. Cloning vectors have also been constructed to isolate regulatory signals such as promoters, ribosome binding sites and terminators. This information could be extremely valuable in allowing the expression of foreign gene in lactic acid bacteria and enhancing the expression of desired phenotypic properties. The development of plasmid integration vectors for lactic acid bacteria offers the possibility of introducing and stabilizing genes of interest by incorporating them into the host chromosomes. The strategy could be extremely useful to tackle the problem of instability in the lactic cultures as a result of frequent loss of plasmid during their propagation. Plasmid integration vectors can also be used to explore and help characterize chromosomal DNA from lactic acid bacteria. Indeed, proteinase and phage resistant determinants have been successfully integrated and stabilized in lactococcal chromosomes where they are amenable to substantial amplification.

2.2.3. Food Grade Biopreservatives

Another potential area for commercial exploitation of lactic acid bacteria through their genetic engineering is the Biopreservative attributes of these organisms. Appropriate food-grade biopreservatives can be developed to control undesirable bacteria and molds in dairy foods by following these strategies for direct application in Dairy Industry.

2.2.3.1. Bacterial Inhibition: Suppression of spoilage and food-borne pathogens by lactic acid bacteria could be extremely beneficial to human health and dairy industry as these attributes can considerably improve the shelf life and safety of fermented foods. The
antimicrobial activity in lactic acid bacteria has been ascribed to a number of factors and the extent of their antibacterial spectrum varies from culture to culture and strain to strain. Among these factors, the bacteriocins appear to be the most promising candidate for exploring the preservative potentials of these bacteria on a large scale. This is precisely due to their proteinaceous nature since the production of these polypeptides could be conveniently manipulated at the molecular level.

The application of such food preservatives in dairy industry to combat undesirable food spoilage and pathogenic organisms could be more relevant to countries like India where the health of consumers is always at risk due to poor hygiene, tropical temperate conditions and inadequate refrigeration facilities. Biotechnological techniques can now be applied to develop strains of lactic acid bacteria capable of enhanced production of these natural food grade preservative and also to combine within a single strain the ability to produce a number of such bacteriocins to extend their antibacterial spectrum. Much of the work being applied to bacteriocins is often based on approaches and protocols originally developed for the analysis of Nisin which has already been given the GRAS status. In this case, the structural genes have been sequenced; protein engineering has been employed to generate the variants of the molecules and engineered strains which can be used to study expression of the compound available.

2.2.3.2. Fungal Inhibition: Molds contamination in dairy foods and feeds is a serious problem in third World Countries like India where tropical humid conditions and unhygienic environment are extremely favourable for the growth and proliferation of these types of organisms. Apart from causing serious spoilage problems in these commodities, many of the incriminating molds could also pose serious health risks to the consumers on account of their ability to produce extremely toxic and carcinogenic metabolites such as aflatoxins. Although, mold contamination in foods can be effectively controlled by the application of some chemical additives, there are serious concerns regarding the use of these unnatural additives in food chain. Exploring the antifungal attributes of lactic acid bacteria could be a very useful strategy to tackle the mold problem in dairy foods.

In this regard, some work has been initiated at NDRI, Karnal. A very promising lactic culture characterized as Lactococcus lactis subsp. lactis CHD 28.3 exhibiting a very strong antifungal activity against a variety of molds has been isolated and studied extensively. Because of its strong broad spectrum antifungal activity, this culture could be a very attractive candidate for exploring its built biopreservative potentials on commercial scale. The nature of the antifungal substance (AFS) produced by the test culture was determined to be proteinaceous which was partially purified and characterized. The production of AFS in the test culture has been found to be encoded on chromosomal DNA rather than a plasmid. A partial nucleotide sequence of open reading frame (ORF) for the AFS has been determined. The nucleotide sequence analysis strongly suggests the involvement of a transposon like element in the production of AFS in the test culture. Efforts are now being made to use a strong nisin promoter for over expression of AFS gene for its commercial application in the development of a food grade bio-preservative.

3.2.4. Food Grade Vector System

Application of Genetically engineered lactic acid bacteria constructed with the help of vector systems having antibiotic/drug resistance markers based on E. coli in foods could raise serious public concerns. Hence, there is a need to develop food grade vector system where only naturally occurring genes such as Bacteriocin production/immunity or lactose/sucrose utilizing
genes from LAB are incorporated into the food-grade vectors as a marker for the selection of the desired recombinants. The use of starter cultures engineered with such food-grade vectors for different desirable characteristics in dairy foods will not raise any controversy and dairy industry will have no hesitation to use such genetically engineered organisms in the manufacture of high quality fermented foods.

3.2.5. NICE System for Over expression of Proteins

Controlled over production of proteins by industrially important micro-organisms is highly desirable. Lactic Acid Bacteria are ideally suited for use as cell factories since they are food-grade, genetically accessible and easy to grow on a low cost substrate. Until now several systems based on broad host range vectors and constitutive promoters have been developed for different LAB. However, a good food grade system was lacking that enable high and controllable expression of target genes. Scientists working at NIZO, the Netherlands have now developed a complete nisin inducible expression system that can be applied in several lactic acid bacteria and also in other gram positive organisms. Some advantages of NICE system include food grade components i.e. plasmids, strains and inducer, all components are tailor made and ready to use, high expression levels (upto 60% of total cellular protein), low costs, versatile and flexible, expression tightly controlled enabling production of lethal proteins, expression levels modulated by varying nisin concentrations and in situ induction is possible in nisin producing lactococci. This system works efficiently for expression of even heterologous proteins in LAB, and hence, can have lot of commercial value in dairy industry.

3.2.6. Dairy Enzymes

Advances in Biotechnology have also made a strong impact on production of several enzymes and proteins used in dairy industry in the processing of milk for the manufacture of some fermented products. Many of the enzymes are by and large of microbial origin. However, their production levels are not very high in the producer organisms and thus their production is not cost effective for commercial exploitation. Food processing has benefited from biotechnologically produced enzymes such as chymosin (rennet), proteases lipases, alpha amylase and lactase which can find lot of application in dairy industry. Many of these enzymes like alpha amylase, chymosin produced by genetically modified organisms have been granted GRAS status by FDA- thereby allowing their use in place of conventional sources of these enzymes in the dairy industry. Genetically engineered enzymes are easier to produce than enzymes isolated from original sources and are favoured over chemically synthesized substances because they do not create by products or off flavours in foods.

3.2.6.1. Recombinant Chymosin: Bovine chymosin traditionally known as calf rennet has been extensively used as milk coagulant during the manufacture of a variety of cheese all over the world. India's cheese industry is also expanding rapidly and hence the demand for calf rennet or its substitutes is also showing a phenomenal increase. However, in India, the cheese made with the help of calf rennet is not acceptable to a large sector of the consumers due to religious sentiments. This has stimulated lot of interest in search for rennet from alternate sources. Although, application of milk clotting enzymes from microbial sources have been in vogue in cheese manufacture, the quality of cheese manufacture, the quality of cheese made with such enzymes does not match with that made from calf rennet. In this context, buffalo rennet could be an excellent alternative to bovine calf rennet on account of its inherent compatibility with buffalo milk for producing high quality cheese.

With the rapid expansion of Fast Foods entering into the Indian market, the demand for specialized cheeses, such as Mozzarella cheese for Pizza etc. has increased tremendously. With
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this, demand for chymosin has also increased several folds. Hence, for large scale production of buffalo calf rennet, application of rDNA technology and Genetic Engineering techniques could be extremely valuable. By cloning the genes of interest from buffalo calves in E. coli and other hosts, it is now possible to produce recombinant Buffalo chymosin for commercial use in cheese industry. Scientists at NDRI have already cloned and expressed buffalo chymosin in E. coli and attempts are now being made to express in a yeast system for commercial application in cheese industry. Similarly other dairy enzymes like proteinases lipases, β-galactosidase, etc. can also be produced through rDNA technology. Proteases and lipases can be used in cheese industry for flavour development whereas Betagalactosidase can find application in the production of lactose free milk intended for lactose intolerant patients.

3.2.7. Functional Foods / Neutraceuticals

Functional Foods produced with the help of genetic engineering methods are regarded as a way to obtain public acceptance for this technology. Functional foods are supposed to have an added value for consumers. The added value may be given either due to health improvements and or minimized risks to develop certain diseases. Internationally apart from functional foods, the terms neutraceuticals, designer foods, healthy foods or pharma-foods are used as synonyms for functional foods so far only, Japan has a legally binding definition for functional foods. Functional foods are primarily targeted at the metabolism of macronutrients, the defense against reactive oxidants, cardiovascular system and physiology of the gut.

Based on the envisaged targets, functional foods are specifically designed to control a number of chronic diseases such as obesity, diabetes, hypertension, other cardiovascular diseases, cancer and enteric disorders. Functional foods are designed and produced by using different approaches i.e. by adding either the probiotic organisms, prebiotics in the food formulations or other ingredients such as anti-oxidants, phytochemicals, fat substitutes, ω-3 fatty acids and bioactive peptides. Out of these, Pro and Prebiotics and bioactive peptides are more popular.

3.2.7.1. Probiotics: There is currently a growing interest in certain lactic acid bacteria that have been suggested and shown to provide health benefits when consumed as food supplements or as food components. Such types of LAB have been invariably referred to as “Probiotics”. Probiotics are defined as living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition. Probiotics may be consumed either as a food component or as non-food preparations. Among the LAB, Lactobacillus species such as L. acidophilus, L. casei and L. bulgaricus and Bifidobacterium bifidum have tremendous probiotic potentials by virtue of which these bacteria can establish in the gastro-intestinal tract of man and animals. After implanting in the GI tract, these organisms do not allow potential pathogens of enteric disorders to inhabit the intestine and hence can play an important role in controlling the enteric disorders. However, this attribute of considerable medical significance is limited to only a few strains of LAB thereby leaving ample scope to manipulate this attribute at molecular level to exploit the full potentials. Apart from the colonization attributes, these probiotic organisms can also exhibit strong anticarcinogenic and anticholesterolemic activities as well and hence can play a vital role in the alleviation of cancer and blood pressure in human beings.

Recently, these probiotic organisms are attracting lot of attention as food grade biotherapeutics and have led to the development of a new concept of functional foods. These functional foods in which viable probiotic organisms are incorporated can find lot of medical applications as a special diet for patients. A number of companies are now venturing into the
development of a variety of probiotic formulations. One such company Probiotics International are leading manufacturers and suppliers of nutritional health care products which include the natural live microbial supplements that are known to be beneficial in the treatment of a variety of digestive disorders and diseases associated with stress. A few of these products are listed below and commercially available:

- **Protexin-Natural Care Tablets** (Maintains healthy nervous and immune system)
- **Protexin Natural Care Powder** (Maintains healthy nervous and immune system)
- **Protexin-Vital (Adults)** (Maintains healthy intestinal flora in adults)
- **Protexin-Vital (Child)** (Maintains natural healthy flora in children)
- **Protexin - Travel** (Diarrhoea and sickness during travel)
- **Protexin - Relief** (Relieves constipation in elderly)
- **Protexin - Lacta** (Lactose intolerance)
- **Protexin - Restore** (Restores intestinal flora in children)

Some of the future targets aimed in this direction include identification of the genetic determinants for probiotic function and regulatory signals to improve the colonization ability of these organisms in different hosts, inter and intraspecies conjugal transfer of this character and understanding the exact mode of action of probiosis.

### 3.2.7.2. Bioactive Peptides:
Research on Bioactive peptides mainly deals with milk proteins such as caseins which are regarded as precursors of bioactive peptides, lactalbumin and lactoferrin. The former is supposed to have anti-carcinogenic effects and latter has antimicrobial anti-oxidants and anticarcinogenic effects. Furthermore, bioactive peptides are said to have regulating and hormonal effects. However, these effects are mainly concluded from in vitro experiments or feeding trials. Their effects on humans are still to be examined.

### 3.2.7.3. LAB as Oral Vaccines:
Lactic acid bacteria possess a number of properties which make them attractive candidates for oral vaccination purposes. These bacteria have been used for centuries in the fermentation and preservation of foods and are considered to be 'safe' organisms with a GRAS status. Apart from this, LAB also has adjuvant properties and low intrinsic immunogenicity. A particularly attractive feature of lactobacilli but not of lactococci is their ability to colonize certain regions of mucosa which permits in principle the induction of a local immune response. An additional benefit of the use of lactobacilli is that some strains are considered to show health promoting activities for humans and animals.

Many of these lactobacilli also produce surface proteins which have been very well characterized. By applying genetic engineering techniques on these organisms, it is now possible to express antigens from potential pathogens such as malarial parasite, *Vibrio cholerae* and Hepatitis B virus either on their surface or extracellularly or intracellularly which eventually can elicit antibody production in the consumers fed with such genetically modified cultures to protect against the targeted pathogen. The elucidation and characterization at DNA level of bacterial surface proteins involved in adherence to mucosal tissues has provided new and challenging opportunities which will allow us to better understand the interaction between intestinal lactobacilli and mucosal tissues. The recent developments of Molecular Biology techniques that permit construction of LAB presenting foreign antigens in different ways to the immune system offers excellent opportunities to further explore the potentials of these food bacteria as vaccine vehicles.
3.2.8. Accelerated Cheese Ripening

Many cheese varieties require long ripening periods at low temperature for characteristic flavour and texture development. This process significantly increases the cost of the product. Controlled use of biotechnological products like genetically engineered proteolytic and lipolytic enzymes can accelerate cheese ripening. Modified/genetically tailored microorganisms and enzymes are now being used for enhancing flavour production in cheese. Enzyme addition is now one of the few preferred methods of accelerated ripening of cheese. The enzymatic reactions are specific and hence undesirable side effects caused by live microorganisms are avoided in the cheese. Enzymes may be immobilized or encapsulated for long term action on the production for quick action and homogenous distribution in the product. The molecular analysis of the lactococcal proteolytic system has created the exciting prospect of being able to use genetic strategies to manipulate these hosts to produce strains with new flavour characteristics. It is also likely that these approaches will allow cheeses to be ripened in a more controlled fashion and an accelerated rate, if desired. This will result in the production of fermented foods with new attractive properties as well as creating economic savings for the producers.

3.2.9. Rapid Detection of Food Pathogens

Although there has been dramatic increase in milk production in the country and more surplus milk is now available to dairy industry for processing, the microbiological quality of such milk and processed products has to be of acceptable quality. The safety of milk and milk products from public health point of view can be ensured by rigorous monitoring of dairy foods especially for high risk food pathogens such as *E. coli* 0157 : H7, *Listeria monocytogenes*, *Salmonella*, *Shigella*, etc. which gain access into these foods due to mishandling during production and processing. Since, the conventional methods currently used in Quality Assurance labs for the detection of these potential pathogens are extremely laborious, lengthy and tedious, they do not serve any useful purpose to dairy industry as by the time results are available, the concerned food lot is already sold in the market and hence no follow up action is possible to avoid possible outbreak of diseases associated with such food pathogens. Hence, there is a need to develop simple and new innovative techniques for rapid detection of pathogens in dairy foods.

2.3.10. Gene Probes

One such approach, which is in vogue these days in most of the developed countries, is the use of molecular techniques for detection of pathogens. Gene probes and immunological assays using poly or monoclonal antibodies, targeted against the specific pathogen are highly specific and sensitive and have turned out to be extremely valuable tool for the reliable detection of pathogens in foods even at low levels. However, application of gene probe requires highly sophisticated procedure, adequate infrastructure and expertise in terms of skilled manpower. Nevertheless, a number of companies are entering into the development of kits based on gene probes and immunological principles for rapid detection of targeted pathogen in the foods. Many such kits are commercially available now.

2.3.10.1. PCR Based Detection of Food Pathogens: Another extremely versatile, reliable, sensitive and practical method for rapid detection of food pathogens directly from foods without following lengthy culturing steps is polymerase chain reaction (PCR). Application of such methods in Food Quality Assurance and Public Health Labs can go a long way in protecting the health of the public. The crucial feature of sample preparation in the direct analysis of food without cultural enrichment is the isolation of target DNA with high
reproducibility and efficiency. Construction of the actual PCR assay requires nucleotide sequence information and knowledge about the chosen target gene to guarantee maximum specificity and sensitivity. By using different formats of PCR, it is now possible to simultaneously detect more than one food pathogen in the food. PCR based detection of food pathogens has completely revolutionized the area of diagnostics as the results are available in less than 24 hrs to enable the food processing units to take appropriate follow up action in case a pathogen is detected in a food to avoid any risk to the consumers.

Molecular Biology group at NDRI, Karnal has recently developed a multiplex PCR assay for rapid detection of *E. coli* 0157:H7 in milk and milk products. The entire test takes just 8 hours to unequivocally prove the presence of *E. coli* 0157:H7 in the given food. Attempts are now being made to extend this technique to other high risk food pathogens like *Listeria monocytogenes*, *Salmonella* and *Shigella*, etc.

### 3.0 REGULATORY CONSIDERATIONS

One of the major concerns in the public mind is whether food prepared with genetically engineered lactic starter cultures or recombinant dairy enzymes or proteins are safe for consumption. To be used in food systems, genetically engineered dairy starter cultures will need to be approved for use by appropriate regulatory agencies. The most important issue which needs immediate attention is the use of artificial drug resistance markers in the construction of genetically engineered organisms since the foods prepared with such organisms carrying drug resistant genes could pose serious health risks to the consumers. This necessitates the need for replacing drug resistant marker genes some natural food-grade markers derived from lactic acid bacteria themselves. In this context, genes encoding bacteriocin production and immunity could be extremely valuable in the construction of food-grade vector systems which can easily replace the conventional *E. coli* based vectors. The application of engineered starters with such food grade vectors in food processing should not pose any problem in getting the approval of regulatory agencies.

### 4.0 CONCLUSION

From the foregoing presentation, it can be concluded that Biotechnology is bound to play a significant role in reshaping the Dairy Scenario in the country. By judiciously applying biotechnological approaches, Indian dairy industry can make a niche and add new dimensions to our economy in the world market by producing high quality dairy products, which are not only nutritious, clean and wholesome but also safe for local consumption as well as for export.

### 5.0 SUGGESTED REFERENCES


1.0 INTRODUCTION

Designer milk is not now a dream but a real possibility. For instance, genetic engineering for secretion of low lactose milk - recently demonstrated - could make whole milk available to the majority of the world's adults currently excluded by lactose intolerance. Modification of milk composition though transgenesis is a promising way for improving existing products and extending the uses of milk components. However, various ethical, legal and social aspects of biotechnological research need to be addressed through public education to bring about greater understanding of the issues involved and to find classical solutions for the existing issues. Until recently, emphasis had been on breeding bigger animals that produced more milk. Now we are turning our attention to adding more value to the milk we produce and studying its health implications.

An interest in understanding how a cow makes milk, what controls the casein’s synthesis, what controls the synthesis of fat in mammary glands, how the biochemical pathways work has led to find ways to produce milk with lower fat and greater amounts of protein or different types of proteins. By combining the two approaches—the genetic work and the diet studies researchers are hoping to develop “designer milk” tailored to consumer preferences or rich in specific milk components that have health implications. There has been a decline in livestock, product consumption viz. egg and egg product—50 per cent, liquid milk—20 per cent, butter—70 per cent and meat—11 per cent over the last two decades. As consumer preference for animal products is likely to continue, it would be important to modify animal products in such a way that dietary risks are minimized while there would be maximum benefits in respect processing of products. Rapid development of genetic technology has placed the dairy processors open to improvement by modern biotechnology, while novel horizons beckon in nutrition, food technology and pharmacology.

2.0 SCOPE AND BENEFITS

Milk is a major component of the diet in the world. For certain purpose, it would be desirable to have the chance to change the milk composition. From the dietary and human health point of view:

- A greater proportion of unsaturated fatty acids in milk fat would be appreciated
- Possibility to reduce lactose content in milk in order to make it accessible also for persons with expressed lactose intolerance
- Milk devoid of β-lactoglobulin.

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From technological point of view: Alteration or primary structure of casein helps to improve technological properties of milk. Dairy producers would have the opportunity to choose to:

- Produce high protein milk
- Milk destined for cheese manufacturing that has accelerated curd clotting time, increased yield, and more protein recovery
- Milk containing nutraceuticals
- Replacement for infant formula

3.0 MODIFICATION OF MILK COMPOSITION BY GENETIC TECHNOLOGY

Milk composition can be dramatically altered using gene transfer. Classical studies have revealed association between major lacto-protein variants and milk production traits. Introduction of DNA technology in the dairy science field has enabled to identify new genetic polymorphism and revealed molecular background of lacto-protein gene expression. Subsequently, several emerging transgenic technologies have focused on the mammary gland.

A transgenic animal has been defined as an animal that is altered by the introduction of recombinant DNA through human intervention (Murthy and Kanawjia, 2002). The process involves that a DNA construct is designed and built to express the desired protein in animal, the construct then introduced into a single cell embryo to allow incorporation of the transgene into animal genome. There are several methods available for this purpose, including retroviral transmission, stem cell transfection, and microinjection into the pronucleus or cytoplasm.

4.0 DESIGNER MILK AND NUTRITIONAL SIGNIFICANCE

4.1. Change in Milk Fat

Composition of milk could be altered by feeding lactating dairy cows a control diet to obtain a naturally modified component. This method is eyed towards altering milk fat. Fatty acids of less than 12 carbon atoms are neutral or actually may decrease cholesterol. Stearic acid (C18:0) acts similarly to oleic acid (cis-C18:1) to decrease cholesterol (Ney, 1991). Only three saturated fatty acids (lauric, C12:0; myristic, C14:0; and palmitic, C16:0) now are considered to be hypercholesterolemic. These fatty acids constitute about 44 per cent of total milk fatty acids. According to a group of nutritionists from industry and academic (O. Donnel, 1989), the “ideal” milk fat for human health would contain <10 per cent poly unsaturated fatty acids, <8 per cent saturated fatty acids, and >82 per cent monosaturated fatty acids. Although it is not likely that the “ideal” milk fat composition could be achieved, manipulation of composition of milk fat is possible through feeding practices for dairy cows (Palmquist et al., 1993)

Studies have demonstrated significant and prompt changes in the iodine number of milk fat when the diet fat was changed from a high to a low degree of unsaturation. The modest increase in unsaturation was probably due to an increase in the oleic acid (C18:1) with little increase in linoleic acid (C18:2). The rumen microflora is apparently able to hydrogenating only one double bond. A “Designer Cow” called “Daisy”, which can produce semi skimmed (half-fat milk) milk and butter that spreads straight from the refrigerator (soft-spreading butter) has been bred by Britain’s Agricultural Development and Advisory Services (ADAS) in collaboration with Samsbury’s, the retail grocer chain (Murthy and Kanawjia, 2002). Cows fed with fish oil, fishmeal or plankton can produce milk rich in ω-3 fatty acids. Restricted quantities of fish products are added to the cow’s diet of grass or silage to produce ω-3 fatty acids.
acids enriched milk. Similarly for half-fat milk, the supplement is dehusked oats and for a spreadable butter is rapeseed oil. In the U.S, researches have a way t boost the whole milk natural cancer fighting ability. Dietary fats such as corn oil are fed to cows in protected form. The cows produce milk with substantially increased levels of conjugated linoleic acid (CLA). A number of laboratory studies have shown that CLA suppresses carcinogens, inhibits proliferation of leukemia and colon, prostate, ovarian, and breast cancers. CLA inhibits cancer growth even in extremely low dietary concentration of 0.5 per cent and is among the mostly potent of all naturally occurring anticarcinogens.

4.2. Changes in Milk Protein

One of the major products of the mammary glands is protein, it is thus legitimate to consider the production of additional proteins for which there is a demand and that introduce desirable compositional changes in the milk several potential changes are listed in Table 1.

Table 1: Potential Changes in Milk through Genetic Engineering

<table>
<thead>
<tr>
<th>Potential Modification</th>
<th>Change in Milk</th>
</tr>
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<tbody>
<tr>
<td>Increase casein content</td>
<td>Increased protein, better manufacturing properties particularly for cheese making</td>
</tr>
<tr>
<td>Engineered Casein</td>
<td>Better manufacturing properties</td>
</tr>
<tr>
<td>Remove a β-lactoglobulin</td>
<td>Better manufacturing properties, decreased milk allergies</td>
</tr>
<tr>
<td>Remove fat</td>
<td>Easier to produce low fat milk products, decrease the butter surplus. Increase solids content, remove lactase lactose</td>
</tr>
<tr>
<td>Produce β-galactosidase, Lactose,</td>
<td>Safer food, mastitis prevention</td>
</tr>
<tr>
<td>Produce antibodies of pathogens</td>
<td></td>
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</table>

4.3. Human like Milk

A human like milk enriched with human lysozyme and lactoferrin might enhance defense against gastrointestinal infections and promote iron transport in the digestive tract. Given the much higher level of lactoferrin in the human milk (10 times more than in cow’s milk), it may be desirable to supplement the level in baby formula to bring it up to the human milk level. A construct comprising human lysozyme cDNA driven by the bovine alpha S1-casein promoter yielded a high level of lysozyme in mice (Maga et al., 1994). Obtention of a transgenic bull carrying a human lactoferrin construct was also reported (krimpenfort et al., 1991). The fore mentioned studies drive towards humanization of non-human mammals’ milk.

4.4 Changes in the Individual Casein Content of Milk

4.4.1. Increasing the α-S1-or α- S2-casein content: A higher content of α S2–casein, which contains 10-13 phosphoserine and two cysteine residues in the bovine species would obviously enhance their nutritional value of casein, which is deficient in sulphur containing amino acids, and would presumably increase micelle stability. The bovine α S1–casein gene and hybrid genes driven by the α S1–casein promoter have been well expressed in mice but attempts to express the bovine α S2–casein gene have been unsuccessful.

4.4.2. Producing Milk Devoid of β-Lactoglobulin: Cow’s milk is an allergic trigger in a significant fraction of infants and β-lactoglobulin, which is not found in human milk, is believed to be one of the culprits. Elimination of this protein from cow’s milk is unlikely to have any detrimental effects, on either to cow or human formula, and might actually overcome
many of the major allergy problems associated with cow’s milk. Presently, a reasonable genetic approach is the systematic search for individuals, which might carry a null β-lactoglobulin allele, which proved successful for the α- and β-casein loci in the goat species. The use of antisense or ribozyme anti β-lactoglobulin mRNA is appealing and is very potent to prevent synthesis of 3 g of β-lactoglobulin in bovine species. The methodology of choice is obviously the knockout of β-lactoglobulin gene, which relies on the availability of the ES cells in dairy species.

4.5. Changes In Milk Sugar

4.5.1. Reduction of Lactose in Milk: Lactose is the major sugar present in milk and is an important osmotic regulator of milk secretion. It is synthesized in the mammary gland by the lactose synthetase complex composed of uridine diphosphate-galactosyl transferase and of the mammary specific α-lactalalbumin. The adult population suffers from intestinal disorders after milk ingestion, as a consequence of lactose maldigestion that results from the normal drop of the intestinal lactose-hydrolyzing enzyme, lactase-phlorizin associated with intestinal pathologies and with rare cases of intolerance can also be limited primarily by dietary changes either by avoidance of dairy products or through the use of low lactose (post harvest) or lactase replacement products (Murthy and Kanawjia, 2002). Each of these management strategies requires dietary supplementation and they vary in their efficiencies.

5.0 DESIGNER MILK AND TECHNOLOGICAL ASPECTS

5.1. Modification of Protein

5.1.1. Increasing the Κ-Casein Content: In the mammary gland, calcium-induced precipitation of α S1-, α S2-, and β-casein is prevented by their association with κ-casein as sub micelles stably bound together through calcium bridges (Rollema, 1992). Higher κ-casein content was found to be associated with smaller micelle size (Rollema, 1992). Further more, heat stability of milk was markedly improved by addition of κ-casein (Singh and Creamer, 1992). Because of the key roles of κ-casein, the relevant gene is one of the most obvious candidates for transgenesis. Transgenic mice carrying a caprine β-casein: κ-casein fusion gene produced milk containing up to 3mg caprine κ-casein per ml (Persuy et al., 1995). This hybrid gene is thus quite suitable for microinjection into eggs from farm animals.

5.1.2. Modification of β-casein in Milk: An increase in β-casein reduces RCT and increases syneresis rate, and also increases curd firmness by 50 per cent. One modification that has been made to β-casein is the deletion of plasmin cleavage site causing prevention of bitter flavour in cheese due to plasmin cleavage. The second alternation is removal of cleavage site for chymosin. The last modification made is the addition of glycosylation sites to the molecule increasing its hydrophilicity (the above modification done to mice milk). Also the native caprine β-casein gene successfully tested in mice, which produced up to 25mg exogenous β-casein per ml of milk, is being used as such for generating transgenic goats producing β-casein. β-lactoglobulin causes aggregation and gelation of milk at higher temperature, and also is potent allergen. This gives the reason to remove this gene by ES cells.

5.2. Modification of Fat

The most significant changes in milk fat quality relate to rheological and organoleptic properties, which influence numerous aspects of character and quality of manufactured dairy products (Mortensen, 1983). The type of fatty acids present in milk fat can influence the flavour and physical properties of dairy products. Sensory evaluation indicated that butter produced from cows fed high oleic sunflower seeds and regular sunflower seeds were equal or
superior in flavour to the control butter. The high oleic sunflower seed and regular sunflower seed treatment butter were softer, more unsaturated and exhibited acceptable flavour, manufacturing, and storage characteristics. Sensory evaluation of Cheddar cheese indicated that extruded soybean and sunflower diets yielded a product of quality similar to that of control diet (Lightfield et al., 1993).

Cheese made from milk obtained with extruded soybean and sunflower diets contained higher concentrations of unsaturated fatty acids while maintaining acceptable flavour, manufacturing, and storage characteristics (Murthy and Kanawjia, 2002). It is anticipated that the total unsaturated fatty acids content could be increased beyond the amounts achieved to date when feeding unsaturated fat sources and without adversely affecting flavour or product processing properties. Milk containing naturally modified fat, obtained either by feeding extruded soybean or sunflower seeds produced blue and Cheddar cheeses enhanced the safety against L. monocytogenes and S. typhimurium due to accumulation of fatty acids, namely C12, C14, C18:1 and C18:2 (Schaffer et al., 1995).

5.3. Other Modifications

5.3.1. Increasing Total Solids and Reducing Lactose and Water Content of Milk:
Mice that produce milk with 33 per cent more total solids and 17 per cent less lactose than normal control mice have been generated by transgene. Mouse milk normally has approximately 30 per cent total solids, while these transgenic mice produce milk that is 40-50 per cent total solids when milk reaches 45-50 per cent total solids it lacks fluidity and becomes difficult to remove from mammary gland. The increase in the total solids is associated with a decrease in total milk volume and a decrease in amount of water in the milk. The activity of lactose synthetase enzyme within the mammary gland decreased by 35 per cent in the transgenic mice. These data indicate that due to a decrease in lactose synthetase activity, less lactose is being produced; less water is being transferred into milk causing a reduction in milk volume. So it appears that the same amount of total milk fat and protein are being produced in a lesser total milk volume.

Using the technology described above and increasing our understanding of these mechanisms may lead to cattle with similar characteristics to the mice that have been produced. Having milk from a dairy cow that contain 6.5 per cent protein, 7 per cent fat, 2.5 per cent lactose and 50 per cent less water would have a number of economic and processing benefits. The main direct economic benefit would be the 50 per cent reduction in the cost of shipping milk. In addition, since the cow would be producing one half her normal volume of milk there would be less stress on the cow and on her udder.

On the manufacturing side, after the removal of fat from this type of milk, a skim milk having twice protein content and have half the lactose content of normal milk could be produced. This type of milk would also make it easier to produce low lactose or lactose free “hard dairy” products. The concentrated milk should lead to better product yields form the same amount of initial input. The lowering of milk volume and lactose content will reduce the total whey output produced during processing. The reduction of stress on the mammary gland of the cow and the more viscous milk may also decrease the susceptibility to obtaining mastitis infection. Organisms that cause mastitis use lactose as their energy source and since lactose would be reduced in the system there would be a decrease in the available food source for these bacteria.
6.0. DESIGNER MILK IN MAKING GM CHEESE

Scientists in New Zealand have created the world's first cow clones that produce special milk that can increase the speed and ease of cheese making. The researchers in Hamilton say their herd of nine transgenic cows makes highly elevated levels of milk proteins i.e. casein-with improved processing properties and heat stability. Cows have previously been engineered to produce proteins for medical purposes, but this is the first time the milk itself has been genetically enhanced. The scientists hope the breakthrough will transform the cheese industry, and if widened, the techniques could also be used to "tailor" milk for human consumption. But opponents of GM foods continue to doubt whether such products will be safe.

6.1. Designer Milk with Higher Levels of \( \beta \)-Casein and \( \kappa \)-Casein-A

"Bio-boom" of Biotechnology

To enhance milk composition and milk processing efficiency by increasing the casein concentration in milk, researchers have introduced additional copies of the genes encoding bovine \( \beta \)- and \( \kappa \)-casein (CSN2 and CSN3, respectively) into female bovine fibroblasts. Nuclear transfer with four independent donor cell lines resulted in the production of 11 transgenic calves. The analysis of hormonally induced milk showed substantial expression and secretion of the transgene-derived caseins into milk. Nine cows, representing two high-expressing lines, produced milk with an 8–20 per cent increase in \( \beta \)-casein, a twofold increase in \( \kappa \)-casein levels, and a markedly altered \( \kappa \)-casein to total casein ratio. These results show that it is feasible to substantially alter a major component of milk in high producing dairy cows by a transgenic approach and thus to improve the functional properties of dairy milk.

6.2.1. Designer Milk to Cut Cheese Costs:

Protein-rich designer milk from modified cows could speed dairy processing. Protein-rich milk from cloned, genetically modified cows could cut cheese-making costs. Dairy manufacturers would need less milk to make cheddar.
firm and ice cream creamy. Two years old and living in New Zealand, the clones produce about 13 percent more milk protein than normal cows. They carry extra copies of the genes for two types of the protein casein, key for cheese and yoghurt manufacture. ‘They allow milk to have high protein content, but to remain watery,’ says study leader Götz Laible of New Zealand biotech company AgResearch. His team must now find out whether the increase improves milk’s calcium content or its ability to coagulate before they seek approval to sell the clones to dairy farmers. Most scientists believe that milk from cloned cows is no different to normal milk. But they are less certain about the safety of milk from genetically modified cows.

7.0 CONCLUSION

The future of biotechnologically derived foods is at crossroads; same being true for milk. Hi-tech milk processing may be more acceptable than transgenesis for altering milk composition. Controversy will inevitably surround all biotechnological manipulations aimed at increasing milk production or altering milk composition. Ultimate acceptability will depend on consideration of four factors:

- Animal welfare
- Demonstrable safety of product
- Enhanced (health) properties of the product
- Increased profitability as compared with conventional practices

Milk for the paramedical industry, i.e., humanized milk is likely to be produced first. In the foreseeable future, with the materialization of genetic game in milk production, herds producing many different types of milk will presumably be available allowing consumers and industrials to choose the milk best adapted to their needs. Aforementioned studies have fueled the debate on weather transgenic animal could be part of our food supply. Perhaps one day we will see designer transgenic herds similar to the organic herds that thrive in the current economic and social climate. Even though these changes may be a decade away, the possible impact on the dairy industry will be great.

7.0 REFERENCES


1.0 INTRODUCTION

The genus *Lactobacillus* is quite diverse and consists of a number of different species with little commonality. A measure of their diversity can be estimated by the range in the G+C per cent content among the *Lactobacilli*. Members of the species have G+C per cent of 32–53 per cent, which is a much wider range than is encountered with other lactic acid bacteria. Their common taxonomical features are restricted to their rod shape and their ability to produce lactic acid either as an exclusive or at least a major end product. In addition, they are Gram-positive and do not form spores. *Lactobacillus* cells are typically rod-shaped with a size range of 0.5–1.2 x 1–10 µm. Under certain growth conditions they can look almost coccoid-like hence this characteristic is not absolutely diagnostic. In fact the former *Lactobacillus xylosus* has been reclassified as *Lactococcus lactis* subsp. *lactis*, although its historical designation as a lactobacillus must have been on the basis of its rod-shape coupled to its ability to ferment xylose.

The *Lactobacilli* are facultative anaerobes that, in general, grow poorly in air, but their growth is sometimes enhanced by 5 per cent carbon dioxide. Because they are auxotrophic for a number of different nutrients, they grow best in rich complex media. It is auxotrophies in some strains that have been exploited to develop bioassays for a number of vitamins and other micronutrients. Their optimum growth temperature is 30–40°C, but they can grow over a range of 5–53°C. They are also aciduric with an optimum growth pH of 5.5–5.8 but in general they can grow at a pH < 5.

The *Lactobacilli* include over 100 unique species, and the first level of differentiation is based on end-product composition; some are homofermentative whereas others are heterofermentative. The former are classified as organisms that produce > 85 per cent lactic acid as their end product from glucose. The latter include organisms that produce approximately 50 per cent lactic acid as an end product, with considerable amounts of carbon dioxide, acetate and ethanol. Notable among the homofermenters are *L. delbrueckii*, *L. leichmannii* and *L. acidophilus*. Heterofermenters include *L. fermentum*, *L. brevis*, *L. casei* and *L. buchneri*. Although they all produce lactic acid as a major end product they differ in the isomeric composition. Some produce exclusively L (+) lactic acid and these include *L. salivarius* and *L. casei*. Others, for example *L. bulgaricus* and *L. jensenii* produce just D (−), and finally *L. acidophilus* and *L. helveticus* produce a mixture of D (+) and L (−) lactic acid. The next major criterion for distinguishing among the *Lactobacilli* is the production of gas from carbon sources including glucose and gluconate. In addition there is a great degree of diversity in the ability of various *Lactobacillus* spp. to ferment pentose sugars including ribose and xylose.
They are precious for the dairy industry, in terms of their well-established role in:

1) Providing typical flavour and aroma to these products, thus enhancing their mass appeal.
2) Making these products more shelf stable & hence are a saviour on economic front in field of food industry.
3) Providing beneficial health enhancement properties when used as live microbial supplement in the fermented foods, such as yogurt.

Besides Dairy products Lactobacilli are also used to ferment vegetables to produce pickles, Olives and Sauerkraut. They also play an essential role in bread making. In these foods, products, the indigenous Lactobacilli are able to overcome other contaminating microflora, largely by thriving under the fermentation conditions, which is the main factor responsible for their predominance in such products. Another property of Lactobacilli that has become more appreciated is their ability to produce bacteriocins. Bacteriocins probably evolved to provide the producing organism with a selective advantage in a complex microbial niche. Incorporation of Lactobacillus spp. as starters or the inclusion of a purified or semi-purified bacteriocin preparation as an ingredient in a food product may provide a margin of safety in preventing pathogen growth.

In essence, some of the important Lactobacillus spp. involved in various beneficial properties may be short-listed as in the Table-1:

<table>
<thead>
<tr>
<th>Important Lactobacillus spp. Involved in Various Beneficial Properties</th>
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<tbody>
<tr>
<td><strong>Lactobacillus spp.</strong></td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
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<tr>
<td>Lactobacillus bulgaricus</td>
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<tr>
<td>L. plantarum, L. casei</td>
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<tr>
<td>L. acidophilus LA 1</td>
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<tr>
<td>L. acidophilus NCFB 1748</td>
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<tr>
<td>L. rhamnosus GG)</td>
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<tr>
<td>L. casei Shirota</td>
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<td>L. gasseri ADH</td>
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<td>L. reuteri</td>
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<td>L. acidophilus</td>
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<tr>
<td>L. brevis</td>
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<tr>
<td>L. delbrueckii</td>
</tr>
<tr>
<td>L. helveticus</td>
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<tr>
<td>L. sake</td>
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<tr>
<td>L. gasseri</td>
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</tbody>
</table>

20
Although a wide range of beneficial aspects of naturally occurring *Lactobacillus* group of spp. pave way towards the possibility of their greater exploitation in these areas and new related fields, lack of correct identification of scheme for species and strains, differentiation and discrimination from other similar species and strains poses a major problem. All the important applications, listed required only a particular strain among the large number of strains, identified in the same single spp. Hence need arises to develop the test or battery of tests which can target individual strains and not only species and genus.

### 2.0 IDENTIFICATION OF LACTOBACILLI

Different classical methods such as morphology, as well as physiological, metabolic/biochemical and chemotaxonomic methods are mostly being used for a long time in order to identify different *Lactobacillus* spp. Physiological methods mostly include growth at certain temperatures and salt levels. While metabolic/biochemical tests often include, production of gas from glucose, production of acids from carbohydrates, hydrolysis of arginine and determination of lactic acid configuration. Chemotaxonomic methods involve cell wall protein analysis, fatty acid composition etc.

Although all these methods are still globally in use but since they are based on the unstable expression of the phenotypic maskeos, which can vary in different environmental and culture conditions, they are not fully reliable. In addition, they are time consuming and often give ambiguous results. Besides one another major problem makes correct identification of lactobacillus spp. and strain difficult i.e. transfer of some characteristics between strains and species via plasmids, leading to altered phenotypic pattern for the same strains within species sometimes. Thus besides genus and species, strain level identification can not be achieved using these traditional methods. Hence focus has shifted towards the more consistent and sophisticated molecular techniques for strain typing now a days.

### 3.0 BASIC OF STRAIN TYPING

Strain typing systems are actually based on the fact that related to microbial isolates are derived from a single clone and share characteristics which are different from unrelated isolates. Clones are basically indistinguishable isolates-derived from the same parent strain. Subsequent generations of organisms often begin to diverge from the parent. As divergence occurs, progeny strains will share most characteristics with the parent, but differences will appear.

Complex typing systems based on methods such as serology, bacteriophage sensitivity and RAPD (Random Amplified Polymorphic DNA) for bacteria such as *Salmonella* and *Listeria* have been developed (Threlfall and Frost, 1990 & Lawrence et al., 1993) But biotyping and serotyping etc. are limited to taxonomic studies only in lactobacillus, due to diversity of species and phenotypic variation in this genus (Shape 1955 & Covett, 1965). Currently rapidly advancing knowledge of new dimensions in molecular biology field has however, allowed proliferation of many new and highly sensitive typing techniques. These typing techniques provide solution to the problem of tracking individual strains, while dealing with mixed consortia of *Lactobacilli* either in foods, feeds and beverages or gastro intestinal tract complex environment.

Still, the typing of *Lactobacilli* has not kept pace with the diversity of techniques available and is still in its exploration phase to identify the best resulting method for distinct identification of individual strains. In general the typing techniques currently in use for lactobacillus may be summarized as follows:

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4.0 NON PCR BASED TYPING TECHNIQUES

A number of molecular techniques can be summarized under these, such as:

1) Plasmid profiling
2) Probe hybridization
3) Total soluble protein content analysis via SDS PAGE
4) Restriction analysis using Pulse Field Gel Electrophoresis
5) Ribotyping, etc.

4.1 Plasmid Profiling

This involves extraction of individual plasmid DNAs and later their comparison with known species through gel electrophoresis for strain differentiation. It has been by far the most favoured technique for strain typing in *Lactobacillus* (Hill and Hill, 1986; Lonner et al., 1990; Tannock et al., 1990 & Dykes et al., 1993). The advantages of this technique are:

- Rapid
- Easy to perform
- Useful, when large no. of strains are to be examined
- Particularly important tool for Lactobacillus strain typing, as most of the Lactobacilli, accruing in natural environment, contain plasmids.

On the other hand it has also some shortcomings, which limit its wider applications such as:

- All strains may not possess plasmids
- Plasmid may be lost, gained during course of time

4.2 Probe Hybridization

This approach although discriminates between species but cannot be used generally for large-scale strain differentiation. It is also time consuming and involved lengthy steps. A probe is a fragment of single stranded nucleic acid that will specifically bind to complementary regions of a target nucleic acid. There are 3 different approaches to the design of nucleic acid probes, randomly cloned probes, probes complementary to specific genes and gene fragments and rRNA probes (Schleifer et al., 1995). Many Researchers have used probes to identify different *Lactobacillus* species, such as *L. delbreuckii* (Delley et al., 1990 & Andrighetto et al., 1998), *L. helveticus* (Pilloud et al., 1990 & Reyes Gavilan et al., 1992), *L. casei* strain (Ferrero et al., 1996), etc.

4.3 Total Soluble Protein Content Analysis via SDS PAGE

Recently SDS-PAGE of cell wall proteins has been developed for differentiation of different Lactobacillus species (Angelis et al., 2001 & Gatti et al., 2001). It is possibly as rapid as plasmid profiling. It gives complex bending pattern, specific. Strain differentiation however, has not been reported through SDS AGE in Lactobacillus. Further more, since expression of protein pattern is not stable always and may vary with the environmental conditions such as growth medium, temperature etc.
4.4 **Restriction endonuclease (REA) and Ribotyping**

Patterns generated from chromosomal DNA are more stable over time than those from extra chromosomal DNA. They are however also more sensitive, allowing the determination of small differences between strains. Simple REA (Restriction Endonuclease Analysis) has been used as a tool for separating different species of *Lactobacilli*. This technique involves first digestion of chromosomal DNA with different restriction endonucleases and then their separation and visualization on agarose gels. The variation in number and size of resulting fragments creates a pattern of bands on gel, which can be interpreted as a fingerprint unique to particular species. However, consistence of hundreds of bands creates problem in interpretation of and it can be minimized using labeled probes targeted against specific spp or strains in the southern blot. Complex profiles of the locations of various restriction enzyme recognition sites, within a particular genetic locus can be polymorphic from strain to strain resulting in bands of different sizes between unlike strains.

Sometimes instead of simple agarose gel electrophoresis use of pulse field gel electrophoresis (which separates large DNA fragments on application of sport pulses of high voltage in two alternate directions gives more discriminating pattern and has been found to differentiate between strains of *Lactobacilli* (Roussel *et al*., 1993). A further modification of this basic scheme makes use of only ribosomal gene restriction pattern analysis i.e. ribotyping. This technique has been successfully applied to *Lactobacilli* (Rodtong and Tannock, 1993 & Roussel *et al*., 1993) and represents a rapid and sensitive strain typing technique. Particular advantage that since rRNA genes exist in various copy numbers so different species can be delineated based on differences in the RFLP of rRNA genes, which have remained well conserved during evolution.

4.5 **16S- rRNA Gene Sequencing:**

With the declining cost of high throughput sequencing operations, straight determination of nucleotide sequence has emerged as a viable option for strain identification. Many researchers have sequenced species specific sequences within conserved 16S rRNA of *Lactobacilli* for correct phylogenetic analysis and identification (Amann *et al*., 1995; Gustler *et al*., 1996 & Vandamme *et al*., 1996).

5.0 **PCR BASED TYPING METHODS**

PCR (Polymerase Chain Reaction) automatically amplifies rapidly any particular stretch of DNA using a pair of primers each complementary to the flanking ends on both the strands, in a thermal cycler, within a short span of time. Thus primers can be designed to specifically amplify only a chosen region, specific to either a particular species or strain or various species simultaneously in a single reaction i.e. (Multiplex PCR). Thus genus, species or even strains can be identified accurately using PCR. Among PCR based methods, RAPD (Randomly amplified Polymorphic DNA, ARDRA (restriction analysis of the amplified rDNA, species-specific PCR all are being used for differentiation of *Lactobacilli*.

5.1 **RAPD**

In RAPD assay, also referred to as Arbitrary Primed PCR, patterns are generated by the amplification of random DNA segments with single short (typically 10 bp) primers of arbitrary nucleotide sequence. The primer is; not targeted to amplify any specific bacterial sequences and will hybridize under low stringency condition at multiple random chromosome locations and initiate DNA synthesis. After separation of the amplified DNA fragments by agarose gel
electrophoresis, a pattern of bands results, which is characteristic of the particular bacterial strain (Williams et al., 1990; Welsh and McCleland 1992 & Maunier and Grimont, 1993). The merits of this method are:

- Requirement of very short time
- Provides good level of discrimination
- Can be applied for large no. of strains (Vincent et al., 1998).

RAPD has been used to estimate the diversity among lactobacillus strains of CNRZ culture collection (Tailliez et al., 1996) and to establish correct classification and nomenclature of strains of Lactobacilli casei (Dick et al., 1996). It was found more discriminatory in comparison to soluble cell protein analysis for strain differentiation (Erica and Dicks 1995). RAPD was also successfully identified closely resembling L. curvatus, L. gramine and L. sakei groups (Berthier and Ehrlich 1999). In a recent study, Nigatu et al. (2001) discriminated 41 types and reference strains of Lactobacilli on the basis of distinctly polymorphic bands specific to strains. Thus, statistical analysis of the RAPD profile using specialized software programme provides a dendrogram, which not only easily differentiates different lactobacillus species but strains also.

5.2 Species–specific PCR

As the name indicates, this type of PCR reaction makes use of species specific primers in for differentiation of different species. These primer sequences usually chosen from highly conserved 16S rRNA, 23S rRNA or 16S-23S intergenic spaces region. The advantage with these generic regions is that all these are part of highly conserved rRNA operon, which has remained well conserved during evolution and is common to all eubacteria; in addition, these also contain highly variable regions unique to each particular species. All these primers are being used for the identification of different Lactobacillus species. (Andrighetto et al., 1998; Drake et al., 1996; Song et al., 2000 & Fortina et al., 2000). Although this is a highly specific technique for species level differentiation, but strain typing is not possible through this methodology. However, straight sequencing of these amplified 16s-23s intergenic spacer regions, in different isolates can easily differentiates even very much closely resembling strains and is readily becoming popular among researchers.

5.3 Amplification and Restriction Analysis of 16s rRNA Gene (ARDRA)

Among other 16s rRNA based techniques, ARDRA combines both PCR and restriction analysis and hence is a powerful technique and is capable of differentiating species as well as strains (Andrighetto et al., 1998 and Kullen et al., 2000). However since it also has limitation of tedious handling steps, it can not be applied for large no. of Lactobacilli simultaneously.

6.0 CONCLUSION

At present time, there are around 120 Lactobacillus species described. Strain typing has been applied to less than a third to these. Typing studies on many other important Lactobacilli particularly those associated with food ecosystems are however lacking. The L. spp, which are strain typed, mostly include L. helveticus, L. acidophilus, L. fermentum (Stahl et al., 1990; Miteva et al., 1992; Tennock et al., 1992; Pot et al., 1999 & Rodtong and Tannock 1993). Other species to which typing has been applied include L. plantarum (Hill & Hill 1986 and Stahl et al., 1990), L. casei (Miteva et al., 1992), L. reuteri (Rodtong and Tannock, 1993), L. sake and L. curvatus (Dykes et al., 1993; Dykes and Von Holy 1993 & Vogel et al., 1993). Typing studies on Lactobacillus have helped in elucidating the role played by Lactobacilli in the complex gastrointestinal ecosystem. Although typing has been demonstrated to be a useful
tool in a no. of fermented food and feed products, silage etc., still application of typing to such complex ecosystems has been limited, with most studies concentrating identification of species rather than strains.

From the above facts, conclusion emerges that the increasing paramount importance of the genus Lactobacillus in both the industrial sphere as well as human & animal health, certainly warrant the extensive development and implementation of strains typing techniques for this genus. Future research in this field should include development of techniques such as RAPD and phage typing. Further more, more comparative studies should be carried on evaluation of different techniques simultaneously to find out the suitable typing method for the more important Lactobacilli.

7.0 REFERENCES


1.0. INTRODUCTION

Milk and dairy products have long been considered as a "healthy food" and an essential component of Indian diet. We know that milk contains an array of bioactive peptides and is repleted with antioxidants, highly absorbable calcium, conjugated linoleic acid and other biologically active components. Furthermore, a wide range of products can be obtained from this nourishing liquid. In future, it will be possible, by introducing gene technology to the animal breeding, to change the milk composition to meet the requirements of modern human nutrition for healthy diet. Increasing scientific evidence has confirmed the connection between many chronic diseases and unbalanced diet. This message has already reached the consumers and food industry has responded. The consumers are increasingly becoming aware now about the relationship of health and diet. The market of so called functional foods is, therefore, expanding worldwide. Within this category, dairy products containing lactic acid bacteria have become of high importance.

Among the beneficial micro-organisms, Lactic Acid Bacteria (LAB) occupy a special significance and includes *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Pediococcus*, etc. The genus *Bifidobacterium* though phylogenetically unrelated to other LAB is often included in LAB on the grounds of similarities. Since antiquity, their ability to inhibit other micro-organisms capable of causing food borne illnesses or food spoilage has found application in a huge range of traditional food fermentation processes helping to increase availability, safety and variety of foods and making an invaluable contribution to the quality and length of human life. This has led to the development of functional foods with positive health attributes. In this context,
Probiotic lactic acid bacteria have been frequently used as active ingredients in functional foods such as bio-yoghurts, dietary adjuncts and other health related products. The health benefits for consumers attributed to probiotic bacteria can be grouped into two categories viz. Nutritional benefits and therapeutic benefits. Nutritional benefits include their role in bio-availability of calcium, zinc, iron, manganese, copper and phosphorus, increasing the digestibility of proteins in yoghurt and synthesis of vitamins. The therapeutic benefits include treatment of gastrointestinal disorders, hypercholesterolaemia, and lactose intolerance, suppression of pro-carcinogenic enzymes, immunomodulation and treatment of food related allergies.

Considerable advancements made in the field of genetic engineering of LAB especially the availability of genome sequences of probiotic strains of lactobacilli and bifidobacteria has opened myriad opportunities for the development of strains with safe and effective health promoting effects. However, food grade genetic modifications are required so that these bacteria can be directly used in dairy products.

2.0. FOOD-GRADE GENETIC MODIFICATIONS

Food-grade genetic modifications are the limited types of modifications that would be considered acceptable for food microorganisms with safety as the chief concern. A food-grade GMO must contain only DNA from the same genus and possibly small stretches of synthetic DNA. A broader definition is gaining acceptance and would allow the use of DNA from other genera of food microorganisms, provided that the donor belongs to the group of organisms referred to as 'generally recognized as safe' (GRAS). By this definition, a Lactococcus GMO contains DNA from Streptococcus thermophilus or other GRAS bacteria. With either definition, it is acceptable to use DNA from non-GRAS organisms during the construction of the GMO provided that this DNA is removed before the GMO is considered to be food-grade. Sensitive techniques such as DNA–DNA hybridization, Polymerase Chain Reaction and DNA sequencing can be used to confirm that the undesired DNA has been successfully removed. A number of genetic modifications which can be done in a food-grade manner as indicated below.

Genes can be deleted from a strain. This is especially relevant when a gene has an undesired property, for example the production of an undesirable metabolic end product or the destruction of a desirable component of the food. A gene in a strain can be replaced with the homologous gene from another strain. If a strain has a number of useful properties but a desirable enzyme activity is at too low a level, this can be corrected by introducing a more active copy of the gene from another strain. Genes can be inserted into a strain, for example genes that result in increased resistance to bacteriophage. Finally, the expression of a gene can be increased by increasing the number of copies in the cell, using food-grade cloning vectors.

3.0. TOOLS FOR FOOD-GRADE MODIFICATIONS
3.1. Homologous Recombination

Most of the food-grade genetic modifications described above are based on the natural ability of bacteria to perform homologous recombination. Simple techniques exist for plasmid curing and the resulting strains are food-grade. To facilitate the integration process, vectors with temperature-sensitive replication and an antibiotic resistance marker have been used.

3.2. Food-Grade Deletions

A food-grade deletion can be made of any dispensable gene of any bacterium using a relatively simple multistep process. Exceptions are strains into which DNA cannot be introduced and those that are incapable of homologous recombination. Deletions are made \textit{in vitro} either using conveniently located restriction enzyme recognition sites or by polymerase chain reaction. The plasmid containing the deletion is introduced into the strain of interest. Homologous recombination results in integration of the plasmid into the chromosome.

3.3. Food-Grade Gene Replacement

Replacement of a gene with another allele is done by deleting the undesired allele as described above and subsequently inserting the desired allele in a similar multistep process.

3.4. Food-Grade Integration into Chromosome

A simple variation of the gene replacement strategy allows the integration of any gene of interest into the chromosome. The gene to be integrated is inserted into a plasmid and flanked on both sides by at least 500 bp of DNA from the integration site. Integration of the plasmid and subsequent excision from the chromosome can result in a strain containing the gene of interest integrated into the chromosome.

3.5. Food-Grade Cloning Vectors

One simple way to increase the expression of a desirable gene is to increase the number of copies of the gene in the cell. This can usually be done by transferring the gene from the bacterial chromosome to a multicopy plasmid. Cloning vectors for \textit{E. coli} usually contain genes for antibiotic resistance and selection with antibiotics is used to kill plasmid-free cells. This is clearly not acceptable for a food-grade cloning vector.

A number of food-grade selectable markers have been developed. In principle, any gene that can be deleted from the chromosome can become a food-grade selectable marker provided that there are growth conditions under which this gene is essential and others under which this gene is dispensable. Inserting the gene onto a plasmid and growing under conditions where the gene is essential selects for the presence of the plasmid. A number of vectors for lactic acid bacteria use the \textit{lac} genes, required for the fermentation of lactose, as selectable marker. The \textit{lac} genes are essential when cells are grown with lactose as the sole carbon source but are dispensable when another carbon source is available. Many lactic acid bacteria produce bacteriocins such as nisin. Nisin based food grade vector ‘NICE system’ is extensively being used for expression of many heterologous proteins in \textit{Lactococcus}.

Currently, relatively few viable genetic tools are available for chromosomal manipulation of probiotic Lactobacilli. Ll.ltrB-directed invasion of target chromosomal genes in \textit{Lactobacillus johnsonii} and other probiotic bacteria have been attempted. The efficiency of
intron delivery is remarkably high, enabling intron-based chromosomal modifications without the need for genetic selection. This is crucial to the future use of this method for modification of commercial starter strains or probiotics. Moreover, the ability to stabilize genetic traits in the genomes of LAB will enable commercial suppliers to design more robust and efficacious starter cultures and probiotics. This in turn will benefit the various dairy products that employ these cultures.

The development of food-grade tools for efficient modification of LAB starter cultures and probiotic strains will enable development of novel, dairy-based, value-added products. Moreover the recent increase in available LAB genome sequences necessitates the development of specific and efficient genetic tools to characterize the multitude of genes available for study.

4.0. DESIGNING LACTIC ACID BACTERIA FOR FUNCTIONAL AND TECHNOLOGICAL BENEFITS

Lactic acid bacteria are used in the production of a large variety of foods and animal feeds. They produce lactic acid and contribute to the flavour, texture and shelf life of the food. Food fermentations are often done on an industrial scale following inoculation with appropriate numbers of lactic acid bacteria either produced in the factory or purchased from a commercial culture supply house. It is of utmost importance that the bacteria complete the fermentation process; otherwise large amounts of raw materials may be lost. One major reason for fermentation failure is the presence of bacteriophages, viruses that infect and kill bacteria. A typical bacteriophage of lactic acid bacteria can produce $10^6$ progeny in the same time that a single cell can produce eight progeny cells. Clearly, even a small number of infective bacteriophage particles can cause a serious problem for industrial fermentations. Lactic acid bacteria contain a number of mechanisms leading to bacteriophage resistance. The presence of individual mechanisms varies from strain to strain. Combining the best bacteriophage resistance mechanisms into superior industrial strains can be most effectively accomplished by genetic engineering. Since the genes are already contained in GRAS organisms, the resulting strains will be food-grade.

4.1. Starters for Better Flavour Development

The flavour of fermented food depends on the presence of desirable flavour compounds and the absence of undesirable flavour compounds. Often, the desirable flavour compounds are further degraded by the culture used to manufacture the food. This results in instability in the quality of the food and a shortening of the shelf life. Genetic engineering can be used to increase the production of the desired compounds and increase stability by prevention of their degradation. Increasing the production of a particular compound can be done by increasing the expression of the genes responsible for its synthesis, elimination of genes responsible for its degradation, or elimination of other biosynthetic pathways that consume a rate-limiting biosynthetic intermediate.

A good example of a flavour compound whose content in food can be changed using food-grade Genetically Modified Organisms (GMOs) is diacetyl, a major flavour component of butter and buttermilk. An important intermediate in the production of diacetyl is pyruvate. Inactivating genes coding for enzymes that use pyruvate as a substrate results in a significant increase in the amount of diacetyl produced by a culture. Likewise, increasing the expression of $\alpha$-acetolactate synthase—an enzyme involved in the conversion of pyruvate to diacetyl-increases diacetyl production. Finally, inactivation of diacetyl reductase, an enzyme that degrades diacetyl to acetoin, results in increased diacetyl levels owing to increased stability.
Combining these modifications in one strain has not yet been accomplished but should not be difficult using the food-grade techniques described here.

Another example is the flavour peptides formed during the degradation of casein in cheese production. Some of the peptides produced by the action of the proteolytic enzymes present in the cheese process have a bitter taste and can result in an unpleasant-tasting cheese. The formation of these peptides can be blocked by inactivating the aminopeptidase responsible, or by increasing the expression of an aminopeptidase that degrades the bitter peptide. Food-grade GMOs are currently being studied to determine which aminopeptidases are responsible for the bitter peptides and which can eliminate them. This knowledge will allow the construction of improved industrial strains for cheese manufacturing.

4.2. Starters for Better Food Preservation

One major role of lactic acid bacteria is food preservation. This is a consequence of the lactic acid and a variety of bacteriocins produced by the lactic acid bacteria. Modification of the genes involved in lactic acid production can lead to a quicker and more efficient lactic acid production. Likewise, the genes for production and resistance to bacteriocins can be moved between strains in a food-grade manner.

4.3. LAB for Producing Mild Shelf Stable Yoghurt / Dahi

One of the pertinent problems in yoghurt / dahi is the post-acidification, which accounts for high acid and bitter taste of the yoghurt and hence can adversely affect the organoleptic quality of the product. This problem can be tackled by regulating the growth and maintenance of _L. delbrueckii_ subsp. _bulgaricus_. One such strategy can be the introduction of mutations in the ‘lac’ gene to create ‘lac’- minus mutants lacking β-galactosidase activity as well as by making ‘proteinasé’ defective mutants. Such lac- and Prt- mutants were not able to grow in milk as a single culture without the supplementation of glucose and peptone. On the other hand when grown in mixed cultures with a lactose fermenting _S. thermophilus_, ‘lac-’ _L. delbrueckii_ subsp. _bulgaricus_ were able to grow in spite the absence of glucose. Once the fermentation process is terminated, growth and lactose metabolism of _S. thermophilus_ and _L. delbrueckii_ subsp. _bulgaricus_ cease resulting into mild yoghurt with enhanced shelf life. The same approach can also be applied to produce mild acidic dahi with aroma which is an extremely popular dairy food served with a place of pride in Indian culinary.

4.4. Starters for Creamier Yoghurt

The texture of yoghurt and other fermented milk products results from the interaction of a number of factors. One factor is the extracellular polysaccharide produced by the bacteria in the starter culture used. The relevant genes have been identified from several strains and GMOs with altered texture-producing properties are being constructed. The use of EPS producing strains increases the viscosity of yoghurt and dahi and decreases susceptibility to syneresis. Further advancements in the understanding of the functions of different glycosyl transferases encoded by the gene would allow the modification of existing EPS constructs. These modifications in starters could be produced by exchanging DNA between strains of a species without the intervention of vector and marker genes used in the genetic shaping of the strain.

4.5. Starters for Controlled Lysis and Flavour Development

Lactococci have very little biosynthetic capacity e.g. for the production of amino-acids. These bacteria utilize amino-acids from milk proteins through the concerted action of proteinases, peptidases and peptide uptake systems. The products of proteolysis are important
cheese flavour components themselves or converted into flavours through chemical or enzymatic modifications.

As all peptidases and a number of other flavour generating enzymes of *L. lactis* are located intracellularly, it is possible to enhance starter cell lysis under industrial fermentation conditions. The lactococcal autolysin gene AcmA provokes cell lysis during stationary phase and manipulating its activity or expressing bacteriophage lysin genes in *L. lactis* in a controllable way holds promise for improving cheese flavour. One of the most pertinent issues is whether to affect rapid and complete lysis or to aim for a gradual time dependent starter lysis. For non cheese applications, it might be preferable to inhibit cell lysis, to prevent extensive flavour formation, thus preserving product’s freshness. The detailed knowledge of the factors involved in starter cell lysis as well as of the components of proteolytic system will lead to developing strains for improved and tightly controlled flavour formation in the near future.

4.7. **Starters for production of L-alanine by Metabolic Engineering**

*Lactococcus lactis* subsp. *lactis*, one of the best studied starter cultures having a single fermentation metabolism converts lactose or glucose to L-lactate as the chief end product with the intervention of L-lactic dehydrogenase which converts the intermediate pyruvate to L-lactate. This step can now be surpassed by rerouting pyruvate to alanine. In this strategy, the L-alanine dehydrogenase gene (alaDH) of a *Bacillus* spp. has been cloned and over-expressed in *Lactococcus lactis* subsp. *lactis* under the control of a nisin inducible food grade promoter system. The application of such a *Lactococcus lactis* subsp. *lactis* biocatalyst for converting cheap sugars into L-alanine presents interesting possibilities for production of amino-acids in a microorganism for application in foods. It is also now feasible to genetically engineer a yoghurt or dahi starter using the aforesaid strategy to produce L-alanine instead of L-lactate during the processing of milk into yoghurt which will have sweeter alanine taste. This is an example of using trans-species genetic engineering in developing novel starter strains for newer product development.

4.8. **Probiotic Cultures for Clinically Safer Functional Dairy Foods**

*Lactobacillus johnsonii* LaI is a well characterized probiotic lactic culture used extensively in commercial preparation of probiotic foods due to its considerable health beneficial effects and positive immunomodulating effect on the host. It is cultured in milk where it ferments lactose into a racemic mixture of D- and L-lactate in 60:40 per cent ratios. Although, the presence of D-lactate in milk fermented by LaI and the ability of the strain to produce D-lactate after ingestion does not pose any problem to a vast majority of the adult population, this situation can cause D-lactic acidosis and encephalopathy in patients suffering from short bowel syndrome and intestinal failures. Hence, it would be appropriate to produce a non-D-lactate producing derivative of LaI that would help patients in reconstituting their intestinal microflora after antibiotic treatment. Another application of a non-D-lactate producing LaI strain would be in the nutrition of infants for building up and regulating their intestinal flora since new borne infants fail to completely metabolise ingested or *in situ* produced D-lactate due to live immaturity.

One of the feasible approaches to tackle the problem of D-lactate induced acidosis attributed to LaI is by introducing a small *in vitro* generated deletion into the coding sequence of NAD^+^ dependent *ldh D* (D-lactate dehydrogenase) sequence that is responsible for the production of D-lactate from pyruvate in LaI, thereby, inactivating the functionality of the gene. A copy of this truncated gene can genetically replace the genomically located original *ldhD* gene of LaI. Thus, the new construct is equivalent to LaI with the exception of a small DNA fragment missing within the *ldhD* gene. The new strain effectively rerouted pyruvate to
L-lactate and does not produce D-lactate at all. This technology based on inactivation of ladhD gene in LaI does not pose any safety concerns and assures the preservation of the desired health promoting attributes of the probiotic strain.

4.9. Overexpression of Lactase for Tackling Lactose Intolerance

Lactose intolerance is perceived by consumers to be a significant and widespread problem. Efforts to alleviate symptoms associated with lactose intolerance have had marginal success. As the lactic acid bacteria are associated both with dairy foods and human intestinal tract, they are ideal candidates for over expression of β-galactosidase. Efforts are going on in several laboratories to create a food grade high copy replicon for lactose overexpression in probiotic lactobacilli. Dairy Foods Research Center in Raleigh, NC is investigating overexpression of lactase in various lactic acid bacteria.

4.10. Lactic Acid Bacteria as Oral Vaccines

By applying genetic engineering techniques, it is possible to express antigens from potential pathogens like malarial parasite, *V. cholerae* and Hepatitis B virus either on their surface or extracellularly or intracellularly which can eventually elicit antibody production in the consumers fed with such modified cultures to protect them against such pathogens.

5.0. LACTIC ACID BACTERIA GENOME PROJECTS

Realizing the practical significance of LAB, a number of representative spp. of LAB have also been sequenced e.g. *L. lactis* IL1403, MG1363, *L. acidophilus*, *L. johnsonii* La1, *Bifidobacterium longum*, etc. and many more are still being sequenced by Dept. of Energy-JGI in collaboration with LABGC. The genomics will help to carry on with comparative and functional genomic analyses of LAB. Genome sequence will provide a critical view of the genetic adaptation to different ecological niches that occurred during their evolution. Outcome of comparative genomics will provide information on conserved and unique genetic functions in LAB that enable core functions like lactose utilization, proteolytic activity, survival at low pH, production of antimicrobials and adaptation to milk environment. Genomic maps will particularly identify “islands of adaptability” critical to the survival and functionality of probiotic organisms in their corresponding habitats.

Genome sequences will revolutionize scientist’s ability to control and exploit beneficial attributes of LAB used in dairy manufacturing and delivered through dairy products and will also help food scientists to enhance preservation and safety of fermented foods. Sequence data will foster newer methods to discriminate and evaluate strains of interest to the dairy industry. Genetic makeup of probiotics will help in prevention and treatment of a variety of gastrointestinal disorders. Future functional genomics (transcriptomics, proteomics, and metabolomics) will aid in defining molecular nature of metabolic networks within LAB. This knowledge aided by development of novel food grade genetic tools will usher in a new era whereby LAB strains are readily and specifically tailored for fermentation and probiotic rationales.

6.0. GMOs AND BOTTLENECKS IN THEIR FOOD APPLICATION

Although, various strains of lactic acid bacteria have been improved by genetic engineering, there is still very limited application of these GMO strains. The problems include lack of robust methods for risk assessment, lack of knowledge on gene transfer systems and undesired side effects of genetic modifications and unpredictability of effect of spreading the modified bacteria in nature e.g. after passage through a human being. Many efforts have been directed to develop tools for self cloning to ensure that no heterologous DNA is present in the GMO to apply for food fermentation or as a probiotic. Consent for application of GMOs depends on exact regulation of GMOs in various countries. Novel methods are also being developed for generation of food grade genetically modified bacteria by site specific
recombination, including site specific DNA resolution of non-food grade DNA used during construction. Use of genomic tools such as DNA microarrays can contribute to devising robust risk assessment procedures which are required by food manufacturers and consumers. Such developments will facilitate food applications of GMOs with improved industrial properties but even more importantly with clear consumer benefits.

7.0. CONCLUSIONS

The extensive knowledge that has now accumulated about the physiology and genetics of LAB has led to detailed understanding of several traits of these relatively simple bacteria. With the advancements made in food grade genetic engineering tools and genomics of lactic acid bacteria, it has now become possible to design LAB for improved as well as new beneficial functions to meet the requirements of consumers.

8.0. SUGGESTED REFERENCES


1.0. INTRODUCTION

The human gastrointestinal tract (GIT) is a complex ecosystem, which contains more than 400 species of bacteria. Some are beneficial, whereas others are potentially dangerous. The mechanisms regulating the number, type and metabolic activity of this diverse array of microbes are quite complex. However, in general, beneficial bacteria can help prevent the pathogenic activity of some invading microbes. These beneficial bacteria include *Lactobacillus* and *Bifidobacterium*. Both of these bacteria are among the indigenous flora in the human gastrointestinal tract, with bifidobacteria dominating during infancy. However, their number diminishes with age, the use of antibiotics, stress, alcohol consumption and other factors. To boost the beneficial attributes of the normal flora, it is theorized that consumers can reintroduce large numbers of these bacteria (probiotics) to their GIT through oral consumption, thus strengthening the body’s defense against potentially harmful bacteria. Metabolic activity by these probiotic cultures, as well as their competitiveness, can positively influence the ratio of desirable bacteria to undesirable bacteria.

2.0. WHY THE INTEREST IN PROBIOTICS?

For centuries folklore has suggested that fermented dairy products containing probiotic cultures are healthful. A list of organisms primarily used as probiotics is detailed in Table 1. Recent controlled scientific investigation has supported some of these traditional views, suggesting the value of probiotics as part of a healthy diet. In addition, the emergence of some new public health risks suggests an important role for effective probiotics. The ability of probiotic bacteria to support the immune system could be important to the elderly or other people with compromised immune function. Some infections, once thought self-limiting or readily treatable with antibiotics, are now recognized as more serious health threats. Prevention of infections before they occur is clearly the better alternative. Vaginitis used to be considered just an annoyance. Now we know it is associated with low birth weight and increased risk of sexually transmitted diseases. New food-borne pathogens have emerged as prevalent and life threatening, including Shiga-like *Escherichia coli* strains. Multiple antibiotic resistances are a continual threat in the battle against once-treatable infections. And in non-industrialized nations, infections such as rotavirus claim the lives of hundreds of thousands of infants yearly. Probiotics may be the avenue to provide a safe, cost-effective, "natural" approach that adds a barrier against microbial infection. Some of the reported benefits of probiotic organisms are summarised in Table 2.
3.0. PROBIOTICS IN FOODS

As there exists a strong inter-relation between the microbial ecology of the gut and the type of food intake, there has been an upsurge of interest in the recent years in health promotion and disease prevention by the incorporation of beneficial bacteria into foods to counteract harmful organisms in the intestinal tract. The Japanese food industry has pioneered R&D in the area of 'functional' foods in general and 'probiotic' foods in particular, and has one of the largest growing markets for such foods. The total sales volume of the Moringa Milk Ind. Co. Ltd. located in Tokyo for fiscal 1997 reached approximately 3.41 billion dollars (Martin, 1998). The Australian probiotic yoghurt segment accounts for 15 per cent of all yoghurt produced in the country and are growing at a rate of 25 per cent compared with total yoghurt, which are experiencing a growth of 3.5 per cent (Jones, 1999). A market survey suggests that the probiotic dietary supplement sales were $28.7 million in the US in 2000.

3.1. Probiotics in Dairy Products

In the recent years manufacturers have addressed themselves to the theme of 'Dairy Food in Health' and have been seeking to promote the role of fermented milk products in positive aspects of human nutrition and health. The most prominent amongst these products are acidophilus yoghurt (Germany), AB milk products (Denmark), bifidus milk (Germany), bifidus milk with yoghurt flavour (UK), Bifilact (USSR), Biokus (Czechoslovakia), Mil-Mil (Japan), Progurt (Chile), etc. (Tamime et al. 1995). With a view of overcoming the unfavourable flavour aspects of traditional fermented products, R&D work has been carried out to develop non-fermented range of dairy products such as sweet acidophilus bifidus milk (USA, Japan), sweet bifidus milk (Japan, Germany) and ice-cream (India and USA).

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus</td>
<td>La2, La5 (also known as La1), Johnsonii (La1; also known as Lj1), NCFM, DDS-1, SBT-2062</td>
</tr>
<tr>
<td>L. bulgaricus</td>
<td>Lb12</td>
</tr>
<tr>
<td>L. lactis</td>
<td>La1</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>299v, Lp01</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>GG, GR-1, 271, LB21</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>SD2112 (also known as MM2)</td>
</tr>
<tr>
<td>L. casei</td>
<td>Shirotta, Immunitass, 744, 01</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>CRL 431</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>RC-14</td>
</tr>
<tr>
<td>L. helveticus</td>
<td>B02</td>
</tr>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>-</td>
</tr>
<tr>
<td>B. longum</td>
<td>B536, SBT-2928</td>
</tr>
<tr>
<td>B. breve</td>
<td>Yakult</td>
</tr>
<tr>
<td>B. bifidus</td>
<td>Bb-11</td>
</tr>
<tr>
<td>B. essensis</td>
<td>Danone, (Bio Activia)</td>
</tr>
<tr>
<td>B. lactis</td>
<td>Bb-02</td>
</tr>
<tr>
<td>B. infantis</td>
<td>Shirotta, Immunitass, 744, 01</td>
</tr>
<tr>
<td>B. laterosporus</td>
<td>CRL 431</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Krishnakumar and Gordon (2001)
The concept of incorporation of probiotic cultures has recently been extended to cheese varieties also in some western countries. Hoping to take advantage of the consumer interest in the improved therapeutic and nutritional attributes of such cultures, two varieties of cheese with added bifidobacteria have been introduced in the Europe-medium mature cheddar and reduced fat cheddar. Some probiotic cultures (e.g. *Lactobacillus rhamnosus*) produce antibacterial substances that act specifically against undesirable microorganisms such as clostridia. The use of these organisms is a possible replacement of nitrate addition to suppress the growth of gas-formers in cheeses like Edam and Gouda. Thus, they promote the natural preservation, with minimized use of chemical preservatives.

**Table-2. Selected Health-Related Attributes of Probiotics Used in Fermented Milks**

<table>
<thead>
<tr>
<th>Type of Effects</th>
<th>Published Health Benefits</th>
<th>Established Health Benefits in Humans*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological effects</td>
<td>• Bile, pH resistant strains, enzymatic activity&lt;br&gt;• Production of bacteriocins&lt;br&gt;• Antagonistic effect against pathogens</td>
<td></td>
</tr>
<tr>
<td>Actions on the digestive tract</td>
<td>• Enhancement of lactose digestion in lactase-deficient people&lt;br&gt;• Prevention of intestinal disturbances&lt;br&gt;• Adherence to human intestinal cell line cultures (in vitro)&lt;br&gt;• Stimulation of intestinal immunity in animal models&lt;br&gt;• Stabilization of Crohn’s disease&lt;br&gt;• Regulation of intestinal motility</td>
<td>Enhancement of lactose digestion in lactase-deficient people</td>
</tr>
<tr>
<td>Alterations of the intestinal microflora</td>
<td>• Balance of intestinal bacteria&lt;br&gt;• Increase in fecal bifidobacteria&lt;br&gt;• Decrease of fecal enzyme activities thought to play a role in conversion of procarcinogens to carcinogens&lt;br&gt;• Decrease of fecal mutagenicity&lt;br&gt;• Colonization of intestinal tract</td>
<td>• Increase in the number of fecal bifidobacteria&lt;br&gt;• Decrease of certain fecal enzyme activities&lt;br&gt;• Alleviation of lactose intolerance symptoms</td>
</tr>
<tr>
<td>Actions on diarrhea</td>
<td>• Prevention and/or treatment of acute diarrhea&lt;br&gt;• Prevention and/or treatment of rotavirus diarrhea&lt;br&gt;• Prevention of antibiotic-associated diarrhea&lt;br&gt;• Treatment of relapsing Clostridium difficile diarrhea&lt;br&gt;• Treatment of persistent diarrhea</td>
<td>• Decreased duration of rotavirus diarrhea&lt;br&gt;• Treatment of persistent diarrhea in children</td>
</tr>
<tr>
<td>Systemic effects</td>
<td>• Immune enhancer&lt;br&gt;• Stimulation of phagocytic activity&lt;br&gt;• Stimulation of γ interferon production by human blood mononuclear cells in culture&lt;br&gt;• Reduction of hypertension in animal models and in humans&lt;br&gt;• Antagonism against carcinogenic bacteria</td>
<td></td>
</tr>
</tbody>
</table>
Developments in Probiotic Foods

- Beneficial effects in superficial bladder and colon cancer
- Reduction in the risk of various cancers
- Alleviation of clinical symptoms in children with atopic dermatitis
- Reduction of serum cholesterol

More than one publication with no conflicting data in humans


4.0. PREBIOTICS AND SYNBIOTICS

Prebiotics are defined as “non-digestible food that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Some oligosaccharides, because of their chemical structure, are resistant to digestive enzymes and therefore pass into the large intestine, where they become available for fermentation by saccharolytic bacteria (Shah, 2001). Such compounds, which are either partially degraded or not degraded by the host and are preferentially utilized by bifidobacteria as a carbon and energy source, are referred to as “bifidogenic factors.” Some bifidogenic factors of commercial significance include fructooligosaccharides, lactose derivatives such as lactulose, lactitol, galacto-oligosaccharides, and soybean oligosaccharides (O’Sullivan, 1996). (The same worker stresses that resistant starch and non-starch oligosaccharides are classified as colonic foods, but not as prebiotics because certain beneficial bacteria do not metabolise them). The majority of yogurts marketed in Australia, the United States, and Europe in recent years contain probiotic bacteria and some form of prebiotics. Such combination products are referred to as “synbiotics.” Japan is the world leader in probiotic and prebiotic products.

The R&D Division of Orafti Active Food Ingredients (USA) has concluded after intensive work, that inulin and oligofructose are the only clinically proven prebiotics. Because inulin and oligofructose are dietary fibers, they also provide the nutritional benefits normally associated with dietary fibers. Both ingredients have been shown to increase calcium absorption by the body and improve bone-mineral density. Attempts at developing foods containing these prebiotics as well as certain probiotics are under way so as to reap benefits derived from them. One product has been developed specifically to enhance calcium absorption. It combines different chain lengths of inulin to aid fermentation along the entire length of the colon, thus providing even more sites for absorption of calcium.

5.0. FUNCTIONAL AND PROBIOTIC FOODS EXPAND BEYOND DAIRY

While the quest to scientifically prove the long-standing hypothesis that dairy foods are the ideal vehicle for delivering viable beneficial bacteria to the human body, newer food products containing new and specific strains of propitious organisms are being continually sought. Many European food companies use grains and candy as a means to expand the probiotic trend. For instance, German firm Schneekoppe markets a probiotic muesli breakfast product, while toffees from Zile Function™ brand in Switzerland and Biolabor Toffitis™ brand in Germany are breaking ground as the first probiotic hard candies.

Probiotics for persons with different blood groups are marketed in the west. One example is a unique formula offering ten strains of probiotic bacteria (Bifidobacterium infantis, B. longum, B. bifidum, B. breve, L. plantarum, L. casei, L. salivarius, L. acidophilus, L. rhamnosus and Streptococcus thermophilus) and meant for people with Type A blood. These organisms are cultured in a base of fruits, vegetables and herbs, and then left in their original
culturing medium to provide the full range of benefits available from the flora and the beneficial foods. The product claims to have 2 billion live cells. Similarly D'Adamo 4 Your Type Probiotic AB is a product meant for people with Type AB blood. The organisms in this are L. casei, L. plantarum, L. salivarius, L. acidophilus, L. rhamnosus, S. thermophilus, B. bifidum, B. infantis, B. longum and B. breve.

6.0 PROBIOCAP FOR PROBIOTICS

Balchem Encapsulates, in collaboration with Institut Rosell uses microencapsulation to coat probiotics to protect them from moisture, high humidity and acidity. The coating allows the probiotic to pass through the gastrointestinal tract without being destroyed by gastric juices and is triggered for release in the intestine based on pH conditions. This technology, marketed as “Probiocap” may triple shelf life for probiotics in certain applications. It was applied to several strains of probiotics from Institut Rosell for use in food applications, including L. acidophilus, L. rhamnosus and B. longum. Encapsulation technology was also applied to two strains geared towards animals, specifically Pediococcus acidilactici and Enterococcus faecium. Gentle Digest™ containing microencapsulated lactobacillus spores (which are resistant to heat and stomach acid) and chicory (serving as a prebiotic) is a commercial product manufactured and marketed for pet animals.

7.0 FREEZE DRIED PROBIOTICS

Freeze-dried pharmaceutical preparations containing bifidobacteria alone or in combination with other organisms are also commercially marketed in several countries. They are primarily used for the treatment of gastrointestinal disorders (e.g. diarrhoea and side-effects of antibiotic and radiation therapy), as special preparations for certain liver disorders and as a protective measure against imbalance in the intestinal microflora (Kurmann, 1993). Some examples of these pharmaceutical preparations are Bifidogene (France), Bifider (B. bifidum, Japan), Infloa Berna (L. acidophilus and B. infantis, Switzerland), Eugalan Toepfer Forte and Lactopriv (bifidobacteria, Germany), Liobif (B. bifidum, Yugoslavia), Lyobifidus (B. bifidum, France) and Life Start II (B. bifidum, USA).

8.0 FINDING SOLUTIONS TO UNANSWERED QUESTIONS

Many attempts are being made to arrive at a better understanding about the intricacies of the gut microflora and their benefits. The Food, GI-tract Functionality and Human Health Cluster PROEUHEALTH has been formed with 64 research partners from 16 European countries in the quest to obtain greater knowledge of the role of the intestinal microbiota in human health and disease and to develop new functional foods and therapies. The cluster aims to provide

- A clearer understanding of the relationships between food, intestinal bacteria and human health and disease
- New molecular research tools for studying the composition and activity of the intestinal microbiota
- New therapeutic and prophylactic treatments for intestinal infections, chronic intestinal diseases, and for healthy ageing
- A molecular understanding of the underlying mechanisms of the inhibition of pathogenic bacteria
- A molecular understanding of immune modulation by probiotic bacteria and examination of probiotics as vaccine delivery vehicles
- Process formulation technologies for enhanced probiotic stability and functionality
- Biosafety evaluation of probiotics used for human consumption
• Commercial opportunities for food and pharmaceutical industries

The projects will, besides dealing with the molecular aspects and genetic engineering of probiotic flora will make special attempts at developing new probiotic foods, particularly for special and vulnerable groups of the population.

8.0. LABELLING OF PROBIOTIC FOODS

The United States Food and Drug Administration (FDA) suggested that manufacturers should use the term direct-fed microbiol instead of probiotics. The shelf life and species of probiotic included in food products are generally listed on product labels. The specific strain and levels of viable cells, however, are not typically shown. As the probiotic industry matures and the consumer requests more detailed information, labelling will likely become more routine, and even, mandatory. A Joint FAO/WHO Working Group has suggested a few guidelines (Fig. 1) for the evaluation of probiotics in food.

9.0. CONCLUSIONS

With the current focus on disease prevention and the quest for optimal health, the use of probiotic market potential is enormous. Already, probiotic food products are popular in Japan, USA, Europe and Australia. The role of probiotics in improving intestinal microbial balance, prevention of cancer, lowering of high cholesterol levels and improving bioavailability of dietary calcium, are the most promising areas for further research. There is also a greater need for selection and developing of starter cultures, especially the mixed cultures, for preparing new probiotic products.

10.0. REFERENCES


http://www.balchem.com


Strain identification by phenotypic and genotypic methods
- Genus, species, strain
- Deposit strain in international culture collection

Safety assessment
- In vitro and/or animal
- Phase 1 human study

Functional characterization
- In vitro tests
- Animal studies

Double blind, randomized, placebo-controlled (DBPC) phase 2 human trial or other appropriate design with sample size and primary outcome appropriate to determine if strain/product is efficacious

Preferably second independent DBPC study to confirm results

Phase 3, effectiveness trial is appropriate to compare probiotics with standard treatment of a specific condition

Probiotic Food

Labeling
- Contents – genus, species, strain designation
- Minimum numbers of viable bacteria at end of shelf life
- Proper storage conditions
- Corporate contact details for consumer
Fig-1: Guidelines for the Evaluation of Probiotics for Food Use
1.0. INTRODUCTION

Cheese, the nature’s wonder food and the classical product of biotechnology, is a highly nutritious food with good keeping quality, enriched pre-digested protein with fat, calcium, phosphorus, riboflavin and other vitamins, available in a concentrated form. Cheese, the most important category of fermented foods, has been reported to have therapeutic, anticholesterolemic, anticarcinogenic and anticariogenic properties beyond their basic nutritive value. They, contributing to a variety in our gustative desire, have been recognized to provide important nutrients and considered superior over non-fermented dairy products in terms of nutritional attributes as the micro flora present produce simple compounds like lactic acid, amino acids and free fatty acids that are easily assimilable. Some of the cheese flora has been reported to inhibit the growth of certain toxin-producing bacteria in the intestine. Cheese is appreciated by consumers for the great interest and variety it adds to the eating experience. It has an excellent image, being perceived as healthy, natural and nutritious. Cheese has, therefore, been truly classified as a value added product and is consumed in various other forms like dietetic foods, snacks fast foods and spreads. With the triumphal achievement in the arena of dairy science and biotechnology since the last two decades, lot of advancement have been made in cheese technology to provide ease in its processing and to gift the mankind with novel kind of cheese with improved flavour and textural characteristics achieved within short ripening period.

The ripening of cheese is a complex process of concerted biochemical changes, during which a bland curd is converted into a mature cheese having the flavour, texture and aroma characteristics of the intended variety. Ripening is an expensive and time-consuming process, depending on the variety, e.g. Cheddar cheese is typically ripened for 6-9 months while Parmesan is usually ripened for two years. Extended ripening period involves increased cost due to refrigerated storage, space, labour apart from considerable loss in weight and higher capital cost. Acceleration of cheese ripening can also be a means of increasing the production of cheese in developing countries where investment in storage facility can be a limiting factor.

2.0. PROSPECTS FOR ACCELERATED RIPENING

There is undoubtedly an economic incentive to accelerate the ripening of low-moisture, highly-flavoured, long-ripened cheeses. Although consumer preferences are tending towards more mild-flavoured cheeses, there is a considerable niche market for more highly flavoured products. While the ideal might be to have cheese ready for consumption within a few days, this is unlikely to be attained and in any case it would be necessary to stabilize the product after reaching optimum quality, eg, by heat treatment, as is used in the production of enzyme-modified cheese. Although the possibility of using exogenous (non-rennet) proteinases, and in
some cases peptidases, attracted considerable attention for a period, this approach has not been commercially successful, for which a number of factors may be responsible:

- Primary proteolysis is probably not the rate-limiting reaction in flavour development
- The use of exogenous enzymes in cheese is prohibited in several countries
- Uniform incorporation of enzymes is still problematic and the use of encapsulated enzymes is not viable at present. Because it can be easily incorporated into cheese curd, is an indigenous enzyme active in natural cheese and has narrow specificity, producing non-bitter peptides, plasmin may have potential as a cheese ripening aid; however, at present it is too expensive but its cost may be reduced via genetic engineering.

Attenuated cells appear to have given useful results in pilot-scale experiments but considering the mass of cells required, the cost of such cells would appear to be prohibitive for commercial use, except perhaps in special circumstances. Selected peptidase-rich, Lac-/Prt- Lactococcus cells added as adjuncts have given promising results but further work is required and they may not be cost-effective.

The selection of starter strains according to scientific principles holds considerable potential. Such selection procedures are hampered by the lack of information on the key enzymes involved in ripening. Preliminary studies on the significance of early cell lysis have given promising results and further studies are warranted; bacteriocin-induced lysis appears to be particularly attractive. The ability to genetically engineer starters holds enormous potential but results to date have been disappointing. Again, identifying the key enzymes in ripening is essential for the success of this approach. It is hoped that current research on cheese ripening will identify the key sapid compounds in cheese and hence the critical, rate-limiting enzymes. Genetic manipulation of Lac-/Prt-adjunct Lactococcus will also be possible when the key limiting enzymes have been identified. Adjunct starters, especially lactobacilli, hold considerable potential. It appears to be possible to produce cheese of acceptable quality without lactobacilli but they appear to intensify (Cheddar) cheese flavour and offer flavour options.

The volume of literature published on starter adjuncts has been rather limited to date; further work will almost certainly lead to the development of superior adjuncts. There is the obvious possibility of transferring genes for desirable enzymes from lactobacilli to starter lactococci. At present, elevated ripening temperatures (15°C) offer the most effective, and certainly the simplest and cheapest, method for the accelerating ripening of Cheddar, which is usually ripened at an unnecessarily low temperature; however, this approach is less applicable to many other varieties for which relatively high ripening temperatures are used at present. The key to accelerating ripening ultimately rests on identifying the key sapid compounds in cheese.

### 3.0. ACCELERATED RIPENING OF CHEESE: UNDERSTANDING BASIC ETHICS

Understanding the reaction mechanisms involved in cheese ripening is a pre-requisite to the development of suitable methods for accelerated cheese ripening. Obviously, the duration of ripening depends on the intensity of flavour sought in the finished product and many varieties are marketed at different stages of maturity, sometimes under different names. The rate of ripening is very strongly affected by the moisture content and aw of the cheese: low-moisture cheeses ripen slowly but are also more shelf-stable than high moisture varieties,
which ripen rapidly. Traditionally, cheese a rind (a low-moisture surface layer) during. Owing to the cost of inventory and controlled atmosphere ripening rooms, the loss of cheese mass through evaporation and other factors and the risk that the quality of the mature cheese may be lower than desirable, cheese ripening is a relatively expensive process and there are economic and technological incentives to accelerate the rate of ripening and reduce costs. However, the quality of the mature cheese must not deteriorate as a result of such accelerating procedures; it is relatively easy to accelerate many or most of the reactions involved in cheese ripening but it is much more difficult to accelerate the complex set of reactions in a balanced way so as to maintain or perhaps to improve cheese quality.

In order to accelerate ripening, it is highly desirable to understand the microbiology and biochemistry of cheese ripening; otherwise, attempts to accelerate ripening will be empirical. Although the ripening of cheese is still not fully understood, very considerable progress has been made on many aspects. The three primary events in cheese ripening, glycolysis, lipolysis and proteolysis, are now quite well understood at the molecular level; the secondary changes are understood in general terms. The texture of cheese is due primarily to its composition (protein, lipids, water, calcium and pH), to changes in pH during ripening (due to catabolism of lactic acid and the production of ammonia) and proteolysis which breaks the continuous protein network. The three primary reactions are also major contributors to, and are certainly prerequisites for, the basic flavour of cheese and are responsible for some major off-flavours, eg, bitterness and rancidity. However, the finer points of cheese flavour result from secondary and even tertiary reactions in which products of the primary reactions are modified, eg, deamination, transamination and decarboxylation of amino acids, cleavage of amino acid side chains by C-C, C-S and C-N lyases, b-oxidation of fatty acids, catabolism of lactic and citric acids and some synthetic reactions, eg, production of esters and thioesters. Several hundred sapid compounds have been isolated from cheese and identified.

The rate and pathways of cheese ripening are determined by:

- Composition of the cheese, especially moisture, pH and NaCl (aw),
- Microflora: primary and secondary starters and adventitious microorganisms from the milk (especially raw milk) and the environment (during curd manufacture and cheese ripening),
- Enzymes: indigenous enzymes from the milk, endogenous enzymes from the starter and adventitious microflora, and exogenous enzymes, especially from rennet,
- Temperature and, for some varieties, humidity.
- Time

Obviously, the control, modification and acceleration of cheese ripening involve one or more of these parameters, some of which are easily adjustable.

4.0. METHODS OF ACCELERATED RIPENING

The characteristic aroma, flavour and texture of cheese develop during the ripening of cheese curd through the action of numerous enzymes derived from the milk, the coagulant, primary and, usually, secondary starter microorganisms and adventitious non-starter bacteria. Ripening is a slow and consequently an expensive process that is not fully predictable or controllable. Therefore, there are economic and possibly technological incentives to accelerate ripening. The principal methods by which this may be achieved are: an elevated ripening
temperature, exogenous enzymes, modified starters and adjunct cultures. At least some of the methods used to accelerate ripening may also be used to modify flavour and in effect to create new varieties/ variants. Slow flavour development and low flavour intensity are major problems with reduced-fat cheese and the methods used to accelerate ripening in general are applicable to low-fat cheese also. Several promising developments have emerged for rapid flavour development in cheddar cheese and other varieties. They can be schematically represented as follows (Table 1):

**Table 1: Different Methods for Accelerated Cheese Ripening**

<table>
<thead>
<tr>
<th>Treatments to Cheese Milk</th>
<th>Treatments to Cheese Curd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased microbial load (starter adjuncts)</td>
<td>Proteolytic enzymes</td>
</tr>
<tr>
<td>Modified starters</td>
<td>Lipolytic enzymes</td>
</tr>
<tr>
<td>Thermal shocked cells</td>
<td>Enzyme cocktails</td>
</tr>
</tbody>
</table>

Accelerated  
cheese  
ripening

Freeze shocked cells  
Lysozyme treated cells  
Mutant strains  
ß-galactosidase  
Microencapsulated ripening agents  
Liposomal enzymes  
Liposomal cell free extracts

4.1. **Adjunct cultures**

Adjunct cultures are specifically selected strains, which are intentionally added to accelerate ripening of full fat cheese and for flavour enhancement of low fat cheese. Adjunct cultures reportedly decrease bitterness and contribute desirable flavour compounds. Significant contribution of non-starter lactic acid bacteria in increasing the rate of casein degradation and flavour development received much research attention. Addition of different *Lactobacillus* spp. (*L. casei* and *L. plantarum*) to cheddar cheese milk to a level of $10^5$-$10^6$ cfu/ml increased the levels of free amino acids to attain highest flavour scores. Augmentation of starter culture with *L. casei* had definite and positive influence on the flavour; body and texture of buffalo milk Cheddar cheese. The flavour development and biochemical changes in buffalo milk Cheddar cheese is faster when *L. casei* is supplemented with cheese. With the objective of improving the control of accelerated cheese ripening, researchers are investigating the role of *Lactobacillus* bacteria and the enzymes involved in cheddar cheese ripening. Considerable effort is being directed towards the identification of bitter and astringent peptides in cheese and the characterisation of aminopeptidases, which have an essential role in eliminating bitterness.

4.1.1. **Lactobacillus Culture as Adjuncts**: The development of a more intense flavour in raw milk cheese has simulated interest in *Lactobacillus* cultures for addition to pasteurized milk as a means of manipulating the chemical and sensory characteristics of modern day commercial Cheddar. Peptidolytic strains of NSLAB may, therefore, be considered for use as adjuncts in cheese making both to manipulate the overall flavour profile of the cheese and to accelerate the rate of flavour formation. Numerous experiments have been carried out to assess
the suitability of various strains of bacteria as adjuncts to the starter culture with the objective of accelerating the rate of development and the intensity of Cheddar cheese flavour.

4.1.1.1. Early Studies on Adjunct Cultures: Significant observations were established from some of the early studies on the ability of various strains of lactobacilli to perform as adjuncts. One of the first such experiments was that mixed cultures of lactobacilli isolated from either good or poor quality cheese were inoculated into the cheese milk and in all cases, caused unclean or fermented flavours. The workers used pure strains or mixtures of several pure strains of lactobacilli isolated from Cheddar cheese as adjuncts; the best cheeses were those for which \textit{Lb. casei} or \textit{Lb. plantarum} were used. Poor results, which included fermented flavour, openness and discolouration, were associated with cheese inoculated with \textit{Lb. brevis}. Other groups of scientists used 8 strains of \textit{Lb. casei} as adjunct cultures for cheese manufacture and monitored the chemical and sensory changes throughout ripening. Enhanced proteolysis was associated with 6 of the 8 strains. Although some of the strains produced cheese with good quality Cheddar flavour, a butter-like flavour was attributable to many of the strains used. It was also reported that the flavour enhancement in cheese to which \textit{Lb.plantarum} was added as an adjunct, whereas objectionable flavours developed in cheeses inoculated with \textit{Lb. brevis} adjuncts.

Another group of researchers also inoculated 10 strains of lactobacilli, isolated from good quality cheese, individually into pasteurized cheese milk; cheeses inoculated with adjunct lactobacilli showed significantly enhanced Cheddar flavour over pasteurized milk cheese without an adjunct. Several early studies on the addition of adjuncts to chemically-acidified cheese failed to generate typical flavour. A reference microflora (RF) was also developed consisting of groups of non-starter bacteria isolated from milk or cheese for addition to experimental cheese manufactured under controlled microbiological conditions. Addition of a RF reproduced the flavour of the commercial cheese from which it was obtained and accelerated flavour development, despite the sporadic occurrence of off-flavours in some RF cheeses. The results of these studies indicate that the effects of adjunct cultures of \textit{Lactobacillus} spp. on cheese quality are both species and strain dependent. It has been suggested that flavour intensity correlates with the number of lactobacilli present at the start of cheese making and in the cheese during ripening.

4.1.1.2. Use of Mesophilic Lactobacilli as Adjuncts: Adjunct lactobacilli were also added to the cheese milk to a level of $\sim 10^5$ cfu ml-1 or g-1 cheese on the day of manufacture. While these initial counts may seem rather high compared to those in modern Cheddar made from pasteurized milk (NSLAB counts ex-press are $10^2$ cfu/g), the level is typical of the number of NSLAB expected in traditional raw milk Cheddar cheese ex-press.

The ripening of Cheddar cheese inoculated with \textit{Lb. casei} ssp. \textit{casei}, \textit{Lb. casei} ssp. \textit{pseudoplanlantarum}, \textit{Lb. Plantarum} or \textit{Lb. brevis} at a level of 105 cfu/ml of milk has been investigated. All adjunct-containing cheeses develop higher levels of free amino acids than the controls (containing only lactococcal starter) during ripening. The cheese inoculated with \textit{Lb. casei} ssp. \textit{casei} L2A receive the best scores for flavour intensity and has been classified as having a “strong Cheddar cheese” flavour after 7 months of ripening. Cheeses inoculated with \textit{Lb. plantarum} 99 also receive high scores for flavour intensity but an onion-like off-flavour develops. The cheeses inoculated with \textit{Lb. Brevis} L35.4 develop off-flavours and gassiness.

Study was also carried out on the ripening of Cheddar cheese to which a mixed adjunct containing 3 strains of \textit{Lb. casei} ssp. \textit{casei} was added at a level of $10^6$ cfu/ml of milk. The
added lactobacilli did not metabolize citrate or produce biogenic amines; the body and texture of the experimental cheese were not affected by the added lactobacilli but they developed higher levels of PTA soluble amino nitrogen, higher levels of free amino acids and had a better flavour than the control cheese after 36 or 48 weeks of ripening. Starter numbers declined faster in the adjunct-containing cheeses which may have contributed to the differences between it and the control. The effects of a number of homofermentative Lactobacillus adjunct cultures (added individually at 105 cfu/ ml of milk) on the physicochemical and sensory characteristics of Cheddar cheese were studied. Again, different strains of the same species produced different effects; Lb. casei ssp. casei LH13 and Lb. casei ssp. pseudoplanitarum 81-10/62, 83-4- 12/62 caused several defects, including high acidity, bitterness, unclean off flavour, and open and crumbly texture during the early stages of ripening, whereas cheese inoculated with Lb. casei ssp. casei 119-10/62 or Lb. casei ssp. pseudoplanitarum 137-10/62 developed a more intense, desirable flavour than the control.

The effects of whole cells or cell-free extract (CFS) from Lb. casei CNRZ 62 on the ripening of Cheddar cheese were also investigated. Greater proteolysis and volatile acid formation occurred in the cheeses containing the CFS than in those containing whole cells of Lb. casei, suggesting that the action of enzymes released from dead cells in the cheese rather than the metabolic activity of viable cells promotes proteolysis and flavour development. However, a bitter flavour developed in the cheese containing the CFS of Lb. casei, which was attributed to altered distribution and activity of the enzymes in the curd. Similarly, the effects of adding liquid or lyophilized cell homogenate and/or live cells of Lb. casei ssp. casei to Cheddar cheese have been observed. It is concluded that the addition of either live cells or lyophilized homogenate at renneting leads to a good quality mature cheese with a 40 per cent increase in flavour intensity compared to the control cheese; no undesirable flavours have been detected in either cheese.

McSweeney et al. (1994) used aseptic cheese making techniques. Using this technique, the effects of a mixed mesophilic Lactobacillus adjunct (strains of Lb. plantarum, Lb. curvatus, Lb. casei and Lb. paracasei) on cheese ripening was investigated. This adjunct significantly improved flavour intensity and acceptability of the cheese. No differences were observed in primary proteolysis but the adjunct-containing cheeses developed higher levels of amino acids than the controls, especially towards the end of ripening. Although the cheese milk was free of lactobacilli, adventitious NSLAB grew to low numbers (< 105 cfu/g) in both control cheeses. In one trial, the control cheese remained free of lactobacilli up to 50 days and differences in NSLAB counts between control and adjunct-containing cheeses were at least 3 log cycles during the first 20 weeks of ripening; therefore, McSweeney et al. (1994) concluded that the differences observed between the control and experimental cheeses were due to the Lactobacillus adjuncts used.

Lane and Fox (1996) compared the contribution of adjunct lactobacilli to cheese made with and without Lactococcus starter; the starter-free cheese was acidified using gluconic acid-δ-lactone (GDL). The mixed adjunct, comprised of strains of Lb. plantarum, Lb. curvatus, Lb. casei and Lb. paracasei, caused a significant increase in proteolysis in the chemically-acidified cheese (i.e., in the absence of starter bacteria), especially at the level of amino acids and small peptides, but their contribution to proteolysis in cheese made with starter was much less. Unfortunately, the sensory properties of these cheeses were not assessed. Elaborate sensory analysis was also used to investigate the effect of 2 strains of Lb. casei on Cheddar cheese.
flavour and ripening. When used with a mesophilic starter, both adjuncts increased aroma and flavour intensity, but reduced creamy flavour in comparison to the controls. One of the adjuncts caused slight bitterness towards the end of a 12-month ripening period. The adjunct-containing cheeses received higher scores for perceived maturity than the controls throughout ripening. It is suggested that the lactic starter has the potential to influence which strains of adventitious NSLAB dominate in the cheese during ripening and affect either favourably or unfavourably the development of cheese flavour.

In general, inoculation with mesophilic *Lactobacillus* adjuncts enhanced flavour and accelerated proteolysis at the level of small peptides and amino acids. Essentially the same volatiles were produced in all cheeses but at very different concentrations. Lynch *et al.* (1997) reported the effect of the mixed *Lactobacillus* adjunct culture (combination of all the above strains) on the ripening of chemically-acidified cheese. Sensory analysis showed considerable intensification of the flavour of the adjunct-containing starter-free cheese; however, the flavour was considered unacceptable and was downgraded. *Lactobacillus* clearly have the potential to modify cheese flavour and accelerate flavour development but further research is required on strain selection if the flavour of raw milk cheese is to be produced in pasteurized milk cheese using adjunct cultures.

4.1.1.3. Thermophilic Lactobacilli as Adjunct Cultures: Species of thermophilic *Lactobacillus* are widely used as starters in several high-cook varieties and in yogurt. These bacteria do not form part of the normal flora of Cheddar cheese made from pasteurized milk and have received little attention as adjuncts for Cheddar cheese. Thermophilic lactobacilli do not grow to a significant extent in Cheddar cheese and essentially serve as an additional source of enzymes. Early cheese making studies on the use of thermophilic lactobacilli as adjuncts indicated that these species contributed little to the development of characteristic Cheddar flavour and some strains caused off flavours and gassiness. It was reported that including a yoghurt culture (mixture of *Lb. helveticus* and *St. thermophilus*) with the normal starter in the manufacture of Cheddar cheese modified the flavour to that of a sweet Swiss-type cheese. However, some workers found that a low inoculum of *St. thermophilus* as adjunct enhanced the flavour of Cheddar cheese. El-Soda *et al.* (1982) accelerated the ripening of Cheddar cheese by treatment with the CFS of *Lb. helveticus* or *Lb. bulgaricus* but a pronounced bitter flavour developed in all experimental cheeses after 2 months of ripening. There has been renewed interest in the use of thermophilic lactobacilli as adjuncts in recent years, partly because of their ability to give a different note to the flavour of Cheddar cheese, although some consumers may regard it as atypical.

The addition of *Lb. helveticus* WSU19 to milk for Cheddar cheese at a level of 102 - 103 cfu/ml accelerated ripening and intensified the flavour; the adjunct increased the nutty flavour and improved flavour acceptability. Similar effects were found with *Lb. helveticus* DPC4571, which dies quickly in the cheese and autolyses, and for *Lb. acidophilus* PF1. In the latter study, adjunct containing cheese underwent accelerated proteolysis and received higher sensory scores than the controls, although some graders considered the flavour to be atypical of Cheddar. In contrast to mesophilic lactobacilli, which grow in Cheddar cheese from low initial levels to dominate the viable microflora of aged cheese, thermophilic lactobacilli die and lyse very rapidly in cheese (Lortal *et al.*, 1997). The difference in growth and longevity between the mesophilic and thermophilic lactobacilli in cheese has not been explained. Another factor contributing to the renewed interest in thermophilic lactobacilli as adjuncts is the fact that these
bacteria produce acid during manufacture; their addition to Cheddar cheese cultures was stimulated, in part, by the need to broaden the range of phage-insensitive, fast-acid producing strains available to industry (Beresford and Cogan, 1997). Consequently, commercial cultures are now available which contain these bacteria blended with normal mesophilic starter lactococci.

4.1.2. Selection of Strains for Use as Adjunct Cultures: The criteria for adjunct selection are quite often not defined. Generally, cultures used as adjuncts have been isolated from a good quality cheese but, usually, no data are available as to the relative abundance of the particular strains in the cheese from which they were isolated. Furthermore, very little information is available as to the homogeneity of the NSLAB population in Cheddar cheese; hence, no correlation has been made between cheese quality and NSLAB diversity. This is probably due to the fact that the NSLAB population normally plateaus at 10^7-10^8 cfu/g and until recently the identification of lactobacilli, even to species level, was a major undertaking. The development of polymerase chain reaction (PCR)-based methods greatly facilitates this task and attempts to correlate cheese quality with the dominant species can be expected. These techniques will aid in the selection of NSLAB as potential adjuncts for Cheddar cheese.

There is a need for the selection of adjuncts to be based on scientifically derived information rather than by random selection or on a “trial and see basis”, which are both costly and time-consuming. Consequently, the biochemical characteristics of NSLAB that could potentially contribute to the overall maturation of cheese need to be identified. Williams and Banks (1997) studied the range and activity of the proteolytic enzymes produced by over 40 NSLAB isolates to assess their potential for proteolysis during ripening and as a selection criterion for strains to be used as adjuncts. Proteolytic activity was detected in all of the NSLAB isolates studied, although both species and strain differences were evident in the range and level of activity present. A wide range of other hydrolytic enzymes were also detected in the NSLAB isolates, which therefore have the potential to contribute to the development of flavour during maturation. The information derived by Williams and Banks (1997) on the hydrolytic activities of NSLAB will aid in the selection of adjunct strains which possess the enzyme potential to complement the activities of the starter lactococci and influence flavour development during maturation.

Some workers advocated peptidase profiling of lactobacilli as an important aid to strain selection. El-Kholy et al. (1998) emphasized the importance of selecting adjunct cells according to their autolytic properties and the necessity to understand the conditions leading to cell autolysis. Therefore, individual strains must be screened for their cheese making properties before they can be used successfully as adjuncts to accelerate ripening and/or improve flavour. The final selection of adjunct cultures must be on the basis of the quality of the cheese manufactured using these strains as adjuncts (McSweeney et al., 1995). Thus, with some exceptions, it appears adjunct lactobacilli inoculated at low numbers accelerate proteolysis in and improve the flavour of Cheddar cheese but high numbers may cause atypical flavour or even flavour defects; however, the upper number is strain-dependent, eg, it was reported that Lb. paracasei ssp. paracasei NCDO 1205 caused no ill effects in Cheddar cheese at ~ 10^9 cfu/g. The optimum level of inoculum is probably 10^3–10^4 cfu/g. Adventitious NSLAB are always likely to gain access to the curd during manufacture in commercial factories. For selected strains of lactobacilli to become widely used as adjuncts in the commercial manufacture of cheese, improved knowledge of these cultures, the effects of their use and interactions of the total microflora of cheese is required.
4.2. GMOs in Accelerated Cheese Ripening: A Novel Cheese-Biotech Alliance

The production of cheese and a range of other fermented foods is one of the oldest manifestations of biotechnology. Many consumers consider cheese to be a delicacy with exquisite taste and aroma characteristics and would recoil at the thought of using Genetically Modified Microorganisms (GMOs) in the manufacturing process. The use of recombinant DNA technology to produce GMOs for accelerated ripening of cheese is one of the most important scientific advances of the 20th century. It has great potential in research, because it allows the development of highly sensitive analytical procedures. It also has potential in industry, leading to processes and products that would be difficult to develop using conventional techniques. These include food and food processing. The use of GMOs in food and food processing is litigious due to a lack of acceptance by consumers; especially in Europe. Recent developments have opened many more possibilities for the use of gene technology. The next generation of bacterial cultures will probably contain strains with properties modified by gene technology, for example with new properties for cheese making created by changing the expression level of one or a few genes.

The considerable knowledge now available on the genetics of cell wall associated proteinase and many of the intracellular peptidases makes it possible to specifically modify the proteolytic system of starter Lactococcus. The gene for the neutral proteinase (Neutrase) of B. subtilis has been cloned in Lc. lactis UC317 by McGarry et al. (1995). Cheddar cheese manufactured with this engineered culture as the sole starter undergoes very extensive proteolysis and the texture became very soft within two weeks at 8°C. By using a blend of unmodified and Neutrase-producing cells as starter, a more controlled rate of proteolysis was obtained and ripening was accelerated (McGarry et al., 1994). An 80:20 blend of unmodified: modified cells gave best results. Since the genetically modified cells were not food grade, the cheese was not tasted but the results appear sufficiently interesting to warrant further investigation when a food-grade modified mutant becomes available.

Since amino acids are widely believed to be major contributors, directly or indirectly, to flavour development in cheese, the use of a starter with increased aminopeptidase activity would appear to be attractive. Two studies (McGarry et al., 1995 and Christensen et al., 1995) have been reported on the use of a starter genetically engineered to super-produce aminopeptidase N; although the release of amino acids was accelerated, the rate of flavour development and its intensity were not, suggesting that the release of amino acids is not rate limiting. In an on-going study, engineered Lactococcus starters harbouring PepG or PepI genes from Lb. delbreuckii have shown increased proteolysis, especially at the level of amino acids; PepG was the more effective. The cheeses have not yet been assessed organoleptically.

Apart from legal aspects, the principal technical problems when engineering starters with improved cheese-ripening properties is the lack of knowledge on the key or limiting lactococcal enzymes involved in cheese ripening. A range of peptidase-deficient Lactococcus mutants (lacking 1 to 4 peptidases) has been developed (Mireau et al., 1996) primarily with the objective of identifying the importance of the various peptidases, alone or in various combinations, to the growth of the organism in milk. Mutants deficient in 1 or 2 peptidases (in various combinations) were also used for the small-scale manufacture of Cheddar cheese (mutants deficient in 3 or 4 peptidases are unable to grow in milk at a rate sufficient for cheese
manufacture). Perhaps surprisingly, cheeses made using the peptidases-deficient mutants, even those lacking both PepN and PepC, did not differ substantially from the control with respect to the level and type of proteolysis or flavour and texture. The results appear to suggest that there are alternative routes for amino acid production.

4.3. Genetically Engineered Starter in Accelerated Ripening of Cheese

Food-grade controlled lysis of Lactococcus lactis for accelerated cheese ripening is an important approach. An attractive approach to accelerate cheese ripening is to induce lysis of Lactococcus lactis starter strains for facilitated release of intracellular enzymes involved in flavour formation. Controlled expression of the lytic genes lytA and lytH, which encode the lysin and the holin proteins of the lactococcal bacteriophage phi-US3, respectively, was accomplished by application of a food-grade nisin-inducible expression system. Simultaneous production of lysin and holin is essential to obtain efficient lysis and concomitant release of intracellular enzymes as exemplified by complete release of the debittering intracellular aminopeptidase N. Production of holin alone leads to partial lysis of the host cells, whereas production of lysin alone does not cause significant lysis. Model cheese experiments in which the inducible holin-lys in overproducing strain was used showed a fourfold increase in release of l-Lactate dehydrogenase activity into the curd relative to the control strain and the holin-overproducing strain, demonstrating the suitability of the system for cheese applications.

4.4. Stimulation of Starter Cells

The growth of starter cells may be stimulated by the addition of enzymes or hydrolysed starter cells to cheese milk. Ripening of Emmental type cheese has been accelerated by using starters grown to high cell numbers in media supplemented with protein hydrolysates and metalloproteinase from Micrococcus caseolyticus.

4.5. Modified Starter Cultures

Addition of modified/attenuated starter culture is to increase the number of starter cells without causing detrimental effect on the acidification schedule during manufacture so that the cells contribute only to proteolysis and other changes during ripening. Modified starter cultures with attenuated acid producing abilities are added with normal starter cultures during cheese manufacture. Selection of starter strains with enhanced autolytic properties and increased peptidase activity would provide a more balanced flavour.

4.6. Heat and Freeze Shock Treated Cells

Mixed strain starters or L. helveticus culture subjected to various heat-shock treatments in an attempt to reduce their acid producing ability but to enhance their rate of autolysis. Some workers used heat-shock cultures to attain large number of a mixed-strain starter, containing Lactococcus, Leuconostoc or L. helveticus strains which were cultivated at a constant pH, followed by heating to 69°C/15 sec. Flavour scores increased with increasing numbers of heat-shocked cells reducing the ripening time to 50 per cent. A combination of neutral proteinase with heat shocked L. helveticus to a final level of 4 x 10^6 cfu/g curd also accelerated the ripening of the cheese. Addition of heat-shocked lactobacilli found to increase peptidolysis and produce good flavour in low-fat semi-hard cheese. flavour acceleration could be significantly improved by augmentation of starter culture with freeze-shocked L. helveticus in buffalo Gouda cheese. The combination of liposome entrapped proteinase and freeze-shocked lactobacilli resulted in development of more intense flavour without bitterness in UF cheese.
The acid-producing ability of lactic acid bacteria can be markedly reduced by a sub-lethal heat treatment while only slightly reducing enzyme activities; heating at 59 or 69°C for 15 sec was optimal for mixed mesophilic and thermophilic lactobacilli cultures, respectively. Most (90%) of the heat-shocked cells added to cheese milk at a level of 2 per cent (v/v) were entrapped in the curd but entrapment efficiency decreased at higher levels. Proteolysis in Swedish cheese was increased and quality improved by addition of the heat-shocked cells to the cheese milk, *Lb. helveticus* being the most effective. The extent of proteolysis increased pro rata the level of heat-shocked *Lb. helveticus* cells added but not for the mesophilic culture; bitterness was not observed in any of the cheeses. Essentially similar results were reported for Gouda cheese. Heat shocking at 70°C for 18 sec and 2 per cent addition were found to be optimal. Of several thermophiles investigated, *Lb. helveticus* gave best results; *Lb. bulgaricus* and one strain of *Str. thermophilus* had negative effects on flavour quality due mainly to bitterness. An acetaldehyde-like or yoghurt flavour was noted in most of the cheeses containing heat-shocked lactobacilli.

Heat-shocked (67°C x 10 s) *Lb. helveticus* cells accelerated amino nitrogen formation and enhanced flavour development in Swedish hard cheese; when added alone, Neutrase accelerated proteolysis but it caused bitterness which was eliminated when both heat-shocked *Lb. helveticus* cells and Neutrase were added to the curd. Trepanier *et al.* (1991) investigated the contribution of live and heat-shocked (67°C x 22 s) cells of *Lb. casei* ssp. *casei* L2A to Cheddar cheese ripening; the addition of 104 live cells /ml or ~ 107 heat-shocked cells/ml of *Lb. casei* ssp. *casei* L2A to the cheese milk yielded a good quality Cheddar cheese with 60% greater flavour intensity than the controls after 6 months of ripening. Asensio *et al.* (1996) studied the effects of adding heat-treated cultures (50°C x 15 s) of *Lc. lactis, Lb. casei* or *Lb. plantarum* as adjuncts to the starter in the manufacture of low-fat goats’ milk cheese. The adjunct did not affect cheese making or the overall composition of the cheese but led to increased aminopeptidase activity and amino nitrogen during ripening which improved the flavour intensity and body of the cheese.

Cheeses containing treated cells were free of bitterness and acid taste; additionally, their general acceptability was greater than that of the controls. Freeze-thawing also kills bacteria without inactivating their enzymes. Addition of freeze-shocked *Lb. helveticus* CNRZ 32 cells to cheese milk markedly accelerated proteolysis and flavour development in Gouda cheese without adverse effects; the greatest flavour difference between the control and experimental cheeses was observed after 5 weeks. Addition of untreated *Lb. helveticus* cells to the curd also accelerated proteolysis but caused off-flavours. Johnson and Etzel (1995) compared different methods for attenuating a strain of *Lb. helveticus*. The extent of attenuation, from least to most, was as follows: spray-dried at low temperature, frozen, freeze-dried, and spray-dried at high temperature (measured by lactic acid production and enzyme activity). The authors concluded that high-temperature spray-dried cells had the best balance between delayed acid production and retained enzyme activity. These attenuated cultures were used as adjuncts for the manufacture of reduced-fat Cheddar cheese with the objective of accelerating ripening (Johnson *et al.*, 1995); adjuncts with low levels of attenuation correlated with increased proteolysis and flavour intensity in the resultant cheeses.

### 4.7. Lysozyme Treated Cells

Addition of lysozyme-sensitised cells to cheese milk at a level of equivalent to $10^{10}$ cells/gm of cheese indicated that intracellular dipeptidases were released and as a result...
concentration of free amino acids significantly increased. However, there was no effect on the rate of flavour development. Economically, the use of lysozyme treated cells may not be viable for large-scale cheese manufacture owing to the cost of the enzyme. It has been suggested the addition of lysozyme encapsulated in a dextran matrix to cheese milk at renneting, which would be released at cheddar cheese pH (5.2-5.4) leading to the lysis of the cells with release of intracellular peptidases.

4.8. Mutant Starter Cultures

Because the rate of acid development is a critical factor in cheese manufacture, the amount of normal starter cannot be increased without producing an atypical cheese. This has led to the use of Lac- mutants, which do not affect the rate of acid development but provide additional enzymes. Like attenuated cells, these mutant cells serve as packets of enzymes but are much easier to prepare and would appear to be the logical choice when it is desired to increase the number of cells without a concomitant increase in the rate of acid production. Lactose negative (Lac⁻) mutants of starter strains have been reported to provide packets of uniformly distributed proteinases/peptidases, enhancing the production of peptides and free amino acids without interfering with acid production during manufacture. Cheddar cheese containing \(10^{11}\) cfu/ml of \(L.\) lactis subsp. cremoris (Lac⁻Prt⁻) cells received highest flavour scores. Some workers have showed advancement in ripening of 4-12 weeks after 6 months storage in Cheddar cheese with mutant starter.

Possibly the first use of a Lac-/Prt- mutant, \(Lc.\) lactis C2, to accelerate cheese ripening was described by Grieve and Dulley (1983). Concentrates containing \(10^{11}\) cells/g were added to the cheese milk to give starter cell numbers in the curd at milling that were 10-60 times higher than in the control cheese. Proteolysis was accelerated in the experimental cheeses, flavour development was advanced by up to 12 weeks and flavour quality was improved. Ripening at an elevated temperature (20°C) increased proteolysis and advanced flavour development in both control and experimental cheeses. Other workers also studied the combined effects of Lac- starter, exogenous proteinase and elevated temperatures on cheese ripening; regardless of the other treatments employed supplementation with Lac- cells accelerated ripening.

It was also recommended to use the Prt- starters to reduce bitterness in cheese. It was claimed that the rate of proteolysis in Cheddar cheese made using Prt- starters was similar to that in control cheese. Lac- Lactococcus strains with high exopeptidase activity are commercially available as cheese additives (Høier, 1998). When inoculated into the cheese milk at 0.002 per cent, individually or as a cocktail, the Lac- cultures enhanced cheese flavour over that of the control. Assessment of proteolysis showed only minor differences between the cheeses with respect to primary and secondary proteolysis but all adjunct-treated cheeses contained higher levels of amino acids than the controls throughout ripening. The current active programmes on the genetics of lactic acid bacteria will probably lead to the development of Lac- starters with superior cheese ripening properties, eg. with increased proteinase and/or peptidase or perhaps other activities that may be important in the rate of cheese ripening and/or quality.

4.9. β-Galactosidase (Lactase)

β-galactosidase (lactase) hydrolyses lactose to glucose and galactose results in stimulation of lactococci and shortens the lag period of growth of lactococci. Lactose
hydrolysed cow and buffalo cheese milks have been reported to reduce manufacturing time, improve flavour and accelerate ripening. It has been found that the lactase from *Kluyveromyces lactis* available as Maxilact contained a proteinase, which was responsible for the increased levels of peptides, and free amino acids.

4.10. **Cell Free-Extracts (CFE)**

Addition of cell-free extracts to accelerate ripening generally leads to the accumulation of small peptides and free amino acids without an increase in gross proteolysis. CFEs of *L. helveticus*, *L. bulgaricus* or *L. lactis* reported to increase primary proteolysis by enhancing 𝛽-casein breakdown. Maturation of cheddar cheese could be reduced to 2 months by synergistic action of Neutrase and CFE of *L. lactis* subsp. *lactis* or *Brevibacterium linens*.

UF retentate could be successfully incorporated into processed cheese. This work was extended by Law and collaborators (Fox *et al.*, 1996) who found that Neutrase, a neutral serine proteinase from *B. subtilis*, was the most effective. A combination of Neutrase and a peptidase-rich cell-free extract (CFE) of *Lactococcus* spp gave better results than Neutrase alone. This combined enzyme preparation was commercialized as “Accelase” by Imperial Biotechnology, London. However, in spite of the claimed success of the Accelase in pilot-scale and commercial-scale studies (Fox *et al.*, 1996b), it has not been commercially successful but is still available; its commercial failure may be due to the prohibition on the use of exogenous enzymes (other than rennet) in cheese making in the UK. Neutrase or other proteinases, alone or in combination with other factors, eg, lactococcal CFE, proteinase-negative/lactose-negative lactococci or elevated temperature have been used with limited and inconsistent success (see Fox *et al.*, 1996b).

It was reported that Neutrase, FlavourAge FR (a lipaseproteinase preparation from *A. oryzae*) or extra rennet added to Cheddar curd at salting accelerated flavour development when the cheese was ripened at 5°C for a relatively short period (4-5 months) but excessive proteolysis and associated flavour and body defects occurred on further storage, especially at a higher temperature. According to the other workers, neither FlavourAge FR nor DCA50 (a proteinase-peptidase blend; Imperial Biotechnology, London) caused substantial acceleration of flavour development and in some cases led to offflavours and textural defects. Reliable data on the commercial use of exogenous proteinases to accelerate the ripening of cheese are not available but it is the authors’ opinion that they are used to a very limited extent for natural cheese; they are used in the production of enzyme-modified cheese.

4.11. **Addition of Exogenous Enzymes**

Cheese ripening is essentially an enzymatic process and hence it should be possible to accelerate ripening by augmenting the activity of key enzymes. However, addition of single enzyme, which accelerates one particular reaction, is unlikely to produce more balance flavour. Hence, the need for addition of mixture of enzymes in proper ratio has been advocated by several research workers. The addition of combinations of various fungal proteases and lipases to Cheddar cheese has been reported to reduce ripening time by 50 per cent. Lipase in combination with proteinase gave good cheese flavour with low levels of bitterness. A lipase/proteinase preparation derived from *A. oryzae* released C6-C10 fatty acids to produce typical cheddar cheese flavour. To achieve more intense and balanced flavour in buffalo milk cheddar cheese, use of mixture 0.001 per cent lipase and 0.01 per cent protease has been
advocated. A number of options are available, ranging from the quite conservative to the more exotic.

4.11.1. Coagulant: The proteinase(s) in the coagulant is principally responsible for primary proteolysis in most cheese varieties and it might, therefore, be expected that ripening could be accelerated by increasing the level or activity of rennet in the cheese curd. Although, it is suggested that chymosin is the limiting proteolytic agent in the initial production of amino N in cheese, several studies have shown that increasing the level of rennet in cheese curd (achieved by various means) does not accelerate ripening and in fact probably causes bitterness. Chymosin produces only relatively large oligopeptides which lack a typical cheese-like flavour and may be bitter. Chymosin-produced peptides are hydrolysed by bacterial (starter and non-starter) proteinases and peptidases and hence it would seem that increased chymosin activity should be coupled with increased starter proteinase and peptidase activities in order to accelerate ripening.

The natural function of chymosin is to coagulate milk in the stomach, thereby increasing the efficiency of digestion. It is fortuitous that chymosin is not only the most efficient milk coagulant but also gives best results in cheese ripening. However, it seems reasonable to suggest that the efficiency of chymosin in cheese ripening could be improved by protein engineering. The chymosin gene has been cloned and expressed in several microorganisms; microbial chymosin is now used widely for commercial cheese manufacture, with excellent results. The gene for the acid proteinase of *R. miehei* has also been cloned and expressed in *A. oryzae*; the product is commercially available (Maryzme GM; Texel, Cheshire, UK). In all these cases, the chymosin gene was not modified but a number of studies on the genetic engineering of chymosin have been published (Fox and McSweeney, 1997 & Chitpinityol and Crabbe, 1998). As far as we know, the cheese making properties of such variants have not been assessed.

Chymosin has very little activity on β-casein in cheese, probably because the principal chymosin-susceptible bond in β-casein, Leu192-Tyr193, is in the hydrophobic C-terminal region of the molecule which appears to interact hydrophobically in cheese, rendering this bond inaccessible. However, *C. parasitica* proteinase preferentially hydrolyses β-casein in cheese (possibly because its preferred cleavage sites are in the hydrophilic N-terminal region) without causing flavour defects. Rennet containing chymosin and *C. parasitica* proteinase might be useful for accelerating ripening.

4.11.2. Plasmin: Plasmin contributes to proteolysis in cheese, especially in high-cooked varieties in which chymosin is extensively or totally inactivated. Plasmin is associated with the casein micelles in milk, which can bind at least 10 times the amount of plasmin normally present, and is totally and uniformly incorporated into cheese curd, thus overcoming one of the major problems encountered with the use of exogenous proteinases to accelerate cheese ripening. Addition of exogenous plasmin to cheese milk accelerates the ripening of cheese made there from, without off-flavour development (Kelly, 1995).

At present, plasmin is too expensive for use in cheese on a commercial scale. The gene for plasmin has been cloned and expressed in a *Lactococcus* host (Arnaud *et al.*, 1997); we are not aware of cheese making trials with this culture. Since milk contains four times as much plasminogen as plasmin, an alternative strategy might be to activate indigenous plasminogen by adding a plasminogen activator, eg, urokinase, which also associates with the casein micelles. This approach is technically feasible (Barrett *et al.*, 1999) but it also may be too
expensive. Since plasmin is a trypsin-like enzyme, trypsin, which is relatively cheap and readily available commercially, may also be suitable for accelerating ripening. Careful use of trypsin has been reported to accelerate ripening but these findings must be confirmed. Since trypsin is more proteolytic than plasmin, greater care is required in its use.

4.11.3. Other Proteinases: The possibility of accelerating ripening through the use of exogenous (non-rennet) proteinases has attracted considerable attention over the past 20 years. The principal problems associated with this approach are ensuring uniform distribution of the enzyme in the curd and the prohibition of exogenous enzymes in many countries. The earliest reports on the use of exogenous enzymes to accelerate the ripening of Cheddar cheese appear to be those of Kosikowski and collaborators who investigated various combinations of commercially available acid and neutral proteinases, lipases, decarboxylases and β-galactosidase. Acid proteinases caused pronounced bitterness but the addition of certain neutral proteinases and peptidases with the salt gave a marked increase in flavour after 1 month at 20°C but an overripe, burnt flavour and free fluid were evident after 1 month at 32°C. Incorporation of the enzyme-treated cheese in processed cheese gave a marked increase in Cheddar flavour at 10 per cent addition and a very sharp flavour at 20 per cent. Good quality medium sharp Cheddar could be produced in 3 months at 10°C through the addition of combinations of selected proteinases and lipases. Upto 60 per cent enzyme-treated (fungal lipases and proteinases).

With the exception of rennet and plasmin (which adsorbs on casein micelles), the incorporation and uniform distribution of exogenous proteinases throughout the cheese matrix poses several problems:

- Proteinases are usually water-soluble, and when added to cheese milk, most of the enzyme is lost in the whey, which increases cost
- Enzyme-contaminated whey must be heat-treated if the whey proteins are recovered for use as functional proteins; the choice of enzyme is limited to those that are inactivated at temperatures below those that cause thermal denaturation of whey proteins
- The amount of Neutrase that should be added to milk to ensure a sufficient level of enzyme in the curd reduces the rennet coagulation time yields a soft curd in which at least 20 per cent of the β-casein is hydrolyzed at pressing and reduces cheese yield.

Consequently, most investigators have added enzyme, usually diluted with salt to facilitate mixing, to the curd at salting. Since the diffusion coefficient of large molecules, like proteinases and lipases, is very low, this method is applicable only to Cheddar-type cheeses, which are salted as chips at the end of manufacture, and not to surface-salted (brine or dry) cheeses which include most varieties. Even with Cheddar-type cheeses, the enzyme will be concentrated at the surface of chips, which may be quite large. Uneven mixing of the salt-enzyme mixture with the curd may lead to “hot spots” where excessive proteolysis and lipolysis, with concomitant off-flavours, may occur. Enzyme encapsulation offers the possibility of overcoming the above problems. The microcapsules, being sufficiently large, are occluded in the curd; the main problem is to achieve the release of the enzymes after curd formation. Several studies on the microencapsulation of enzymes for incorporation into cheese have been reported (Wilkinson, 1993 & Skeie, 1994). Although microcapsules added to milk
are incorporated efficiently into cheese curd, the efficiency of enzyme encapsulation is low, thus increasing cost. As far as is known, encapsulated enzymes are not being used commercially in cheese production.

4.11.4. Exogenous Lipases: Lipolysis is a major contributor, directly or indirectly, to flavour development in strong-flavoured cheeses, eg, hard Italian, Blue varieties, Feta. Rennet paste or crude preparations of pre-gastric esterase (PGE) are normally used in the production of Italian cheeses. *R. miehei* lipase may be used for Italian cheeses, although it is less effective than PGE; lipases from *P. roqueforti* and *P. candidum* may also be satisfactory. The ripening of blue cheese may be accelerated and quality improved by added lipases. A Blue cheese substitute for use as an ingredient for salad dressings and cheese dips can be produced from fat-curd blends by treatment with fungal lipases and *P. roqueforti* spores. Although Cheddar- and Dutch-type cheeses undergo little lipolysis during ripening, it has been claimed that addition of PGE or gastric lipase improves the flavour of Cheddar cheese, especially that made from pasteurized milk; several patents have been issued for the use of lipases to improve the flavour of “American” or “processed American” cheeses.

The enzyme mixtures used by Kosikowski and collaborators to accelerate Cheddar cheese ripening contained lipases. Law and Wigmore (1985) reported that the addition of PGE or *R. miehei* lipase, with or without Neutase, to Cheddar cheese curd had a negative effect on flavour quality. FlavorAge contains a unique lipase from a strain of *A. oryzae* which has an exceptionally high specificity for C6-C8 acids and forms micelles, 0.2 μm in diameter, in aqueous media as a result of which 94 per cent of the enzyme added to milk is recovered in the cheese curds. According to these authors, FlavourAge accelerates the ripening of Cheddar cheese; the formation of short-chain fatty acids parallels flavour intensity. In contrast to the FFA profile caused by PGE, which liberates high concentrations of butanoic acid, the FFA profile in cheese treated with FlavourAge is similar to that in the control cheese except that the level of FFAs is much higher. There are reports in the literature on the successful application of lipases to accelerate the ripening of, or intensify the flavour of several other varieties of cheese, eg, Feta, Samsoe, Kesseri, Ras, Domiati or Queso Blanco (Fox *et al.*, 1996). However, the traditional use of PGE in certain Italian cheese varieties and the recently developed application of lipases in EMCs appear to be the principal or only commercial applications of lipases in cheese.

4.11.5. Microencapsulated Enzymes in Cheese Making: Addition of enzymes to cheese milk leads to a partitioning of the enzyme between curds and whey, representing an economic loss of the enzyme (only 5-10% of enzyme retains in the curd). Alternative method of dried enzyme addition with the salt at milling is not applicable to brine-salted varieties. Therefore, addition of encapsulated enzymes, enclosed in liposomes to cheese milk offers best possible method for enzyme addition till date. Liposomes are composed of materials, which are normal food components, and degradation of the vesicle membrane in cheese releases the enzyme into the curd. Lipid encapsulation also ensures uniform distribution of the enzyme in the curd as the liposomes are entrapped in the matrix at renneting.

Liposomes can be prepared using three different methods, which produced multilamellar vesicles. Small unilamellar vesicles and reverse phase evaporation vesicles. The enzyme encapsulated was highest for reverse phase evaporation vesicles and when added to cheese milk, neutrase vesicles gave significant advancement in ripening with no textural defects or bitterness. Successful use of multilamellar vesicles of Neutrase for accelerated...
Ripening of Cheddar cheese has been reported. Addition of both goat milk and microencapsulated enzymes exhibited synergestic effect on flavour development. Incorporation of liposomes, namely Flavorage and Naturage at the rate of 2.5 g/100 liters of milk were found to accelerate ripening in buffalo milk Cheddar cheese. The texture of cheese containing liposomes of Neutrase + CFE of *L. lactis* was normal even after 8 months while that of cheese with free enzyme was deteriorated. It has been stated that the liposomes engineered to have a phase-transition within the pH range of most cheese varieties (pH 5.0-5.5) so as to release the encapsulated enzymes would provide a simpler method for controlled release of enzymes.

Much research has been directed towards the acceleration of cheese ripening because of the obvious economic and technological advantages to be gained in shortening the ripening process. Scientific literature claims that exogenous enzymes are effective in accelerating ripening of cheese but their commercial application is yet to take off, possibly due to their high cost, difficulties in distributing them uniformly in the curd and the possible danger of over ripening of cheese. The use of starters with enhanced flavour producing ability by genetic manipulation together with increased autolytic properties to affect the early release of these enzymes may offer the best method of accelerating cheese ripening.

### 4.12. Other Looms to Accelerating Ripening

If the key flavour compounds in cheese were known, it should be possible to greatly accelerate flavour development by adding them or their precursors to cheese, although the problem of body and texture would remain. Such an approach was attempted by Wallace and Fox (1997) who added various combinations of amino acids, which are considered to be important flavour precursors, to Cheddar cheese curd at salting. Methionine, the precursor of methanethiol, which is commonly regarded as the characteristic flavour compound of Cheddar cheese, was the most effective of the amino acids although less effective than might be expected. The results of a recent study (Yvon et al., 1998) have suggested the limiting factor in the development of cheese flavour may be ketoglutarate, which acts as an acceptor of amino groups in transamination reactions, leading to the formation of a range of organic acids from amino acids. These acids can be reduced to carbonyls and alcohols. Conformation and extension of this very interesting piece of work is awaited.

### 5.0. CONCLUSION

Many cheese varieties have need of long ripening periods at low temperature for characteristic flavour and texture development. This process significantly boosts the cost of the product. Recent developments in biotechnology and food science have brought a renaissance in the field of cheese technology. Now technologies are in our clasp to design cheese with desired textural and flavour characteristics that can be achieved within short ripening period. Much research has been directed towards the acceleration of cheese ripening because of the obvious economic and technological advantages to be gained in shortening the ripening process. Scientific literature claims that exogenous enzymes are effective in accelerating ripening of cheese but their commercial application is yet to take off, possibly due to their high cost, difficulties in distributing them uniformly in the curd and the possible danger of over ripening of cheese. The use of starters with enhanced flavour producing ability by genetic manipulation together with increased autolytic properties to affect the early release of these enzymes may offer the best method of accelerating cheese ripening.
The unprecedented accomplishments of biotechnology and genetic engineering since the past decade have added a new margin in to the approaches of accelerated ripening. Controlled use of biotechnological products like genetically engineered proteolytic and lipolytic enzymes can accelerate cheese ripening. Modified/genetically tailored microorganisms and enzymes are now being used for enhancing flavour production in cheese. Enzyme addition is now one of the few preferred methods of accelerated ripening of cheese. The enzymatic reactions are specific and hence undesirable side effects caused by live microorganisms are avoided in the cheese. Enzymes may be immobilized or encapsulated for long term action on the production for quick action and homogenous distribution in the product. Today’s’ research should be directed to demonstrate how the sorority of cheese technology with modern biotechnology and molecular natural science can have a positive impact on the cheese making process through the generation of "state of the art" starter strains with novel properties, which in turn could potentially be exploited to produce cheeses with enhanced characteristics and at a reduced cost.

6.0. REFERENCES


1.0.  INTRODUCTION

Milk fatty acids had always had a bad literature for its relative high content of some saturated fatty acids, which may increase plasma cholesterol associated to LDL. However, recently much attention has been directed toward an unusual fatty acid, conjugated linoleic acid (CLA), which is naturally present in milk and dairy products. The formation of CLA is part of an overall process called biohydrogenation that takes place in the rumen, which converts linoleic acid, or less efficiently other 18 Carbon PUFAs with double bonds in 9 and 12 positions, to stearic acid (1). Furthermore, it has been demonstrated that manipulating cow’s dietary PUFA intake it is possible to modulate milk CLA content. Therefore, ruminants are the major reservoir for this fatty acid. During this process vaccenic acid is also formed, which may be converted to CLA in all organisms that possess $\delta$-9 desaturase (2). Therefore vaccenic may serve as a precursor of CLA.

Even though the presence of CLA in milk was known since the ’30s, only 60 years later it has been discover that it possesses biological activities (Table-1). Since the first report of Pariza’s research group of CLA antimutagenic activity (3), there has been an explosion of interest in CLA research (Table-1). In fact, among 28 possible positional and geometrical isomer of CLA, only two have been thoroughly studied (c9, t 11 and t10, c12). Studies on pure single isomers showed that they might have some differences in biological activities in regards to decrease body mass and in general on adipogenesis (4). On the other hand, the anticarcinogenic (5) and the antiatherogenic effects seem to be exerted by both isomers.

2.0.  POSSIBLE MECHANISMS BY WHICH CLA EXERTS ITS ACTIVITIES

CLA may act through different pathways (Table-1) and probably influencing each tissue differently. In the early reports it was hypothesized an antioxidant activity as a mechanism of action, which has successively been confuted (6). The discovery that CLA can be elongated and desaturated as a regular PUFA to conjugated (CD) 18:3, CD 20:3 and CD 20:4 (10, 11) brought a new possibility that the activity of CLA may be related to its metabolism and possible competition with the other PUFA families and in particular way with n-6 for the formation of arachidonic acid (7). Its incorporation into rat liver lipids is similar to that of oleic acid, and occurs preferentially into neutral lipids (8). Furthermore, CD 18:3 and CD 20:3 are incorporated similarly to CLA and very differently from their non-conjugated parent compounds 18:3n-6 and 20:3n-6 (8). On the other hand CD20:4 is mainly incorporated into phospholipids (8). The preferential incorporation of CD 18:3 and CD 20:3 into neutral lipid fraction allows these metabolites to be stored in adipose tissue. This may give an advantage in the competition with arachidonic acid since its precursors 18:3n-6 and 20:3n-6 is mostly incorporated into phospholipids (9). Interestingly, in mammary and adipose tissues it seems that the decrease of 20 carbon atom polyunsaturated fatty acids i.e. 20:4 and 20:3, substrates of cyclooxygenase and lipoxygenase pathways, are replaced by CLA isomers CD 18:3 and CD 20:3 (10), that have been
demonstrated to inhibit both pathways of eicosanoid biosynthesis (11). It would be reasonable to expect that the biosynthesis of eicosanoids will be affected by the reduced availability of arachidonic acid. In addition to the activity of cancer prevention, CLA is known to modulate immune functions, and atherogenesis (Table-1).

It has also been shown that CLA is a good ligand of nuclear receptors called peroxisome proliferator activated receptors (PPARs); in particular way CLA seems to be active on two isoforms alpha and gamma. The isoform alpha is mainly located in liver and works as transcription factor for many enzymes linked to fatty acid oxidation and particularly to peroxisomal beta-oxidation. In fact induction of key enzymes of peroxisomal beta oxidation by CLA has been reported as a consequence of activation of peroxisome proliferator activated receptors (PPAR) alpha by CLA (12).

Table-1: Major CLA Activities on Experimental Models “in vivo”

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<thead>
<tr>
<th>Type of Activities</th>
<th>Scientific Findings</th>
</tr>
</thead>
</table>
| **Biological Activities of CLA** | • Antimutagenic activity (1987) (3)  
• Anticarcinogenic activity in mouse forestomach (1990) (24)  
• Mammary cancer prevention in rat (1991) (25)  
• Anticarcinogenic activity in colon (1995) (26)  
• Anticarcinogenic activity in skin (1996) (27)  
• Antiatherogenic activity in rabbits (1994) (28)  
• Reduction of body fat (1996) (28)  
• Antidiabetic activity in rat (1998) (29)  
• Inhibition of metastasis in mice (2000) (30) |
| **Biological activities linked to possible mechanisms of action** | • Metabolism similar to linoleic acid (1995) (31)  
• Decrease of arachidonic acid (1997) (32)  
• Activity as PPARs ligand (1997) (33)  
• Inhibition of proliferation of mammary epithelial cells (1997) (34)  
• Inhibition of δ-9 desaturase (1998) (15)  
• Modulator of immunoglobulins (1998) (35)  
• Increase of free retinol in rat tissues (1999) (13)  
• Induction of apoptosis premalignant lesions in the rat mammary gland (2000) (16)  
• Induction of both proliferation and apoptosis in rat liver (2002) (36) |
| **Studies on single isomers** | • Anticarcinogenic activity of CLA enriched butter in rat (1999) (37)  
• Vaccenic acid is desaturated to CLA in mice (2000) (22)  
• t10,c12 isomer inhibits δ-9 desaturase in rats (2000) (23)  
• Suppression of development of premalignant lesions in the rat mammary gland by vaccenic acid (2001) (22)  
• t10,c12 and c9,t11 are both effective in mammary cancer prevention in rats (2002) (5)  
• Both t10,c12 and c9,t11 are both anti-atherogenic in rabbits (2002) |

One more biological effect that may be linked to PPAR alpha activation is the ability of dietary CLA to induce an increase of free retinol in different tissues (13). This increase may be due to an increase of the level of the carrier protein of retinol (CRBP), mediated by an activation of PPAR alpha known to be a transcription factor for CRBP. The activation of PPAR gamma, which is mainly present in adipose tissue, may well explain its activities on adiposity and diabetes. In fact PPAR gamma regulates adipocyte differentiation and glucose tolerance (12). Furthermore, it has recently been shown that blocking the δ-6 desaturase the CLA activity on PPAR gamma was significantly lower, implying
that some CLA metabolites could contribute in a substantial way on PPAR gamma activation (14). The inhibition of δ-9 desaturase by CLA (15) has also important implication in terms of triacylglycerol synthesis and thereby on accumulation of triacylglycerol in adipocytes (4). It is now demonstrated that one specific isomer t10,c12 is able to inhibit δ-9 desaturase in vivo, even though it has been recently shown that on human breast cancer cells both isomers c9,t11 and t10,c12 have inhibitory activity.

Other biological activities which may be a consequence of the biochemical changes described above, and that may easily explain the preventive activities in several pathological states, especially on cancer, are the inhibition of proliferation with concomitant induction of apoptosis of mammary epithelial cells (16). Also the very recent report that dietary CLA is able to inhibit angiogenesis in vivo in mice (17), may well explain the capacity of CLA to inhibit metastasis, showing that CLA may inhibit each phase of carcinogenesis.

3.0. HUMAN STUDIES

Human studies are not numerous as animal studies even though many are in progress. From human studies is emerging that CLA may be beneficial in preventing some diseases by modulating lipid metabolism and immune function, thus establishing stable conditions which may delay or inhibit the onset of important disease such as cancer, atherosclerosis and diabetes. However no significant changes have been detected by CLA administration in normal conditions (18), while data are more encouraging when CLA is given to patients showing risk factors such as obesity (19) or hyperglycaemia (20). Probably, in some pathological states where lipid metabolism is impaired such as diabetes or obesity, it is likely that CLA is more effective.

Another important discover is that CLA is metabolized similarly in humans and rats (21), therefore, if CLA metabolism is able to influence lipid metabolism in humans as it has been demonstrated in rats, it should exert similar activities. By measuring plasma levels of CLA and metabolites in humans taking 6 g/d of CLA and comparing them to plasma levels of rats fed increasing amounts of CLA from 0-2 per cent, resulted that 6 g in humans corresponded to about 0.4 per cent of dietary intake in rats, slightly less than the range of 0.5-1 per cent within which CLA exerts most of its activities. It is not surprising therefore that lower dietary intake to healthy humans showed no significant effects (18). However, in particular chronic pathological conditions where lipid metabolism is impaired probably lower levels but for very long periods may exert beneficial effects. Another important finding is that an increase in CLA level is possible by way of δ-9 desaturase activities that convert vaccenic acid to CLA, thus determining an increase of tissue CLA. This should be taken into account when planning intervention studies on CLA or vaccenic acid activities.

4.0. ENRICHMENT OF DAIRY PRODUCTS WITH CLA

The dairy products can be enriched with CLA through *in vivo* and *in vitro* approaches. An eight fold increase CLA content of milk has been reported by feeding cows with diet containing 5.3 per cent sunflower oil (37). Alternatively, CLA can be synthesized in the laboratory by refluxing linoleic acid with sodium hydroxide dissolved in ethylene glycol at 180°C under the atmosphere of N₂ for 2h (25). While 80-90 per cent CLA present in milk is c9, t11-isomer, the CLA prepared by alkali treatment contained 42.5 per cent of c9, t11- and t9, c11-isomers and 43 per cent t10, c12-isomer.

5.0. CONCLUSIONS

At least in experimental models CLA has been clearly shown to be powerful as a preventive agent against different pathologies. Furthermore, the inclusion of CLA among “dangerous” trans fatty acids is not substantiated by any references on possible adverse effects that may justify any limitation of CLA intake. The same is true for vaccenic acid, which has been recently discovered that is efficiently converted to CLA either in humans or rodents (Table-2) where it has been shown to be able to reduce preneoplastic lesions in rat mammary gland (22). Major CLA activities in humans include the followings:
Changes Induced by Dietary CLA

- Reduction of body fat mass in overweight and obese (2000) (19)
- Reduction in serum lipids and body fat (2001) (40)
- Reduction of abdominal adipose tissue in obese middle-aged men with signs of metabolic syndrome (2001) (41)
- Reduction of body fat in healthy exercising humans (2001) (42)
- Effects on fatty acid and glycerol kinetics (2001) (43)
- Maternal supplementation with CLA decreases milk fat (2002) (23)
- Increase of immune response (2002)

Conversion of Vaccenic Acid to CLA

- Increase of CLA by dietary trans fatty acids (1998) (44)
- Vaccenic acid is converted to CLA (2000) (38)
- Conversion of vaccenic acid to CLA is about 20 per cent (2002) (45)

The use of t10, c12 may have some adverse effect in lactating women for its activity in decreasing milk fat (23). The supplementation of t10, c12 CLA for decreasing body weight may be advised in people that already possess risk factors such as obesity, hyperlipidemia and diabetes (20). The c9, t11 CLA can safely be vehicled with food, since no adverse effects have been reported so far. For this reason, naturally CLA enriched dairy products could represent a good way to deliver CLA and vaccenic acid in a certain amounts.

6.0. REFERENCES


PROBLEMS WITH BIOFILMS AND THEIR ELIMINATION

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1.0 INTRODUCTION

The dairy industry used a great deal of water and with out it, would be hard pressed to stay in business. Miles and miles of pipes carrying it are found everywhere around a dairy plant. But while, we all known about the problems of furring up pipes by mineral deposits much less is known about the deposition of organic substrates and the subsequent formation of slimy layer known as biofilms.

The term “Biofilm” refers to the biologically active matrix of cells and extracellular substances in association with a solid surface (Bakke et al., 1984). On most of the occasions where biofilms are a nuisance, the term biofouling is generally implied. Biofouling refers to the formation of living microorganisms and their decomposition products as deposits on the surface in contact with the liquid medium i. e milk. The matrix of the biofilm consists largely of water 98-99 per cent and remainder is an assortment of extrapolymers (polysaccharides, glyco proteins) which are referred to as extracellular polymeric substances (EPS) (Christensen and characklis, 1990). The micro-organisms initially are deposited on the surfaces and actively multiply to form of colony of cells. These cells become large enough to entrap organic and inorganic debris, nutrients and other microorganisms leading to the formation of microbial biofilms.

Milk is a complex emulsion containing mineral components and organic compounds and many of these are simply deposited on the contact surfaces forming residual build up which serve to protect the associated bacteria from cleaning procedures and disinfectants and at the same time provides a source of nutrients which triggers bacterial population. The factors affecting the formation of biofilms are:

- pH
- Temperature
- Micro topography of milk contact surface
- Type of organic matter processed (Whether it is milk, skim milk or whey etc)
- Water activity
- Characteristic of organisms
- Flow rate
- Oxygen content

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Problems with Biofilms and their Elimination

1. Availability of nutrients

1.1 pH and Temperature

pH and temperature of the contact surface also have an influence on the degree of adhesion of micro-organisms. *Ps. fragi* shows maximum adhesion to stainless steel surfaces at the pH of 7 to 8 (Stanley, 1983). *Y. enterocolitica* adhered better to stainless steel surfaces at 21°C rather than at 35°C or 10°C (Herald and Zottola, 1988).

1.2 Micro-Topography

Micro-organisms always attach to contacted surfaces. In this context, the micro-topography of the milk contact surface is important to flavor bacterial retention, particularly, if these surfaces consist of deep channels and crevices to trap bacteria. The nylon and teflon surfaces are smooth and the microorganisms appear to be attacked. However, stainless steel surfaces have rough appearances due to cracks and crevices and exhibit a sponge like appearance. Such topography allows the escape of entrapped bacteria from the shear forces of the bulk liquid and even the mechanical methods of cleaning would be inadequate (Wirtanen et al., 1996).

1.3 Type of Organic Matter Processed

The adsorption of certain protein surfaces plays an important role in biofilm formation. Milk and its components such as casein and β-lactoglobulin have also been found to inhibit the attachment of *L monocytogenes* and *S. typhimurium* (Helke et al., 1993). However, in the presence of whey proteins an increase in attachment of several milk associated micro-organisms to stainless steel, rubber and glass surfaces was observed (Speers and Gilmour 1985).

Different micro-organisms involved in biofilm formation in different dairy processing are shown in Table 1. In the dairy industry improperly cleaned and sanitized equipment and air borne micro-flora are usually considered to be the major sources of contamination of milk and milk products (Koutzayiotis, 1992). The limitation of CIP is the accumulation of micro-organisms on the equipment surfaces resulting in the formation of biofilms (Mattila et al., 1990). The common sources involved in the biofilm formation in dairy plants are the floors, waste water pipes, rubber seals, bends in pipes, conveyor belts etc. Buna-N and teflon seals have also been implicated as important sits for biofilm formation (Blackman and Frank, 1996). In recent years, membrane technologies like UF and RO have been widely used in the dairy industry. Even a small degree of adsorption causes pore blockage and as a result the filters get clogged and phenomenon called fouling. This fouling of the membranes may also favour the formation of biofilms (Cheryan, 1986).

Bacterial attachment and subsequent survival involve interactions between a bacteria cell, a surface and the surrounding microenvironment. *Lb. Curvatus*, even at low levels on equipment surfaces can potentially cause quality defects in the final cheese product. *L. monocytogenes*, a pathogen which has been recorded in cheese, ice-cream and milk to stainless steel surfaces at various pH and temperatures. The bacterial adhesion was highly promoted by...
the presence of lactose and non casein protein solutions. The consequence of the presence of psychrotropic bacteria in raw milk and dairy products is an important problem in the dairy industry. These are mainly derived from the surfaces of the milking machine reveals the attention especially those concerned with the initial binding and the formation of bacteria colonies at the contact surfaces. Milk soil may provide harbours for survival of bacteria in areas not adequately cleaned or hard to clean, such as gaskets, joints and crevices. *L. monocytogenes* and *S. typhimurium* is two pathogens of major concern to the dairy industry in the biofilm formation. The attachment of these two pathogens altering the microenvironment leading to physiological changes affecting the cell surface profile by producing extracellular matrix which may trap the other cells.

2.0 **MECHANISM OF BIOFILM FORMATION**

If one considers a clean, inert surface is a current of liquid containing organic and mineral molecules and micro-organisms, the formation of biofilm is a dynamic process goes through four stages (Characklis, 1981). They are:

- Transport
- Adsorption of molecules
- Adhesion of cells
- Formation of micro-colony and biofilm formation

2.1 **Transport**

First, molecules are carried towards the surfaces by diffusion or in some cases by turbulent flow. The large particles, including the micro-organisms, are slowed down by the laminar boundary layer; other mechanisms also intervene to explain how the micro-organisms are carried right to the surface (gravity, thermophoresis and chemotaxis, fluid dynamic forces). The nutrient transfer is also more rapid in biofilm than for the bacterial cells in the aqueous phase. The increase in nutrient level favours biofilm formation and is also dependant on the type of competitive culture associated with the biofilms.

2.2 **Adsorption of Molecules**

Molecules are almost instantly adsorbed at the surface and play a part in the events that follow. This “conditioning” (The accumulation of molecules at the solid liquid interface often referred to as the conditioning film) of the surface alters its physico-chemical properties (surface free energies, electrostatic charge) and contains higher concentrations of molecules of nutrients than is found in the mass of the fluid. Nutrient transfer is more rapid within the biofilm than in aqueous phase, towards the bacterial cells in suspension. Thus where the medium is poor in nutrients, copiotrophic cells (those require high nutrient levels) grow better at the interfaces.

2.3 **Adhesion of Cells**

Adhesion is due to various types of forces that operate between the inert surface and the living cell. These are vanderwaal attraction forces, electrostatic forces, hydrophobic interactions, which are generally so called when attractive and are called hydration pressures when repulsive and, steric forces between polymer-coated surfaces.
The physico-chemical properties of the bacterial cell surface are important in determining the adhesion of cells during initial attachment phase (Van-Loosdrecht et al., 1990). The adhesion of cells takes place mainly in two stages:

(i) A reversible adhesion followed by

(ii) An irreversible adhesion

Initial weak interactions developed between the bacterial cells and the substrata are referred to as reversible adhesion. The interaction forces influencing the process are vanderwaal attraction forces, electrostatic forces and hydrophobic interactions.

The irreversible attachment of the cells is the next crucial step in the biofilm development. The repulsive forces mainly prevent the bacterial cells in direct contact with the surface, however, through surface appendages by bacteria such as flagella, pilli and the exopolysaccharide fibrils. In reversible adhesion, various short range forces involved include dipole-dipole interactions, hydrogen bonding, ionic and covalent binding and hydrophobic interactions. The polymeric fibrils form a bridge between the bacterial cell and the substratum and this enables the irreversible association with the surface. In this process the removal of cells requires much stronger forces. Spores exhibit a greater rate of adhesion than vegetative cells to milk contact surfaces. This process is mainly facilitated by the relatively high hydrophobicity, in addition to hair like structures on the cell surface (Ronnoer et al., 1990). On adhesion to surface, spores may germinate and the vegetative cells multiply and produce exopolysaccharides.

3.3 Formation of Micro-colony and Bio-film Formation

The irreversibly attached bacterial cells grow and divide by utilizing the nutrients present in the conditioning film and the surrounding film environment. This leads to the formation of micro-colonies. This colonization results in the formation of biofilms. It is fairly slow process depending upon the culture conditions; it takes from a few hours to several days or even several months to reach a state of equilibrium. During the period, their attached cells also produce additional polymer (EPS), which helps in the anchorage of the cells to surface and to stabilize the colony from the fluctuations of the environment (Characklis and Marshall, 1990).

The continuous attachment of the bacterial cells to the substratum and its subsequent growth along with the associated EPS production forms a biofilm. The micro-organisms within the biofilm are not uniformly distributed. As the biofilm ages, the attached bacteria, in order to survive and colonize new niches, must be able to detach and disperse from the biofilm. On the outer surface of the biofilm, micro-organisms both peel off and adhere. The cells that peel off are mainly daughter cells and are for more hydrophilic than the cells remaining in the biofilm. (Allison et al., 1990). On the cell division, the mother cells remained attached and the daughter cells break away and become plankotinic. Sloughing is discrete process where by periodic detachment of relatively large particles of biomass from the biofilm occur. This can be due to various factors such as fluid dynamics and shear effects of the bulk fluid (Applegate and Bryers, 1991). Presence of certain chemicals in the fluid environment or altered surface properties of the bacteria or substratum. The released bacteria may be transported to newer locations and again restart the biofilm process (Marshall, 1992).
3.4 Extracellular Polymeric Substances

The EPS produced by the micro-organisms plays major role in the biofilm formation as well as firm anchorage of bacteria to solid surface (Christensen, B.E, 1989). It is reported that within the biofilm matrix, many other organic and inorganic substances and particulate matter may get entrapped along with the microbial products and other micro-organisms which join to form a consortium protected by the glycocalyx (Marshall, 1992). EPS production increased with attachment of bacteria to a solid surface and that increase was not due to preferential attachment of a genotypic subpopulation with increased EPS production, as reinoculation of biofilm bacteria into liquid medium resulted in the reduction of EPS production to the level previously found in planktonic cells. These EPS also helps in trapping and retaining the nutrients for the growth of biofilms and protecting the cells from the effects of antimicrobial agents.

4.0 PROBLEMS CAUSED BY BIOFILMS

Biofilms create a number of serious problems for industrial fluid processing operations. Mechanical blockage, impedance of heat transfer processes and biodeterioration of the components of metallic and polymeric systems results in billions of dollars in losses each year. Biofouling in heat exchangers and cooling towers has been a major problem in the dairy industry. The bacterial attachment greatly reduces operating efficiency of the processing equipment (Lehman et al., 1992).

4.1 Detrimental Effects of Biofilm Formation

- Impairment of heat transfer: It has been estimated that 0.05 mm depth of biofilm can reduce heat transfer by about one third.
- Increased fluid frictional resistance: Raising the energy (and therefore cost) needed to maintain a given flow rate.
- System blockages: The growth and or subsequent detachment of biofilm causes blockage of filter traps, dead-legs and distribution channels and can increase sedimentation fouling.
- Enhancement of corrosion: This is due to physical presence of the biofilm and the metabolic activity of biofilm organisms particularly the sulfate reducing bacteria or acid producing bacteria. The micro-organisms create anodes and cathodes on metal surfaces, the unequal distribution of ions causes currents resulting in a metal loss (Costerton and Lappin-scott, 1989).
- Reduction of efficiency of water treatment chemicals, particularly inhibitors and biocides.
- Process contamination: The loss of product integrity due to micro-organisms from the system biofilm, for example deterioration in quality of potable water in distribution systems, intrinsic microbial contamination of formulated iodophor antisepsics.
- Potential health hazards: The incorporation of Legionalla organisms within biofilms in water circulation systems/ the colonization of prosthetic devices by opportunistic pathogens.
- In filtration systems bifilms upped 500μ thick greatly reduces the permeability of the membrane.
5.0 METHODS FOR THE STUDY OF BIOFILMS

Until now the evaluation of bacteria adhesion process in the dairy industry context has tended to employ either microscopy techniques (particularly fluorescence microscopy and electron microscopy) or indirect techniques, such as the determination of the no of colony forming units in bacteriological rinses. More recently, different methods in microscopy, especially scanning electron microscopy (SEM) in direct observation of the various forms of bacterial attachment to milk contacting surfaces (Zottala, 1994).

Interference reflection microscopy, atomic force microscopy and conical laser microscopy and some other techniques that have attracted considerable interest in the study of biofilms (Ladd and Costerton, 1990). Moreover, in the recent past, environmental scanning electron microscopy (ESEM) has also been widely used for biofilm enumeration (Hodgson et al., 1995). This technique helps in visualizing sample without the need of conventional microscopic procedures like dehydration, fixation and staining. Very recently, cellular automation models have found applications for the study of biofilms.

5.0 ELIMINATION OF BIOFILMS IN THE DAIRY INDUSTRY

Since removal of biofilms is a very difficult and demanding task, a complete and cost effective cleaning procedure should be developed (Zottala and Sasahara, 1994). The equipment designs are very essential in achieving better cleanability of the milk contact surface on which biofilms has formed. With time, stainless steel exhibited better hygienic properties by resisting damage caused by the cleaning process when compared to the glass, nylon and poly-vinyl compounds. The biofilms in the dairy industry can be eliminated by adopting different strategies like physical, chemical and biological methods.

5.1 Physical Methods

The new physical methods used for the control of biofilms include:

- Super high magnetic fields
- Ultra sound treatment
- High pulsed electric fields on their own and in combination with organic acids
- Recently, a low electric current in combination with antibiotics was successfully employed for biofilm control. It was observed that the bioelectric effect generated by the combination of antibiotics with low levels of electric current proved more effective in controlling the biofilms. This also suggested that the biofilm age and activity were limiting factors in the antibiotic effectiveness (Jass et al., 1995).
- Mechanical action (Brush, medium- and high pressure jets) are recognized as being highly effective in eliminating biofilms and this is probably the best means we have at our disposal
- Air injected CIP for milking system: Air is injected to increase the circulating velocity and mechanical cleaning action of the wash solutions (Reinemann and Grasshiff, 1993). The wall shear stress that develops can be 10 to 20 times higher than those in flooded CIP system. More effective cleaning was achieved by applying high shear level (114N/m²) and temperature about 60°C.
5.2 Chemical Methods

It is speculated that before application of disinfectant, it is essential to eliminate as many micro-organisms as possible.

- Low concentrations of sodiumhypochloride in the range of 0.5 to 5 ppm are only inhibitory to biofilms associated with stainless steel surfaces.
- With chlorine compounds, $p^H$ is an important treatment variable. The $p^H$ shows acidic side to provide maximal efficiency. High $p^H$ favours the hypochlorite ion promotion of detachment of matured biofilms and low $p^H$ enhances chlorous acid disinfects thin films.
- The isothazolone microbicide, 2-methyl 5-chloro-2-methyl isothazolone has been employed at 10mg/ltr for the successful control of $L$.monocytogenes that is associated with conveyor system in a dairy processing and packaging operation.
- Chlorine sanitizers are using extensively in the dairy industry questions have arisen concerning environmental effects of chlorine sanitizers. Chlorination can pose health risks to human and wild life due to formation of biohalomethanes and other carcinogenic halo compounds. A possible alternative for this is ozone or ozonated water. Like chlorine it is a powerful oxidizing agent. Bacterial membrane lipids, carbohydrates and proteins are oxidized resulting in cell death. Recently, small ozonization units have been developed that can be used in dairy and food industry.

5.3 Biological Methods

By the adsorption of bioactive compounds like bacteriocins onto milk contact surfaces we can control the biofilm formation. Bacteriocins are proteinaceous anitmicrobial compounds exhibiting bactericidal properties. Nisin, the most applied anitmicrobial peptide has proved to be an effective controller of $Cl$.botulinum spores in processed cheese spreads. Enzymes have also proved to be effective in cleaning the extracellular polymers which form the biofilm matrix and thus help in removal of biofilms (Kumar, 1997 & Potthoff et al., 1997). The specific enzyme requirements mostly vary according to the type of microflora making up the biofilm. The mixture of $\alpha$-amylase, $\beta$-glucanase and protease was the most effective. Very recently, an enzymatic preparation comprizing of EPS- degrading enzymes, derived from streptomycin isolate was reported for the removal and prevention of biofilm formation (Ganesh Kumar, C and Anand S.K, 1998).

The important aspects in controlling biofilm formation and/or minimization include proper choice of equipment, materials and accessories, correct construction, process layout and process automation. In addition, the quality and smoothness of equipment were found to be equally important.

6.0 CONCLUSION

The controls of biofilms represent one of the most perisistent challenges within food and industrial environments where the microbial communities are problematic. Recently, acquired data on biofilm give a better understanding of:
- How biofilms build up on moist surfaces even if the nutrient supply is poor and irregular.
- How micro-organisms are protected from cleaning operations in the biofilms.
- How the physiological state of micro-organisms in the biofilm may explain.
- Why it is so difficult to count them and to destroy them.

In practical terms, no guidelines can yet be given to dairy industry on the choice of materials or the consequences of fouling/cleaning/disinfection cycle. However, we can recommend the following:

- Use of materials smooth, nonporous and resistant to wear and or surfaces and equipment without inaccessible corners.
- Mechanical operations to detach biofilms, while avoiding dispersing microbes into other undesirable places (Careless use of water jets).
- Use of peracetic acid and chlorinated alkalines, as established disinfectants.
- Cleaning at short intervals to prevent excessive build-up of biofilms; and as water is indispensable for biofilm production and dry work shop is biofilm-free work shop.
- Thorough drying whenever possible.

It is important to understand the interactions between bacteria and the surfaces in specific dairy processing and food service environments and the impact of surface associated bacteria on cleaning and sanitizing to provide more effective measures for prevention of biofilm formation and for biofilm removal in dairy plants.

Table-1: Microorganisms Involved in Biofilm Formation in Different Dairy Processing

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>Occurrence</th>
<th>Adhesion surface</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Str thermophilus</em></td>
<td>Milk pasteurization</td>
<td>Plate exchanger</td>
</tr>
<tr>
<td><em>Bacillus Spp</em></td>
<td>Milk RO and UF</td>
<td>Membranes</td>
</tr>
<tr>
<td>Thermophilic nonspore forming bacteria</td>
<td>Milk or whey evaporators</td>
<td>Pre-warming section</td>
</tr>
<tr>
<td><em>L.monocytogenes</em></td>
<td>Laboratory test</td>
<td>Steel chips</td>
</tr>
<tr>
<td><em>Y.enterocolitica</em></td>
<td>Laboratory test</td>
<td>Steel chips</td>
</tr>
<tr>
<td>Unspecified microflora</td>
<td>Water desalination</td>
<td>Ion exchange resins, RO membranes</td>
</tr>
<tr>
<td><em>Acinetobacter</em></td>
<td>Milk transfer</td>
<td>Stainless steel pipe line</td>
</tr>
<tr>
<td><em>B.subtilis</em></td>
<td>Laboratory tests</td>
<td>UF membranes</td>
</tr>
<tr>
<td><em>Ps.aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: (Speersand Gilmour, 1985).

7.0 REFERENCES
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Problems with Biofilms and their Elimination


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1.0 INTRODUCTION

Protein hydrolysis is an ancient technology used for centuries all around the world. Today, Protein hydrolysates have assumed a new dimension in food industry. They find extensive use in wide ranges of soups, gravies, sauces, drinks, vegetable and fruit juices, flavourings and nutritional, dietetic and formulated foods. Protein hydrolysates are boon to people who are suffering from protein allergy or stomach disorders and to those who require easily digestible foods. The production of hydrolyzed protein provides an opportunity for the dietary management of patients with various digestive disorders as a result of pancreatic malfunction, pre- and post-operative abdominal surgical patients, patients on geriatric and convalescent feeding and others who for various reasons are not able to ingest a normal diet. Protein hydrolysates also have pharmaceutical applications in intensive care foods, anemia treatment, prevention of blood cholesterol, treatment of dental diseases and in administration of amino acid mixture intravenously.

Enzymatic hydrolysis of protein is preferred over acid or alkali hydrolysis as the former causes no destruction or racemization of the amino acids and produces hydrolysates with low salt content (Deeslie and Cheryan, 1988). The manufacture of protein hydrolysates is a well-established process for the utilization of protein rich food by-products, wastes and non-conventional food proteins. Historically, soy sauce was the first protein hydrolysat. The fundamental flavour characteristic of a protein hydrolysate is suggestive of meat flavour. Therefore, hydrolysates are used by packer, canners and other manufacturers, who wish either to accentuate or to suggest the flavour of meat. Fish protein concentrates and egg albumin are two popularly used animal products as substrate for hydrolysates, while soy protein is the extensively used vegetable source. Casein is an important substrate for the preparation of milk protein hydrolysates. The hydrolysate of each protein has distinct flavour characteristic. Different proteins may be blended to give a specific flavour characteristic. Lighter and more delicately flavoured hydrolysates are derived from casein, corn and rice proteins and are preferred for fish and pork as well as poultry. Darker and heavier hydrolysates, on the other hand, are derived from soy, yeast and gluten and are preferred for beef and mutton.

2.0 PROTEOLYTIC ENZYMES

Proteolytic enzymes have the ability to hydrolyse proteins into peptides and amino acids and could be obtained from plant (Papain, ficin, bromolain), animal (Pepsin - from stomach secretion, trypsin - from pancreatic secretion, rennin - from calf stomach) or microbe (Neutrase, Alcalase, Esperase, Pronase, etc.). As per their specificity of action they can be classified into exopeptidases and endopeptidases.
- Exopeptidases refer to enzymes which split terminal amino acids from one end of the chain by hydrolysis of peptide bond - mostly microbial enzymes. One can distinguish the Carboxy (exo) peptidases which act on the terminus of chain carrying free carboxyl group and the Amino (exo) peptidases which start from the other end i.e. the terminus of chain carrying free amino group.

- Endopeptidases have preference for certain side chains on amino acids adjoining peptide bond and are usually divided into three groups: (i) The pepsin type of protease is characterized by a preference for amino acids with free carboxyl groups, (ii) Trypsin types are characterized by a preference for amino acids with basic group, and (iii) Chymotrypsin types are characterized by a preference for amino acids with aromatic or bulky chains. Papainase type enzymes (Papain, chymopapain, ficin, bromelain) are endopeptidases, but are difficult to classify under this scheme.

Choice of the protease for protein hydrolysis depends mainly on its specificity and also on its pH optimum, heat stability and the presence of activators or inhibitors. The production of protein hydrolysates for flavour purposes requires enzymes of broad specificity which lead to extensive hydrolysis into low molecular weight peptides and amino acids. Individual endopeptidases do not split all or even a majority of the peptide bonds in a protein system leading to the formation of bulky, hydrophobic acid chains which give bitter taste. Exopeptidases are reported to hydrolyse carboxyl and amino terminal amino acids of such peptides, thus eliminating the bitter taste.

3.0 ENZYMATIC PRODUCTION OF PROTEIN HYDROLYSATES

The extent of protein hydrolysis, which represents the extent of protein breakdown to peptides and amino acids, is expressed either as percent amino nitrogen or as degree of hydrolysis (DH). Degree of hydrolysis is the ratio of the number of peptide bonds cleaved and the total number of peptide bonds in the intact protein. In the manufacture of protein hydrolysates, per cent DH is one of the important controlling factors, which reflect on the product quality.

Hydrolysis of protein has been carried out both as a single stage process (where enzyme is added once during the hydrolysis period) and a two-stage process (where two or more enzymes are added at subsequent intervals of hydrolysis). The general steps in the manufacture of protein hydrolysates include suspension of protein in appropriate amount of water, incubation with enzyme at appropriate pH and temperature in the presence of preservative like chloroform, removal of insoluble material by centrifugation, pasteurization to inactivate enzymes, concentration, drying and packaging in moisture proof container.

3.1 Single Stage Hydrolysis

In single-stage hydrolysis process, the common enzymes used have been pepsin, papain, trypsin, pancreatin and microbial proteases. Kodjev et al. (1974) used 0.4 per cent pancreatin as the proteolytic enzyme at pH 8.2-8.7, temperature 65°C, yeast preparation 0.2 per cent and sodium triphosphate 6 per cent as the optimal conditions for the manufacture of casein hydrolysate with improved flavour qualities. In other study three enzymes - pancreatin, proteinase of Aspergillus oryzae and Lactobacillus helveticus were added to 10 per cent casein solution and incubated at 50°C for 24 h. Inactivation of proteolytic enzymes was done by heating at 85°C for 15 min (Takase, et al., 1979). The solution was cooled to 5°C and centrifuged at 1,200 x g for 20 min to remove the precipitate. About 91 per cent of the total casein nitrogen remained in the supernatant.
Vegarud and Langsrud (1989) manufactured hydrolysates from commercial sodium caseinate using various commercial proteolytic enzymes: Corolase PS, Corolase L10, Maxatase LS 400,000 and Novozym 257. Hydrolysis was carried out in a pH-stat at constant pH using IM NaOH for 5 h at 40°C at pH 6.7-7.0 in a vessel, with enzyme substrate ratio in the range of 0.1 to 5 per cent. The hydrolysis process was terminated by inactivating the enzyme by heating at 100°C for 5 min.

### 3.2 Two Stage Hydrolysis

In a two step process, peptic predigestion of protein followed by hydrolysis with pancreatin or trypsin, papain followed by leucine aminopeptidase and pancreatin followed by pepsin have been tried.

A process was described for the production of casein hydrolysate on commercial scale with 12 Kg casein suspended in 220 l water at pH 6.2-6.3 and digested with papain at 40°C for 18 h and then with a pig kidney homogenate (serving as a source of exopeptidases) at pH 7.8-8.0 for 24 h. The hydrolysate produced was passed through a separator to remove insoluble material followed by heating at 83-85°C for 3-5 min, concentration upto 20-23 per cent TS and spray drying (Clegg et al., 1974). However, this was not an efficient process for production of debittered hydrolysate, as it is costly, time consuming and results in production of significant amount of free amino acids (Clegg et al., 1974; Fujimaki et al., 1970 & Helbig et al., 1980). Clegg and McMillan (1974) used first endopeptidase papain (4%) and then an exopeptidase leucine aminopeptidase (0.015%) to obtain casein hydrolysates with reduced bitterness and high (46%) free amino acids.

Cogan et al. (1981) suggested use of papain and pepsin. Treatment with Rhozyme enzymes was performed with specified enzyme concentrations under the following conditions of pH and temperature: Rhozyme P-11 and Rhozyme 41 at pH 8.5, 50°C, Rhozyme P-33 at pH 7.5, 60°C and Rhozyme 62 at pH 8.3, 60°C. Khanna and Gupta (1996) reported enzymatic production of casein hydrolysates in a short hydrolysis period of 8 h. Sodium caseinate solution (10% TS) at pH 7.0 was observed to be optimum for hydrolysis with papain. Four per cent papain in the first stage and 0.4 per cent pancreatin in the second stage, each stage with 4 h of hydrolysis, were found to give optimum degree of hydrolysis. However, the casein hydrolysate so produced was definitely bitter. The bitterness was removed using activated carbon treatment.

### 4.0 MANUFACTURE OF WHEY PROTEIN HYDROLYSATES

Whey protein concentrate was hydrolysed using the technical food grade enzyme Corolase 7092 in order to eliminate the allergenicity of whey proteins. Ultrafiltration of the hydrolysates appeared to be necessary to obtain a hypoallergenic product. The minimum molecular weight capable of eliciting immunogenicity and allergenicity of whey protein hydrolysates appeared to be between 3000 and 5000 Da, so the molecular weight cut off value of the filters required must be in this range. Although there was no evidence that extensively-hydrolysed whey protein is nutritionally inferior to casein, the slightly bitter taste might reduce food intake (Beresteijn et al., 1994).

A process has been developed for hypoallergenic and low-bitterness hydrolysates from whey proteins that are suitable for use in infant formula. Of the proteinases tested, Alcalase 2.4 L, papain W-40 and proleather were found to be effective in reducing the antigenicity of WPC. The hydrolysate prepared from papain W-40, had no bitterness and was thus selected as the most suitable treatment for reducing antigenicity (Nakamura et al., 1993). Ziajka et al. (1994) prepared whey protein concentrate by adding trypsin or pepsin to dried whey protein and dis-
tilled water at enzyme:substrate ratios of 0.1, 0.2, 0.3 and 0.4 and incubating for 6 h at 37°C followed by heating at 85°C for 20 min and spray drying. The bitterness in all hydrolysates was not strong, probably because of the masking of salts formed during hydrolysis. The hydrolysates could be used for the production of specific dietetic formulae or for protein fortification of acidic beverages.

5.0 CONTINUOUS HYDROLYSIS OF PROTEINS IN MEMBRANE REACTOR

The development of membrane technology has led to a new concept: continuous reaction and simultaneous separation of the product from the reaction mixture (Mannheim and Cheryan, 1990). A membrane module with the appropriate pore size and physicochemical properties is incorporated into the reactor which contains the enzyme. Feed is continuously pumped into the reaction mixture while product is continuously withdrawn as the permeate. The molecular size of the product can be controlled by proper selection of the pore size of the membrane. The enzyme is recycled and reused, thus improving enzyme utilization and overall productivity. Chiang et al. (1995) worked on producing casein hydrolysate continuously by hydrolysis of bovine casein with protease type XXIII (from *Aspergillus oryzae*) in a pilot scale formed-in-place membrane reactor. A high percentage (>99%) of TCA-soluble nitrogen in the hydrolysate (product) was achieved after 45 min at 37°C and pH 7. The product was completely soluble over pH range 2-9. Water sorption increased 4-6.5 times at water activity of 0.35-0.95 as compared with intact casein. The immunologically active casein and immunologically active whey proteins in the product were reduced 99 and 97 per cent, respectively.

6.0 DEBITTERING OF PROTEIN HYDROLYSATES

Although the hydrolysates manufactured by enzyme process have distinct advantages, bitterness problem is the main obstacle for their use in food preparations. Several attempts have been made to overcome this fundamental problem and to make the product more palatable. The practice include further hydrolysis of bitter peptides by exopeptidases (carboxy-peptidase A, leucine aminopeptidase), addition of cyclodextrins to hydrolyzed proteins, application of hydrophobic chromatography, absorption of bitter peptides, plastein reaction and masking the bitterness.

Tossavan et al. (1983) eliminated the bitter peptides by exopeptidase or absorption by resin. Umetsu et al. (1983) successfully made use of carboxypeptidases for debittering of bitter peptides from milk casein. Minagawa et al. (1989) reported that aminopeptidase T hydrolyse hydrophobic amino acid residues at N-terminal of peptides and proteins and hence remove bitter components from bitter peptides. Roland et al. (1978) described application of hydrophobic chromatography to peptide solutions for debittering casein and soy protein hydrolysates. It has been explained that during chromatography, binding forces occurring between the structurally similar phenolic resin and peptide amino acid residues (containing artomatic/heterocyclic side chains) delay the emergence of these bitter components, permitting selective preparation of a non-bitter peptide hydrolysate. Debittering by hydrophobic chromatography on hexyl Sepharose 6B was described by Lalasidis and Sjoberg (1978) who also showed that solvent extraction of hydrolysates with azeotropic secondary butanol is very effective in reducing bitterness.

The activated carbon treatment method has been found safe, promising and most effective in the elimination of the bitter taste. Cogan et al. (1981) successfully eliminated the bitterness in the hydrolysates obtained from Rhozyme 62 by employing 0.5 g of activated carbon per
g protein and storing it for 60 min at 25°C. However, such treatment was accompanied by a selective loss of tryptophan (63%), phenylalanine (36%), arginine (30%) and 26±2 per cent of protein nitrogen due to adsorption of peptides and amino acids on to the activated carbon. As a remedial measure, supplementation of the treated hydrolysates with the proper amounts of tryptophan and phenylalanine was suggested for the production of casein hydrolysates of acceptable taste and high nutritive quality. Helbig et al. (1980) obtained soluble casein hydrolysates for use in acid beverages by stirring 5 per cent casein hydrolysates solutions with 10 per cent carbon of various kinds and mesh sizes at 22°C for 2 h or at 90°C for 10 min. Khanna and Gupta (1996) observed that minimum 15 per cent activated carbon treatment was necessary for debittering the casein hydrolysate, though this treatment resulted in 40.90 per cent N loss through adsorption on activated carbon. The yield and recovery of liquid casein hydrolysate were 47.98 per cent and 46.23 per cent, respectively. The liquid product had 10.25 per cent TS, 1.93 per cent nitrogen, 1.19 per cent ash and a low viscosity of 1.99 cP at 20°C.

The debittering of skim milk and casein hydrolysates (Produced by various proteinases) by a variety of hydrophobic and absorption chromatographic media was assessed by Helbig et al. (1978). Among different adsorption methods employed, activated carbon and β-Cyclodextrin were found to be more effective. Suzuki et al., (1981) claimed reduction while Szejthl (1982) reported removal of bitterness from casein hydrolysates through addition of 10 per cent β-Cyclodextrin. The saltiness of the dibittered hydrolysates was decreased by ion-exchange chromatography on Amberlite IR-45. Hydrophobic chromatography based on delay of the emergence of bitter compounds was successfully employed by Roland et al. (1978). However, Ma et al. (1983) reported this method to be impractical and costly for application to casein hydrolysates.

The plastein reaction has been claimed to eliminate bitterness present in hydrolysate and to possibly incorporate limiting essential amino acids. Cheeseman (1981) described the plastein reaction as a process in which proteins are broken down by proteolytic enzymes into a mixture of peptides and amino acids and are then resynthesized enzymatically into products. Plastein has been defined as the product formed during the interaction of concentrated peptide solutions with proteinases which is insoluble in 10 per cent TCA, 70 per cent ethanol or acetone (Fujimaki et al., 1977) and as almost water insoluble polypeptides having an ill-defined variable structure, the formation of which involves a peptide chain elongation process. During plastein synthesis, a high concentration of hydrolysate (30-50%) is incubated with an enzyme or heated to form viscous gel like material.

Farr and Magnolato (1981) debittered the whey protein hydrolysates by making contact of hydrolysate for 5-10 min. at 10-60°C with an absorbent consisting of desugared carob particles. Stanle (1981) tried masking of bitter flavour by cohydrolysing chicken protein with gelatin, the active agent in gelatin is thought to be glycine. Arai (1980) masked the bitterness of the peptic hydrolysate of soyglobulin and the tryptic hydrolysate of casein by acidic oligopeptides.

6.0 REFERENCES


1.0. INTRODUCTION

The major impact of biotechnology in the field of food and agriculture has primarily been seen in relation to agricultural production practices as a determinant of the quality of produce. Bioprocess technology is only an extension of this view. However, quality assessment of agricultural products including animal products employing biological principles is no less important. And it is in this context that dairy and food industries stand to benefit a great deal from biotechnological applications, where biosensors have an important role to play as product-quality monitoring tools based on biochemical and/or microbiological mechanisms. With their high degree of specificity or selectivity for the analyte, biosensors have become particularly relevant in the food industry trying to catch up with the ever-growing need of quality assurance and product safety. The present discussion briefly includes what biosensors are and further deals with how they could be useful for the dairy and food industries whose quality and safety concerns are extending from the laboratory to the processing premises.

2.0. NATURE AND TYPES OF BIOSENSORS

A sensor is a device to transform a quantity to be detected or measured into another quantity accessible to human senses or to an acquisition system. It comprises an energy transfer mechanism, which converts a physical, chemical or biochemical signal into a physical one, which is suitably amplified and displayed. For example, a pH meter senses hydrogen ions in terms of potential difference between electrodes and displays the pH value. In a biosensor one or more biological events mediate the energy transfer, which results in analyte recognition and quantification. Thus, a biosensor comprises a biological catalyst (biocomponent or biorecepter), usually in intimate contact with a suitable physical transducer (a device converting one form of energy into another) such as an electrode or optical detector, and electronics (for amplification and display) (Fig. 1). The bioreceptor gives the biosensor its
selectivity or specificity. The analyte may be biological in nature such as a certain pathogen, or a biochemical or a chemical entity e.g. an antibiotic or glucose. The biocatalyst (a) converts the substrate (S) to product (P). This reaction is determined by the transducer (b) which converts it to an electrical signal. The output from the transducer is amplified (c), processed (d) and displayed (e).

The bioreceptor involved in rapid recognition and detection of specific molecules causes a chemical change (e.g. production of chemical entities such as hydrogen peroxide), or produces physical effects (e.g. changes in temperature, fluorescence, etc.) in the medium that can then be monitored by the physical transducer. While, enzymes, organelles, whole cells (of bacterial, fungal, plant or animal origin), tissues, nucleic acids, antigens or antibodies serve as bioreceptors, the effects of the biocatalytical reaction will determine the type of physical transducer to be employed. Thus, a bioreceptor, a physical transducer and suitable electronics all put together into a handy tool is what we call a biosensor (Fig. 2).

Fig.-2: A Biosensor for Depth Profile of Glucose Concentration as a Function of Spoilage of Meat

Source: Scott (1998)

The biocatalyst may be entrapped or immobilized on to a membrane of selective permeability and the membrane is assembled in a close contact with the physical transducer, which in turn is in intimate proximity with the electronic component(s). Miniaturization of biosensors has been achieved by direct adsorption of the bioreceptor onto the transducer surface, thereby eliminating the membrane layer (Fig. 3). Further attempts in this direction aim
at fixing the biocatalyst right onto an electronic element, which would directly sense and amplify the changes in the analyte. Such ‘biochip’ sensors, if produced economically, could be particularly valuable in in-package monitoring of product quality.

A variety of physical transducers have been used in biosensors. In optical transducers signal transduction is effected through measurement of fluorescence, absorbance, refractive index, interferometry, diffraction or polarization. In mass transducers change in density, elasticity or conductivity is transduced by acoustic or piezoelectric principles. Electrical transducers work on the principles of potentiometry, amperometry, conductance, capacitance, impedance, etc. Thermal transducers used in conjunction with certain heat-evolving reactions monitor temperature changes by thermistor devices. Other types of physical transducers involve the use of semiconductors, or electrochemical-sensitive transistors e.g. Field Effect Transistor (FET), which could be coupled with an ion-selective membrane to give an ISFET.

Biosensors are generally classified on the basis of the type of bioreceptor used. The major types are enzymic biosensors, immunosensors, nucleic acid sensors (DNA probes), microbial sensors, etc. The type of biosensor will determine the application, but biosensors have been developed to handle several analytes without losing sensitivity. Actual and potential applications of different types of biosensors are discussed in the following sections.

3.0. ENZYMIC BIOSENSORS

Enzyme-based biosensors were the first to be developed, and enzyme electrodes are still among the most commonly used biosensors. Depending on the analyte, either a single-enzyme or multi-enzyme system is used. For instance, single-enzyme systems consisting of glucose oxidase or lactate oxidase are commercially available and are routinely used in bio-processes for determination of β-D-glucose:

\[
\text{Glucose + O}_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

The enzyme is either coupled to a preactivated synthetic membrane e.g. polyamide membrane or entrapped in a polymer matrix, which can be used for direct coating of the transducer to form the enzyme electrode (Fig. 4). A glucose electrode on glucose could detect as low concentration as \(10^{-8}\) M with a response time of 30 seconds and an operational stability of more than 6 months. A self-contained, semi-automatic analyzer employing lactate oxidase...
of more than 6 months. A self-contained, semi-automatic analyzer employing lactate oxidase has been reported to provide accurate measurement of L-lactate in several dairy products including cream cheese, yoghurt and whey. A fructose dehydrogenase-based sensor has recently been tested for sucrose determination in sweetened condensed milk.

A multi-enzyme electrode may use two or more enzymes e.g. β-galactosidase co-immobilized with glucose oxidase or galactose oxidase for determination of lactose in milk:

\[
\text{Lactose} + \text{H}_2\text{O} \xrightarrow{\beta\text{-galactosidase}} \text{Glucose} + \text{Galactose}
\]

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

Enzyme biosensors, often amperometric, have been studied for application in determination of lactose in milk and milk products, lactulose in heat-treated milk, L- and D-lactates in milk, L- and D- amino acids in aged milk, antibiotic residues, enzyme-inhibiting pesticide residues, and urea in milk. Flow injection analysis employing enzyme electrodes for determination of pesticides, sugars, etc. has been tested.

Application of an immobilized enzyme gel layer over the ion-selective membrane of an ISFET results in an enzyme FET (ENFET), which enables potentiometric measurement of specific ions produced by enzyme action in the medium. The thermal enzyme probe (TEP) is a calorimetric biosensor employing an enzyme immobilized onto a thermistor, which measures the heat released as a result of the enzymic reaction. For example, action of the respective oxidases on cholesterol and glucose, and that of catalase, penicillinase, trypsin, amylase, invertase, urease, and uricase on hydrogen peroxide, penicillin G, peptides, starch (or amylose), sucrose, urea and uric acid, respectively are all exothermic reactions, and therefore represent potential applications of TEPs.

4.0. IMMUNOSENSORS OR BIOAFFINITY SENSORS

Biosensors relying on the high degree of specificity of the antigen-antibody reaction have recently been developed employing a piezoelectric crystal. An alternating electric field applied across a piezoelectric oscillator, such as a quartz crystal, results in small mechanical deformations. At a certain frequency mechanical and acoustic resonance is induced. The resonant frequency being a function of the crystal mass, adsorption of molecules on the crystal surface leads to a frequency change, which forms the basis of the analyte measurement.
Surface acoustic wave (SAW) immunosensors react to changes in the acoustic properties of the surface when an analyte e.g. an antigen binds to a particular binding agent such as an antibody. Such devices are useful for detection of, say, pathogens but can also be designed for assaying of aroma compounds by fixing a film having specific adsorbing properties onto the surface of a sensor. When this adsorbing film carries antibodies specific to the odour molecules as proposed by Unilever scientists in the Netherlands, the sensor is truly a ‘biosensor’.

However, often the odour sensors are based on non-biological receptors such as metal oxide semiconductors, or organic polymers. These latter sensors, devoid of biocatalysts, can more appropriately be designated as ‘bio-electronic devices’. Of course, e-nose and e-tongue in the form of bench-top analyzers or handheld devices have a considerable potential in quality assurance in the dairy and food industries. Typical immunosensors have also been tested for detection/determination of antibiotic residues such as penicillin and sulphamethazine, and enterotoxins in milk. Recently determination of biotin and folic acid in infant formula using an immunosensor has been reported. Polyclonal and monoclonal antibodies have been used for detection of Salmonella groups B, D & E with the help of an optical biosensor. Having 100 PER CENT specificity and sensitivity comparable to that of ELISA and PCR methods.

5.0. **NUCLEIC ACID PROBES AND MICROBIAL BIOSENSORS**

Nucleic acid sensors involve detection of a unique sequence of nucleic acid bases through hybridization. Upon bringing together of a DNA probe with DNA from an unknown sample, combination of the probe with the unknown nucleic acid through pairing of complementary-bases enables detection and identification of the organism. Commercially available DNA probes for the detection of Salmonella, Listeria, *E. coli* and *Staphylococcus aureus* can go a long way in successful implementation of safety assurance programmes of the dairy industry.

Biosensors in which microbial cells are coupled to the physical transducer are used to measure the concentration of a substrate, which the microorganisms utilize. The metabolic activity of the immobilized cells is measured by an electrochemical change, which is proportional to the substrate concentration. An ammonia gas sensor employs nitrifying bacteria immobilized on an oxygen electrode. As the bacteria oxidize the ammonia present, oxygen concentration goes down in direct proportion to the substrate ammonia. Microbial biosensors have considerable potential in chemical analysis of milk and related products. In a very recent application of a microbial sensor, cobalt and zinc concentration were related to the growth of *Saccharomyces cerevisiae* on an amperometric biosensor.

6.0. **CONCLUSION**

Biosensors represent an important area of biotechnological applications in the product quality monitoring for the food industry. As compact, convenient instruments based on biological or biochemical catalysts, biosensors represent a valuable biotechnological application in food product assessment. The potential advantage of using biosensors in food analysis is a rapid, specific quantification without the need for extensive sample preparation. Biosensors also offer the possibility of measuring an analyte in its natural microenvironment thus avoiding analyte modification during sample preparation. Convenience and simplicity of use are other merits of biosensors. Real-time measurement in a process line on account of rapid response suggests the potential application of biosensors in effective on-line and off-line product/process monitoring. Biosensors representing a group of instrumental devices based on biological interactions/catalysis coupled with physical transducers and electronics are emerging as a revolutionary analytical technique having diverse applications in the area of dairy and food processing.
Among various biosensors, enzyme-based biosensors have been most frequently reported for dairy and food applications e.g. determination of lactose, lactulose, lactic acid, amino acids, pesticide residues, antibiotic residues, etc. in milk and milk products. The ability of immunosensors to detect pathogens makes them very valuable for the food industry. Immunosensors have been found applicable also in detection of antibiotic residues and enterotoxins. There are many potential applications of biosensors including determination of triglycerides, cholesterol, starch, etc. in dairy and food products. However, before the potential of biosensors as a tool of quality and safety assurance is realized problems associated with their use e.g. short life, limited commercial availability, etc. would have to be overcomed. With the pace of current developments in biosensors, it should nevertheless be possible for the dairy and food industries to enhance their quality management programmes with the help of biosensors in days to come.

7.0. REFERENCES


1.0. INTRODUCTION

Biological conversions employing enzymes in free and soluble form, as an catalyst, have been used in food products, pharmaceuticals and vitamins. Crude enzyme preparations have been used in food processing since prehistoric times for processes like meat tenderization, brewing and distilling and cheese manufacture. The increased availability of commercially pure enzymes has expanded and diversified the range of applications, such as (1) those in which enzyme treatments form an essential part of the process; (2) those in which the enzymes are used to improve the product quality where alternative methods may or may not be available; (3) those in which enzymes are used to improve the economic or technological aspects of a process. In the recent past, added enzymes have been perceived but as food not as a food additive, the application of which is govern by stringent food legislations for consumer protection. This has led to the development of immobilized enzyme systems for food usage, overcoming the disadvantages of soluble biocatalysts.

Immobilization is the techniques, which confine a catalytically active enzyme within a reactor system and prevents its entry into mobile phase carrying the substrate and the product. It can be recovered after usage or it can be continuously used and not lost in the product. Classically the term immobilization has been used to describe an enzyme that has been physically and chemically attached to a water insoluble matrix, polymerised into water insoluble gel, entrapped within water insoluble gel matrix or water insoluble microcapsule. Some of the enzymes used as catalysts in food processing and their functions are listed in table-1.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Functions</th>
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</thead>
<tbody>
<tr>
<td>Amylases</td>
<td>Liquefied starches</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Lactose hydrolysis</td>
</tr>
<tr>
<td>Catalase</td>
<td>Destroying hydrogen peroxide in milk used for Cheese manufacture</td>
</tr>
<tr>
<td>Invertase</td>
<td>Invert syrups</td>
</tr>
<tr>
<td>Papain</td>
<td>Beer clarification</td>
</tr>
<tr>
<td>Pectinase</td>
<td>Juice clarification</td>
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<tr>
<td>Rennin</td>
<td>Cheese curd formation</td>
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IMMOBILIZATION OF ENZYMES FOR FOOD PROCESSING

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2.0. TECHNIQUES OF ENZYME IMMOBILIZATION

Various physical and chemical techniques have been used to immobilise different enzymes on a variety of inert support materials that are described hereunder.

2.1. Adsorption

This technique involving attraction of opposite electrical charges and other physico-chemical bonds is probably the cheapest and least complicated system. The enzyme is localised through physical forces viz electrostatic interaction, ionic bond formation, protein-protein interaction etc. However, the immobilised enzyme may be displaced from the support during product processing as food products usually contain relatively high levels of ions and may vary in pH. Adsorbed enzymes may find applications in simple food system such as dilute sugar solutions.

2.2. Entrapment

Enzymes can also be entrapped in semi-permeable membranes or in pour gels. It is necessary that the substrate and/or product molecules to be small enough to diffuse rapidly in and out of the membrane or gel and there should be no electrostatic repulsion between the membrane and the substrate. However, entrapped enzymes are either unable to act or show very low activity against large molecules like protein and starch. The most widely used polymer is polyacrylamide while entrapping material may include silica gel and starch.

2.3. Covalent Bonding

Immobilization of enzymes by covalent bonding to activated support matrix is the most studied method. Although often tedious, it provides an immobilized enzyme that is firmly bound to its polymeric support. This method is based on the formation of covalent bond between the residue of the enzyme molecule and the functionalised polymer. Chemical bonding involves preparation of reactive sites on the support matrix by chemically activating itself or by linking reactive molecules to the support. Enzymes are then added which forms the covalent bond with the reactive site on the support. The support material should be hydrophilic and should not have any electrostatic charge. The particle size should be large enough to allow flow through the bed of particles in a column. The processes for immobilization can be classified on the basis of coupling reactions involved namely–acylation, alkylation, carbodamylation, aminidenation, diazo reaction, bifunctional reagent, acid chloride coupling, etc. Covalent bonds are stronger than bonds involved in adsorption.

3.0. BIO-REACTOR TYPES FOR IMMOBILIZED SYSTEMS

Various types of bio-reactors have been developed both batch and continuous where immobilized enzymes systems can be used for purpose of specific reactions.

3.1. Stirred Tank Reactor

It is a kind of vat with an agitator to keep the immobilized enzyme uniformly suspended throughout the tank. Therefore, it is necessary that the enzyme action is rapid to bring about instantaneous depletion of the substrate so that the level of substrate removed with the product is minimum. The most common reactor type was dead end stirred cell while the other type is cross flow or flow through system and operates as continuous stirred tank reactor.
3.2. Tubular Reactor

Tubular reactor may be semi-permeable membrane, semi-permeable fibres or porous sheets. The enzyme is usually trapped in the membrane or fibre and the substrate diffuses into while the product diffuses out of the membrane. It is possible to have both the enzyme and the substrate within the semi-permeable membrane and the product diffusing out. The porous sheet is generally coiled to form a cylinder and the substrate flows parallel with the sheet. Ultra filtration membranes can also be used as a tubular reactor. Hollow fibre devices are available in two basic configurations- beaker and tubular type of which later is most suited for large scale operations.

3.3. Packed Bed Reactors

These are generally columns which are filled with immobilized enzyme preparation and remain static. The product flows down through the bed of enzyme or is gently pumped. This kind of reactor is most commonly used for experimental studies on foods. The enzyme support material is usually densely packed and must be sturdy for use in large columns.

3.4. Fluidized Bed Reactor

Such reactors are also in column form where substrate is pumped up through the bed of immobilized enzyme preparation. The rate of flow is sufficient to keep the particles in suspension and thereby fluidised. The supporting material for such reactors must be able to withstand the movement during fluidization.

4.0. Applications of Immobilized Enzymes in Food Processing

4.1. Lactose Hydrolysis

Whey, the chief lactose containing by-product, poses challenging problems for technologists and the dairy industry world wide for its economic utilisation. Fractionation of whey into lactose and whey proteins involve cost intensive techniques that are economically difficult to implement. However, bioconversion of lactose into more sweeter and soluble carbohydrates is an attractive avenue for whey utilisation.

Enzymatic hydrolysis of lactose using the enzyme B-galactosidase (Lactase) from various sources has always been preferred because of its specific reaction pathways with no side effects. Hydrolysis of lactose into monosaccharide, glucose and galactose, can be achieved by use of exogenous lactase either in soluble or immobilised form. In the recent past several carrier matrixes have been used for the immobilisation of lactase from fungal, bacterial and yeast sources. Amongst the inorganic carriers, porous glass and formaldehyde resin have shown promise with reference to their application in dairy industry. A semi commercial preparation of lactase immobilised on a resin has been used to treat lactose solutions, milk and whey. A commercial lactase preparation from yeast has been entrapped with cross linked polyacrylamide using bead polymerisation technique, was used to treat milk.

The application of lactase enzyme in the food industry can be outlined as for (1) production of low lactose milk for lactase deficient persons, 2) production of low lactose concentrated milk, 3) manufacture of modified milk powders for bread making, 4) accelerated cheese and yoghurt fermentation, 5) production of food syrups and sweeteners.

4.2. Milk Clotting Enzymes

In the manufacture of cheese, clotting milk with immobilized enzymes like rennin, chymotrypsin etc., offers a number of advantages over traditional methods. An immobilised
system can fit into continuous, semi-continuous or batch processes depending upon requirements. The immobilised enzyme does not remain into the curd permitting separation of milk clotting function with that of cheese ripening functions. Consequently, the immobilised protease could be chosen for optimum effect on clotting and cheese yield while small amount of soluble protease could be selected for optimum ripening action. However, there has been no success in treatment of milk for clotting action, on a commercial scale, as the casein tend to adhere on the immobilised enzyme molecule reducing its activity by blocking reactive sites.

4.3. Glucose Isomerase

This enzyme has found commercial application in the enzymatic production of glucose-fructose syrup from starch, and glucose-galactose syrup from whey. The glucose-galactose syrup is 70 times sweeter than sucrose. This sweetness could be increased to 170 per cent using glucose isomerase to yield glucose-fructose-galactose syrup. Glucose isomerase has been entrapped into polyacrylamide gel and coupled to porous glass beads covalently. Lactose hydrolysed syrups have been made from whey but superior products are made from permeate. It greatly reduces undesirable reactions that may result from presence of unwanted protein substrates. An 80 per cent hydrolysis is preferred because of cost factor and more so at this level of hydrolysis there are 4.66 fold increases in sweetness and 2.73 fold increases in solubility, compared to un-hydrolysed lactose. The isomerization of glucose to fructose enhances the quality of hydrolysed syrups.

Hydrolysed lactose syrups have been used as sweeteners in ice-creams, soft drinks, bakery goods, fermented beverages, flavoured drinks and confectioneries. It is used as a replacement of corn syrup solids in many applications. It has been successfully used in production of isotonic sports drinks.

4.4. Glucose Oxidase

Glucose oxidase has four main applications in the food industry removal of residual glucose, removal of residual oxygen, production of acid and generation of hydrogen peroxide. Normally for the production of Cottage and Mozzarella cheese by direct acidification method, acidogen (GDL) or combination of acidogen and food grade HCl or lactic acid is added. For better control of acid production in situ formation of GDL by glucose oxidase has been suggested. Glucose oxidase has been immobilised onto variety of inorganic carriers like porous glass, titanium, alumina and silica. The immobilised preparation is readily applicable for the removal of glucose and oxygen from liquid products and could be used in milk at 30–40 °C to lower its pH to 5.5. At lower temperatures the pH could be further reduced to 4.6 and when the temperature is increased it induces coagulation.

5.0. APPLICATIONS OF IMMOBILIZED ENZYMES IN INDUSTRIAL PROCESSES

Despite large volume of research work carried out on the potential applications of immobilised enzymes, relatively few industrial processes using immobilised enzymes actually exist.

5.1. Resolution of DL-Amino Acids

The growing use of amino acids in both medicine and human foodstuffs has led to large scale increase in demand. The industrial production of L-Methionine by Aminoacylase immobilised on DEAE-Sephadex in a packed bed reactor was set up by Tanabe Seiyaku Co. of Japan. Its great virtue lies in its relative simplicity and ease of control. The half life of the
enzyme reactor was 65 days at an operative temperature of 50 C. The productivity of a 1,000 litre column is 200 and 700 kg / hr depending upon the amino acid used.

5.2. **High Fructose Syrup**

The recent controversy over the safety of artificial sweeteners and reduction in the use of Saccharin in food industry has placed more emphasis on traditional sweetener sucrose. While sucrose in sweet, fructose is one and half times sweeter and consequently a large quantity of invert sugars are produced commercially using soluble enzyme. The first commercial production of high fructose syrup using glucose isomerase immobilised on cellulose ion exchange polymer in a packed bed reactor was initiated by Clinton corn products, USA. The potential market for high fructose syrup is large and can be obtained from any cheaply available starch source.

5.3. **Chill Proofing of Beer**

The consumer of beer demands that his purchase must not only taste right but must also look right (completely clear). Proteins present in untreated beer causes haze to develop when the beer is chilled. A process has been described whereby papain immobilised on chitin has been used to remove chill haze from the beer. The low cost of chitin in comparison of most other polymers makes its use economically possible also chitin is poor absorbent and is therefore unlikely to remove any other flavour from the beer.

5.4. **Lactose Hydrolysis**

Attempts have been made to hydrolyse cheese whey using immobilised Lactase (B-galactosidase). The great attraction in developing a process for continuous hydrolysis of lactose in whey remains the abundance and cheapness of the raw material and the potential value of the product. Immobilised enzymes in the form of droplets of Lactase trapped inside cellulose triacetate fibers have been designed. The fibres are arranged in skeins and packed loosely in longitudinal bundles in a reactor column. Such an arrangement provides both low residences to fluid flow and high surface area of immobilised enzymes.

6.0. **SUMMARY**

Enzymes, the biological catalysts, have been used in modification and production of many foods. However, uses of soluble enzyme preparations are costly. The possibility of use of immobilised enzymes as re-usable catalysts has been recognised. Various techniques have been used for immobilisation of different enzymes for their food usage. One aspect of immobilised enzyme process that makes them highly practical is their potential for automation. The pertinent consideration in using immobilised enzyme systems are their cost effectiveness, reactivity and possibility to use with other systems. Till date only few IME systems are in commercial usage in spite of enormous research carried out in this area.

7.0. **REFERENCES**


1.0.  INTRODUCTION

Genetic engineering is one of the most powerful biological technologies yet developed. It is providing and will provide tremendous opportunities for several sectors, such as agriculture, food processing and pharmaceuticals. Currently there are hardly any genetically modified food products in the market as public confidence in such products is very low. Recent developments in biotechnology and genetic engineering have brought a renaissance in the field of cheese technology. Now technologies are in our clasp to design cheese with desired textural and flavour characteristics. Considerable developments have been made to shorten the long ripening period, increase the cheese yield, reduce the bitterness in cheese, produce desired flavour level within short maturation period, etc. The unprecedented accomplishments of biotechnology and genetic engineering since the past decade have added a new margin in designing transgenic cheese.

The production of cheese and a range of other fermented foods is one of the oldest manifestations of biotechnology, handed down generation to generation. The cheese technology had been modified time to time in order to surmount the processing challenges and to gift the mankind with novel type of cheese with improved physico-chemical and functional properties. With the triumphal achievements in genetic engineering in the field of food, agriculture and health sector, recent developments in cheese technology have emerged with a new margin as the unprecedented consequence of genetic games alike the conventional and gradual amelioration in cheese making technique as usually expected with the passing generation by the virtue of evolution in order to satisfy the more and more requirements. Instead of the buzz, as front page news story in several European dailies, over the controversy concerning the biosafety of Genetically Modified (GM) food, development of Genetically Modified Cheese (GME) as the outcome of good cheese-biotech sorority seems to curtail or minimize a number of processing challenges for cheese manufacture.

2.0. WHAT IS GENETIC MODIFICATION?

Genes are organized into chromosomes which are found in all living cells. They are a coded form of instructions to make proteins. Most of the proteins manufactured by living cells are enzymes. Enzymes regulate the functional activities. Genetic information mainly is stored in a coded form in deoxyribonucleic acid (DNA) molecules present in genes. So, DNA is a kind of molecular blue print where it stores all the information needed to make a new cell. Therefore, the phrase, “genetically modified” is commonly used to describe the application of recombinant deoxyribonucleic acid (rDNA) technology for the genetic alteration of microorganisms, plants and animals. This advanced molecular technology allows for effective and efficient transfer or alter genetic material from one organ to another. An identified single gene responsible for a particular trait can be inserted / transferred among all living organisms. The technology is now well developed and can be applied to any living organism.
3.0. GENETICALLY MODIFIED FOODS/ CHEESES

Foods obtained / processed by the application of directly or indirectly DNA technology which offer the opportunity not only of better control of existing food processing but also to improve food quality with minimum production and post harvest losses, are referred to as GM foods. However it is obligatory to look into the safety factors of this applied technology.

Cheese obtained or manufactured by the application of genetic engineering by adopting the following three approaches can be called as genetically modified cheese. These three approaches may be adopted individually and or in combination to obtain cheese with approved vegetarian sentiment, improved functional attributes, increased nutritional/ therapeutic value, accelerated ripening activities and enhanced sensory quality. These approaches are:

- Modification in milk composition
- Addition of Recombinant coagulating enzymes
- Application of Modified starter culture

Among the three approaches, considerable amount of work have been carried out on application of genetically modified microorganism to obtain improved starter culture and recombinant coagulating enzymes for the cheese production. Work on alteration of milk composition by Genetic manipulation in animals results in the improvement of milk production system which can even produce dairy products with functional characteristics. In this context, main emphasis for genetically modified cheese is now on the processing aspects i.e. on starter culture and coagulating enzymes.

3.1. Modification in Milk Composition

It is established that genetic variation in animal causes the different composition in milk. Transgenic cows with altered genetic make up to produce milk with 2 per cent fat with a greater proportion of unsaturated fatty acids in milk fat, higher levels of milk protein, β- and κ-casein and reduced lactose content in milk have been developed. Such milk is suited ideally for people suffering from lactose intolerance and also to produce improved varieties of specialty cheeses with effective cost price efficiency. Genetically modified bovine somatotrophin (BST) also play a role in the regulation of milk yield, growth rate and protein to fat ratio of milk which results in milk composition alteration.

3.2. Recombinant Coagulating Enzymes

One of the success stories for the application of genetic engineering is the manufacture of recombinant chymosin and its use as milk coagulating enzyme for commercial cheese production. Concern over the supply of chymosin from traditional source (suckling calves) has led to efforts over the past three decades to develop a recombinant source. Cheese industry has been the major beneficiary of this technological work.

The gene coding for the chymosin enzyme has been cloned in the bacteria Escherichia coli, the yeast Kluyveromyces lactis and the mould Aspergillus niger. The enzymatic properties of the recombinant enzymes are indistinguishable from those of calf chymosin. The cheese making properties of recombinant chymosin produces very satisfactory results and its use in commercial plant have been approved by many countries. Three recombinant chymosins are now marketed commercially which have taken more than 35 per cent share of the total market. The three brands are as follows:

- **Maxiren**: From K. marxianus var. lactis produced by Gist Brocades, Netherlands
- **Chymogen**: From A. niger produced by Hansen’s, Denmark.
- **Chymax**: From E. coli by Pfizer, USA.
All the three chymosin available are identical to calf chymosin, considered as vegetarian source and accepted by religion group. More knowledge on genetic engineering combined with better understanding of protein structure might one day give us chymosin with higher activities, lower cost and with flavour enhancing properties of cheese during ripening.

3.3. Modified Starter Culture

The application of gene cloning technology to lactic acid bacteria is the potential process in the generation of enhanced starter cultures for the manufacture of cheese and yoghurt. These starter cultures are mainly made of species of *Lactococcus*, *Lactobacillus* and *Streptococcus*. Modifications of these microorganisms were achieved mainly on three directions for cheese making process. These are:

- Development of phage resistant cheese culture
- Organisms with Probiotic Activity for cheeses
- Acceleration of cheese ripening

3.3.1 Phage Resistant Cheese Culture: The major cause of slow acid production in cheese plants today is bacteriophage (phage). This can significantly upset manufacturing schedules and, in extreme cases, result in complete failure of acid production or “dead vats”. Phages are viruses that can multiply only within a bacterial cell. They have a head, which contain the DNA and a tail, which is compared of protein. Morphologically, there are three types of phage for *lactococci* as mentioned below:

- Small isometric headed (spherical headed) phage - most common
- Prolate – headed (oblong – headed) phage
- Large isometric – headed phage

Phage multiplication occurs in one or two ways, called the lytic and lysogenic cycle. Multiplication of phage is very fast in their hosts. Microbiologists have enhanced cheese culture performance by genetically engineering bacteria increasing the viability of the culture during cheese making. The new strain of bacteria resist phase contamination and are suitable for prolonged use in milk fermentation. Several phase resistance mechanism, including inhibition of phage adsorption, restriction–modification mechanism and abortive infection mechanism are found in LAB. All of these are commonly encoded on plasmids.

A phage resistant starter culture of cheddar cheese, *L. lactis* DPC 5000 have been developed which was shown to embody three effective phage resistant mechanisms. Cheddar cheese manufactured with DPC 5000 compared favorably in term of composition with cheeses manufactured using commercial starter. Two phage resistant thermophilic starter strains DPC 1842 and DPC 5099 have been developed in Cork, Ireland which performed well in commercial plants for Mozzarella cheese preparation. The new cultures provide more predictable performance and reduce the chance of vats failure.

Cheese with Increased Yield

Scientists in New Zealand have created the world's first cow clones that produce special milk that can increase the speed and ease of cheese making. The researchers in Hamilton say their herd of nine transgenic cows makes highly elevated levels of milk proteins i.e. casein-with improved processing properties and heat stability. Cows have previously been engineered to produce proteins for medical purposes, but this is the first time the milk itself has been genetically enhanced. The scientists hope the breakthrough will transform the cheese industry, and if widened, the techniques could also be used to "tailor" milk for human consumption. But opponents of GM cheese continue to doubt whether such products will be safe.
3.3.2 Probiotic Organisms for Cheese: Ripened variety of cheeses may offer certain advantages as a carrier of probiotic microorganisms with specified health benefits. Growing public awareness of diet-related health benefits has fuelled the demand for probiotic foods. Dairy foods including fermented milks and in particular yoghurt are the best accepted food, which can carry probiotic cultures. Among the most important criteria when considering cheddar cheese as a probiotic food is that the microorganism be able to survive for relatively long ripening time of at least 6 months and / or that they grow in the cheese over this period although the pH is higher in comparison to traditional probiotic dairy foods. The potential health promoting effects achieved by the consumption of dairy foods containing probiotic organisms, such as *Lactobacillus* and *Bifidobacterium* spp., have resulted in intensive research efforts in recent years.

Even though small number of probiotic cheese are currently in the market world wide, a few reports are available concerning cheese as a carrier of probiotic organisms. European manufactures have introduced different varieties of cheese with added *Lactobacillus paracasei* NFBE 338. Dinakar and Mistry (1994) incorporated *Bifidobacterium bifidum* into cheddar cheeses as starter adjunct. This strain survived well and retained a viability of $2 \times 10^7$ cfu/gm of cheese even after 6 months of ripening, without adversely affecting cheese flavor, texture or appearance. This suggested that cheddar cheese can provide suitable environment of probiotic organisms. *Bifidobacteria* were used in combination with *L. acidophilus* strain Ki as starter in Gouda cheese manufacture. There was a significant effect on flavour development in the cheese after 9 weeks of ripening (Gomes *et al*., 1995). Cheddar cheese was manufactured with either *Lactobacillus salivarius* NFBC 310, NFBC 321 and NFBC 348 or *L. paracasei* NFBC 338 or NFBC 364, isolated from small intestine, as the dairy starter adjunct. Using randomly amplified polymorphic DNA method, it was found that both *L. Paracasel* strains grew and sustained high viability in cheese during ripening, while each of the *L. salivarius* species declined over the ripening period. These data demonstrate that Cheddar Cheese can be an effective vehicle for delivery of some probiotic organisms to the consumer (Gardiner *et al*., 1998).

Therefore, though the reports on probiotic cheeses are few but there is promising scope to exert probiotic effect on the cheese by enhancing the existing characteristic of probiotic organisms with genetic engineering. They may be called as second-generation probiotics (Rastall and Maitin, 2002). These might include the incorporation of attachment molecules to facilitate colonization, increased production of antimicrobial compounds directed against common food pathogen, cholesterol metabolism, enhanced immune stimulation and viability which contribute beneficial effect on human health. Attempts are also being made to identify new strains of probiotic culture by identifying the genes involved in such attributes and exploring the advanced molecular technique such as PCR, RFLP, RAPD and DNA finger printing etc. All these modifications in dairy starters can be brought also with the help of genetic engineering with the sole objective of improving their functionality so that cheese industry could benefit from value addition in cheeses/dairy foods through the intervention of these genetically modified organisms.

Another desirable property might be the generation of probiotic genetically modified organism to digest especially prebiotic carbohydrates. The combinations of probiotic and a prebiotic are known a synbiotic and the prebiotic is thought to enhance the survival of the probiotic. Several strains of *Lactobacillus* and *Bifidobacterium* sp. have been engineered to metabolize unusual carbohydrate, but the potential to form enhanced synbiotic has not yet been evaluated.
3.3.3 Acceleration of Cheese Ripening

The objective of acceleration of cheese ripening is to accelerate the proteolytic process and related events that occur in naturally ripened cheese as closely as possible. The methods used to accelerate ripening fall into six categories:

- Elevated ripening temperature
- Exogenous enzymes
- chemically or physically modified bacterial cells
- Genetically modified starters
- Adjunct culture
- Cheese slurries and enzyme–modified cheeses

In this context genetically modified starter cultures is of special interest.

3.3.3.1. Genetically Modified Starters: Genetically modified starter cultures with enhanced complements of proteinase and / or peptidase, which could be released early and evenly distributed in the curd would be an ideal method of accelerating cheese ripening. Modified / genetically tailored microorganisms, genetically engineered proteolytic and lipolytic enzymes are now being used for enhancing flavour production in cheese. Enzyme addition is now one of the few preferred methods of accelerated ripening of cheese. Enzyme may be immobilized or encapsulated for long term action on the production for quick action and homogenous distribution in the product. Cheese manufactured with an amino peptidase N-negative clone strain of *Lactococcus* produced bitter off flavour (Baankreis, 1992). The possible role of amino peptidase as a debittering agent confirmed by Prost and Chamba (1994) after making the Emmetal cheese with *Lb. helveticus* strain L₁ ( high amino peptidase activity), L₂ or strain L₃ ( clones selected for lack of  amino peptidase activity). They also explained the bitterness in ripened cheese made with *Lb. delbrueckii subsp. lactis* to be due to their very low amino peptidase activity.

The gene for the neutral proteinase (neutralase) of *B. subtilis* has been cloned in *Lc. lactis* UC317. Cheddar cheese manufactured with this engineered culture as the sole starter showed very extensive proteolysis, and the texture became very soft within 2 weeks at 8°C (Fox et al., 2000). Cheddar cheese made with *Lc. lactis* subsp. *cremoris* Sk11 (cloned with proteinase) revealed that starter proteinases are required for the accumulation of small peptides and free amino acids in cheddar cheese. The strain in which the proteinase remained attached to the cell wall appeared to contribute more to proteolysis than the strain that secreted the enzyme. Cheeses made with proteinase positive starter produce more pronounced flavour than those with proteinase negative strain during ripening. The inactivation of genes in a metabolic pathway can be used to alter end products that accumulate from a given pathway. Application of regulated promoters (in genetic engineering) is the controlled expression of lytic genes resulting in autolysis of the starter culture. This would result in rapid release of enzymes (i.e. peptidase) into the cheese matrix and potentially accelerate cheese flavour development.

Therefore, through genetic engineering, specific genes can be implanted / removed to increase or decrease the activity of specific property of the existing strain of LAB. It can be stated from the utilization of genetically modified starter as: i) Cloning of exogenous proteinases in starter cells leads to enhanced proteolysis ii) Debittering action of amino peptidase is now well recognized iii) Starter peptidases and proteinases produce small peptides and amino acids in cheese but may not have a direct impact on flavour.

4.0. CONCLUSION
Scientists in New Zealand have created the world's first cow clones that produce special milk that can increase the speed and ease of cheese making. The researchers in Hamilton say their herd of nine transgenic cows makes highly elevated levels of milk proteins i.e. casein-with improved processing properties and heat stability. Cows have previously been engineered to produce proteins for medical purposes, but this is the first time the milk itself has been genetically enhanced. The scientists hope the breakthrough will transform the cheese industry, and if widened, the techniques could also be used to "tailor" milk for human consumption. But opponents of GM cheese continue to doubt whether such products will be safe.

Application of gene technology for genetic modification of organisms involved in food production and processing is highly sensitive issue. Most of the applications of genetic engineering developed so far only provide economic benefits and business opportunities to the producers. Understanding the technology is likely to aid in gaining acceptance. But it will depend on high quality risk assessment and convincing results from long term experiments whether the GMOs will find broad acceptance in the food industry.

5.0. REFERENCES


1.0. INTRODUCTION

The heart of a fermentation process is the fermenter. It is a container in which is maintained an environment favorable to the operation of a desired biological process. Historically, the main tool for fermentation laboratory was the shake flask or flat bed bottle. The next stage was the introduction of a glass vessel with a stirrer, and this was followed more recently by stainless steel vessels in various sizes and forms. In recent years, microbiologists and engineers have developed several sophisticated mini-fermenters capable of specializing in different types of fermentation.

The environment maintained in a fermenter is a combination of several favorable parameters considered essential for biological process. The biological environment is considered favorable to a biological process when only desirable organisms, contributing to process are present and undesirable, destructive, non-productive are excluded. The chemical environment involves the composition of the microbial growth medium, which should contain the desired concentration of substrate or microbial nutrients free from inhibitory substances. The physical environment refers principally to the temperature of the system and maintaining the uniform conditions throughout the fermenter which involve mixing, agitation and foam control. For maintaining a favorable environment, the environmental parameters are not necessarily held constant with time. A fermentation process passes through different phases of growth which may have different environmental optima to favour the growth. The design characteristics of a fermentation system should thus meet the objective of operation and its applicability.

2.0. PRINCIPAL CATEGORIES OF FERMENTERS

Several different types of vessels are used for large scale biological processes and their degree of sophistication in design, construction and operation is determined by the sensitivity of the process to the environment maintained in the vessel. In the simplest case, the fermenter is a vessel in which reagents, substrates and organisms are brought in contact with provision for their addition or removal. The fermenters can be classed into two broad groups, the liquid fermentation and the solid state / substrate fermentation.

2.1. Liquid Fermenters

Mostly industrial fermentations are carried out in liquid medium using bioreactor system, which range from simple stirred tank or non-stirred containers to complex, aseptic, integrated systems. These are run as batch or continuous type of fermentation process.
2.2. **Solid State/Substrate Fermentation**

There are many biotechnological processes that involve the growth of microorganisms on solid and insoluble substrates in the absence of free water. The bioreactor design for solid state fermenters is simpler than the liquid fermenters. Such systems can be without mixing, with occasional mixing or with continuous mixing. The use of solid state fermenters is limited to those cultures that can grow comfortably at low moisture levels. These fermenters have advantages of using simple media with cheaper, natural and abundantly available components. Low moisture content required for the process gives economy of bioreactor space, low effluent treatment, less microbial contamination, often no need of sterilization and easier downstream processing. Aeration requirement can be met by simple gas diffusion or by aerating intermittently. Two major advantages of this fermentation method are high yields of products and low energy expenditure compared to stirred tank bioreactor.

3.0. **FERMENTER CONFIGURATIONS**

There are numerous fermenter types. These include stirred tank reactors, air-lift fermenters, tower fermenters, fluidized bed reactors and rotating disc fermenters. Each of these configurations of fermenters may offer advantages in certain applications.

3.1. **Stirred Tanks**

Basically stirred tank fermenter consists of a cylindrical tube with a top-driven or bottom driven agitator. The stirred tank with a top drive assembly is the most commonly used fermenter because of its ease of operation, neat design, reliability and robustness. For smaller mini-fermenters borosilicate glass is used as the cylindrical tank and a top plate of stainless steel clamped on it. A motor is fixed above the top plate and is attached to the shaft. The vessel, medium and probes are usually sterilized together, minimizing the number of aseptic operations required. The glass vessel can be protected by a removable stainless steel mesh or jacket. The stirred tanks are available in stainless steel also. They are more expensive than the glass vessels but they are more robust, reliable, and designed to last a lifetime.

3.2. **Air-lift Fermenters**

In all aerobic fermentations, air is an essential requirement. Air-lift fermenters have no mechanical agitation system but utilize the air circulating within the fermenter to bring about the mixing of the medium. This is a gentle kind of mixing and is ideal for plant and animal cell cultures as there is no shearing action because of absence of mechanical mixer or agitator. The air-lift fermentation is based on the difference in specific weight between the air-enriched volume and low-air volume. As the fermenter is aerated, the lower density broth (air-enriched medium) creates an upward thrust which results in the circulation of the both. The type of circulation depends upon the vessel configuration. The basic design for a laboratory scale air-lift fermenter is an outer glass hollow tube with an inner stainless tube. A tubular loop type fermenter is designed to increase the volume of the fermentation while maintaining the residence time.

3.3. **Tower Fermenters**

These types of fermenters are designed for continuous yeast fermentation process. Continuous brewing of beer or lager can be carried out successfully in a tower fermenter. The design of these fermenters is simple and they are less expensive than the conventional stirred tank fermenters. Also due to lack of any complex mechanical agitation system they are easier to construct.
3.4. Fixed-bed Fermenters

There are several systems developed with a tubular packed bed reactor for laboratory scale applications. The main problem associated with these reactors is getting the fixed bed fully aerated. If air is restricted then anaerobic micro-organisms take over. The type of fermentation in fixed beds is a heterogeneous reaction, whereas it is homogeneous in stirred tank fermentation. The fixed bed reactors are widely used in waste water treatment plants.

3.5. Fluidized-bed Fermenters

These fermenters are hollow chambers in which dense particles containing a microbial film or microbial mass is recycled. One of the oldest fermentations known to man, vinegar fermentation utilizes this principle.

3.6. Rotating-disc Fermenters

They have circular discs which rotate through the medium at slow speed. The micro-organisms adhere to the discs and the microbial film is exposed to both the nutrient solution and the air. These types of reactors are being used for waste water treatment.

4.0. DESIGN AND ANALYSIS OF BIOREACTORS

It is essential to understand the analysis of flow and mixing patterns of different streams and mass transfer between different phases in the reactor for its design and scale-up. A batch reactor is a close thermodynamic system with respect to exchange of matter with its environment, while a continuous reactor is an open system with mass exchange across its boundaries. Unstructured models comprising of cell numbers, mass or concentrations are employed to characterize the biophase in the steady state or the unsteady state operation of the reactor.

4.1. Steady-state Analysis

In the steady state, all concentrations within the vessel are independent of time and the following mass balance is applied to the components of the system.

\[ F (X_0 - X) + V R_x = 0 \quad \text{----------- (1)} \]

Where,
- \( F \) = volumetric flow rate of feed and effluent stream.
- \( X \) = viable-cell concentration in tank and effluent stream.
- \( X_0 \) = viable-cell concentration in feed stream.
- \( V \) = volume.
- \( R_x \) = rate of cell formation, cells per unit time per unit volume.

If we neglect the cell death and let ‘\( \mu \)’ denote the specific growth rate (\( R_x / X \)), it follows from Eq.(1) that

\[ D X_0 = (D - \mu) X \quad \text{----------- (2)} \]

Where, the parameter ‘\( D \)’ is called as the Dilution rate and is equal to the number of tank volumes which pass through the vessel per unit time. It is the reciprocal of the mean residence time. Often the liquid feed stream to a continuous culture consists only of sterile nutrient, so that \( X_0 = 0 \), which can only be maintained when \( D = \mu \), that is when the culture has adjusted so that its specific growth rate is equal to the dilution rate.

4.2. Unsteady-state or the Dynamic Analysis

For dynamic studies, the general conservation equation is modified to give following unsteady-state mass balance;
\[
\frac{d}{dt} (VX) = F(X_0 - X) + VR_x \quad \text{(3)}
\]

Where ‘\(R_x\)’ denotes the rate of biomass production per unit volume. Since ‘\(V\)’ is constant, for the sterile feed the mass balance equation becomes

\[
\frac{dX}{dt} = -DX + R_x \quad \text{(4)}
\]

If exponential growth exists in the continuous culture or in other words, \(R_x\) is prescribed by Malthus’ law as ‘\(R_x = kX\)’ then the solution to Eq (4) with the initial condition as \(X(0) = X_i\) is

\[
X(t) = X_i e^{(k-D)t} \quad \text{(5)}
\]

Consequently, \(X\) increases without bound if the organisms grow more rapidly than they are washed out \((k > D)\), and washout occurs in the converse situation \((k < D)\). If \(k = D\), the population remains at its initial level; a steady state is achieved only under these conditions.

**4.3. Residences-Time Distributions**

Determination of the distribution of residence times in the exit stream is a valuable indicator of the mixing and flow patterns within the vessel. It is determined by conducting a stimulus-response experiment using a tracer. At some datum time designated \(t = 0\), we introduce tracer at concentration \(C^*\) into the feed line and maintain this tracer for \(t > 0\). The system is monitored for this specific stimulus. The tracer must not react in the vessel and should be detectable at small enough concentration. The response to a unit-step tracer input is expressed in terms of \(F\)-function and the tracer concentration \(C^*\) as follows;

\[
F(t) = \frac{C(t)}{C^*} \quad \text{(6)}
\]

The residence-time-distribution function \(C(t)\) is then obtained as

\[
\frac{d}{dt} \frac{F(t)}{F} = \frac{C(t)}{V} = \frac{C(t)}{V} e^{-\frac{Pt}{V}} \quad \text{(7)}
\]

The RTD however does not characterize all aspects of mixing. It indicates how long various ‘pieces’ of effluent fluid have spent in the reactor.

**5.0. INSTRUMENTATION AND PROCESS CONTROL**

Increasingly complex control systems are finding application in commercial fermenters for maximizing process productivity and batch-operating-cycle reproducibility. The limiting factor in the possible sophistication of the control system is the limited availability of steam-sterilizable sensors. Various parameters of interest in the process of fermentation could be grouped into two categories, the physical parameters and the chemical parameters.

**5.1. Physical Parameters**

The physical parameters for which suitable instrumentation exists today include temperature, flow, pressure, agitator shaft speed and power, foam and liquid level.

**5.1.1. Temperature:** Fermentations are run either in the mesophile range (20-45°C) or thermophile (> 45°C) range. The optimum temperature must be chosen to achieve maximum...
growth on one hand and optimal product formation on the other hand. In some fermentation, higher temperatures are used to obtain increased growth of culture and then the temperature is decreased at the onset of the idiophase. The temperature programming of the process facilitates the optimal temperature policy and corresponding growth and production profiles of product manufactured by fermentation. Temperature is usually measured with a Pt-100Ω probe and is controlled with the heat exchange facilities. Because sterilization of the culture medium and removal of heat are vital for successful operation, the fermenter generally has an external cooling jacket through which steam or cooling water can be run. For very large fermenters, insufficient heat transfer occurs through the jacket, and so the internal coils must be provided for circulation of steam or cooling water.

5.1.2. Pressure: Hydrostatic pressure influences the solubility of O₂ and CO₂ in the nutrient solution, especially in the large fermenters. Usually in the fermenters an over pressure of 0.2-0.5 Bar is used in order to minimize the risk of contamination. The elastic deformation sensor elements, such as, diaphragms have employed to sense and control this variable.

5.1.3. Flow: The peristaltic pumps are used for supplying different fluids to the fermenter. The rate of aeration is adjusted to the amount of O₂ required and is usually maintained at 0.25-1.0 vvm (air volume / liquid volume / min). The flow of different fluids is made to pass through motorized valves controlled by the pO₂ regulator.

5.1.4. Agitation: The installation of a continuous drive system is desirable in industrial fermenters in order to be able to precisely adjust the stirring rate to the process. The speed of the impeller varies between 50 to 450 rpm depending upon the fermenter size and is controlled with the help of a dynodrive. The agitator assembly is designed to meet the mixing and aeration requirements.

5.1.5. Foam Control: Foams are produced in any aerated and agitated culture vessel. Mechanical foam breakers or the addition of chemical antifoam agents such as corn oil, cottonseed oil are the foam controlling methods. Antifoam agent can be directly added to the medium before sterilization or at the fermentation system through a feed line.

5.2. Chemical Parameters

The chemical parameters which can be monitored continuously are limited; only the broth pH, redox, NAD level, dissolved O₂ and CO₂ concentrations and exit gas composition permit direct measurement. Other chemical variables such as RNA and ATP concentrations are also of potential importance, their measurement require increased sophistication and are not employed at industrial level. Of all those mentioned now, pH measurement and control is by far the most common Modern practice is the controlled metering of an acid or base into the fermenter to maintain the desired pH. During the course of operation often pH falls due to depletion of an ammonia nitrogen source. Use of added ammonia as the controlling base then serves the twofold purpose to maintain the desired pH and preventing nitrogen limitation of the culture. Monitoring of dissolved oxygen and controlling of air flow rate allows controlling dissolved oxygen concentration as an independent fermentation parameter.

Process control got a boost with the development of biosensors. These are basically the enzyme electrodes and currently hold about 90 per cent of the market, mainly for detecting substrates and metabolites such as glucose, lactate, ATP, ethanol, etc. Recently new techniques have been developed such as Near Infra Red (NIR) spectroscopy analysis, fibre optical methods Flow Injection Analysis (FLA) sensors based on conductivity modifications of a polymer which reacts to vapor. It is possible to measure on-line directly in a bioreactor the concentration of biomass by means of optical density (OD) or by acoustic resonance
densitometry (ARD). But very often, problems are encountered with these sensors with regard to reproducibility, drift, response time and sterility.

5.3. Process Control

Fermentation monitoring and control is generally a three-stage process: the first step involves acquisition of data through in-line, on-line or off-line measurements, in the second step these informations are analyzed and processed, and finally in the third step this processed information is used to control the process in the desired manner. Reliable and accurate monitoring of a process is of great importance in an automated control strategy. Due to slow fermentation process dynamics, in many cases the manual process control is the easier choice.

Based upon the data acquired through monitoring devices, process variables are identified. Variables may be the directly monitored ones or they may be the derived ones, e.g. oxygen uptake rate, carbon dioxide evolution rate, respiratory quotient, etc.

The measuring devices or the sensors sense the value of a process variable. Indicators give a read-out of the variable and recorders log a hard copy of it. The automatic controller compares the measured value of the variable with a prefixed set-point value, computes the error and gives out an error signal. The error signal then actuates the final control element to produce the desired result. This set of sensor, indicator, recorder, controller and the final control element constitute a control loop. The most effective control loop is feed back closed control loop and the common control algorithm being used in the bioprocess industry is PID (Proportional-Integral-Derivative) control algorithm. It is able to give response based on size of error (proportional), length of time the error is present (integral) and the rate of change of error (derivative). Any combination of the three components can be used depending upon the bioprocess requirement.

5.4. The Software

A microcomputer could be coupled to the reactor to automatically pilot the data acquisition during the different phases of the process. Different softwares have been developed to facilitate the repetitive calculations. The user can choose the values of the set points, the parameters of each regulation loop, the frequency of data acquisition, the scale on the coordinates axes for the graphic etc. During the different phases of a process, all the acquisitions are on-line displayed. It is also possible to display the flow sheet of each reactor and the working of the regulators. The software also allows checking different parts of the bioreactor equipments.

6.0. REFERENCES


1.0 INTRODUCTION

The main membrane systems that are commercially available for dairy and food applications and in descending order of tightness of membrane are: reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF) and microfiltration (MF). These pressure driven processes are based on the ability of semi-permeable membranes of appropriate physical and chemical nature to discriminate between molecules—primarily on the basis of size and to a lesser extent on shape and chemical composition. Generally the membrane will split the liquid feed into two streams: retentate stream the part of the feed retained by the membrane and concentrated in one or more constituents/solutes; and permeate stream—that part of the feed which passes through the membrane. The capacity of the component passing through the membrane (permeate) is called the “flux rate” and is usually referred to as the unit volume passing unit area of membrane in unit time (normally expressed on L/m²/h).

2.0 CHARACTERISTICS OF MEMBRANE PROCESSES

The distinction between RO, NF, UF and MF is somewhat arbitrary and has evolved with time and usage. The separation characteristics of different membrane processes have been shown in Fig. 1 (Cheryan, 1998).
**Fig.1 Membrane Separation Processes**

In a broader sense RO is essentially a dewatering technique, NF a demineralization process, UF is a method for simultaneously purifying, concentration and fractionation of macromolecules or fine colloidal suspension, and MF is a clarification process that separates molecules and particles on the basis of size and solubility. Table 1 shows the detailed features of the membrane separation processes.

**Table-1: Characteristics of Principal Membrane Separation Processes**

<table>
<thead>
<tr>
<th>Process</th>
<th>MWCO (Daltons)</th>
<th>Operational Pressure (psi)</th>
<th>Membrane Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Osmosis</td>
<td>Ions and most organics</td>
<td>200-800</td>
<td>5-20 50 Asymmetric, thin film composite</td>
</tr>
<tr>
<td>Nanofiltration</td>
<td>Most organics over 500</td>
<td>100-500</td>
<td>10-50 50 -do-</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Most organics over 1000</td>
<td>25-200</td>
<td>10-100 60 Asymmetric</td>
</tr>
<tr>
<td>Microfiltration</td>
<td>Large suspended particles, some emulsion, most bacteria</td>
<td>1-25</td>
<td>2.0 μm 70 Several types</td>
</tr>
</tbody>
</table>

**3.0 MEMBRANE MATERIALS AND THEIR CONFIGURATION**

Through a large number of membrane materials are available, all of them do not have commercial application in food processing. Cellulose acetate (CA) membranes were first used for industrial applications and still occupy a prominent place. In spite of several advantages, CA membranes, however, are plagued with certain limitations, for example, poor thermal stability (maximum operating temperature 35°C) and narrow pH range (3-8). They do not have good resistance against chlorine. Polyamide (PA) membranes are also used for RO applications but they are very sensitive to chlorine and provide very low permeate flux. Polysulphone (PS) membranes are most widely used in UF applications. They are highly desirable for food and biological applications because of higher operating temperature (up to 75°C), wider pH range (1-13) and adequate resistance to chlorine. These properties confer good cleaning and sanitization ability to PS membranes.

Recently, membranes based on more stable non-cellulosic acetate (non-CA) polymers have replaced CA membranes for food applications. These “thin-film- composite” (TFC) membranes are considered “second generation membranes since their asymmetric structure
arises from a series of a layers of non-CA polymers, usually, a polyamide “skin” on a polysulphone support. They have superior properties to CA in every respect except chlorine tolerance and are gaining rapid interest in RO and NF applications. The newest membranes, called “third generation membranes” are made from mineral or ceramic materials (usually alumina, zirconia, silver or carbon). They have great mechanical strength, withstand pressure up to 20 bar without rip, tolerate the entire pH range as well as very high temperature (up to 400°C for sterilization, has been claimed by some manufacturers). The ceramic membranes are now being used for both UF and MF systems (Pal and Cheryan, 1987 a).

The membrane element is the central part of the total system and is usually supplied on some form of supporting structure. There are four types of membrane systems available in the market, namely spiral wound, plate and frame, hollow fibre and tubular (Pal, 2003 a). Each configuration has its own benefits and limitations, and should be selected carefully keeping in view the feed to be handled, application, porosity and rejection co-efficient of the membrane, ease of cleaning, cost, etc (Bird, 1996).

4.0 APPLICATIONS OF MEMBRANE PROCESSES

4.1 Reverse Osmosis

Since RO is the most energy efficient dewatering process, it can be used for pre-concentration or final concentration of liquid feed for different purposes. RO concentrates can be used in fluid form either as pasteurized or UHT processed milk and for the manufacture of spray/roller powders, yoghurt, ice cream, etc. (Pal and Cheryan, 1987 a) In India, RO concentration has tremendous scope in the preparation of many concentrated traditional dairy products namely khoa (Pal and Cheryan, 1987 b), rabri, basundi, and kheer. Concentration of fruit juices by RO is also gaining lot of signature in food industry. In this write up main focus is given only to selected applications.

4.1.1. Concentration of Milk/Skim Milk by RD: The maximum concentration that can be obtained for whole milk is 38 per cent TS and for skim milk 28 per cent TS (Gupta and Pal 1993 a), but these levels can not be considered as economical because the flux is almost negligible at this stage. The economic levels of RO concentration for skim milk is 22 per cent TS and for whole milk upto 30 per cent TS. Several factors influence the performance of RO unit and the quality of product. The feed in RO process is pumped at very high pressure (30-35 bars) that causes the damage of fat globules. This is probably due to the shearing action of the high pressure pumps causing distinct homogenization action on milk fat, resulting in decrease in the size of fat globules and reduction of the free fat content. Hence, if lipase enzyme is not inactivated before RO process, the hydrolysis of fat take place leading to increase FFAs and spontaneous development of rancid flavour in milk. The microbial load also increases along with the milk constituents in RO processing of milk; therefore, an appropriate pre heat treatment is essential to retain the quality of milk (Drew and Manners, 1985). The RO concentrated whole milk, preferably up to 2-fold, can be used either as such or after dilution in the manufacture of certain fluid products like plain milk, flavoured milk, skim milk and high protein milk (Gupta and Pal, 1993 b).
The transportation of milk in “single strength” is very expensive. The feasibility of transporting raw milk from rural chilling centres in concentrated form to city dairies has been explored by many workers. Gupta and Pal (1993 b) reported that more than 25 per cent saving on transportation cost can be achieved by concentration of buffalo milk up to 2 fold at the collection center and its subsequent transportation to processing plant located at a distance of 400 Km with no appreciable change in the organoleptic and chemical properties in RO concentrated milk up to 48 hours at 4 ±°C.

4.1.2 Concentration of whey: RO may be used (a) as a pre-concentration step prior to transportation of whey and (b) for its concentration for using as such or for making whey powder and other products (Jayaprakash et al., 1995). Whey can be easily concentrated to about 18% total solids on a batch type RO plant, but with the use of multistage recycle designs (MSR), concentration up to 26-28 per cent total solids is now technically and economically feasible (Pepper and Orchard, 1982). Flux for RO of whey is normally higher than that for milk mainly because of lower total solids in the former.

4.1.3 Concentration of UF Permeates: Application of UF process in concentration and fractionation of whey, skim milk and whole milk are increasing rapidly for various purposes, particularly for cheese making. Consequently, a large quantity of permeate is produced as a by-product. The UF permeate contain 5.5-6.5 per cent solids of which approximately 90 per cent is lactose alone and 8-9 per cent are minerals. Such a high concentration of lactose obviously favours the use of UF permeate as a potential raw material source for manufacture of lactose. RO can play an important role in pre-concentrating UF permeate with minimum energy requirements and least thermal degradation. It is possible to concentrate UF milk permeates from initial total solids of 5.8 per cent to about 25 per cent by RO and maintain a reasonable flux (Pal and Cheryan, 1987a). Higher concentration than this may not be economically feasible with the present generation of membranes and current energy costs. The quality of lactose can be improved by demineralization of permeate using nanofiltration process before the RO processing.

4.2 Ultrafiltration

Applicability of ultra-filtration has widened considerably because of substantial decrease in cost of membranes, availability of better-designed units and decrease in fouling problem of membrane. It is now being used in diverse fields such as water treatments, chemical processing, food processing and biotechnology. Dairy applications probably account for the largest share of installed membrane capacity (Pal, 2003b). Some of the important applications of UF in food processing areas, particularly the dairy field, are discussed here.

4.2.1. Protein Standardization by UF Technique: Standardization of protein has now become an important international issue mainly because of the following reasons (Puhan, 1996):

- Seasonal and regional differences in protein contents of milk are inherent features, which affect the quality and composition of products made there from, hence the need for equalizing protein exists.
• Fortification of milk with total protein to increase the nutritional value.
• Manufacture of total milk protein concentrates for use in special and dietetic foods. Ultrafiltration can be used with a great success for upward standardization of milk proteins in all the above mentioned situations.

4.2.2. Cheese Making with the UF Process: Several varieties of cheese are commercially produced employing UF process (Lelievre and Lawrence, 1988 & Pal and Cheryan, 1987c) because of several benefits offered by this technology, particularly a higher yield. Based on the levels of concentration achieved, the methods of manufacturing cheese by UF can be broadly divided into three groups, viz. low concentration factor (LCF), high concentration factor (HCF) and precheese method. In case of LCF process, milk is ultrafiltered to about two fold level and cheese made there from employing traditional method. Since drainage of whey in this method takes place as for traditional cheese making, the benefit of higher yield is not achieved. In the HCF process, milk is concentrated between three and six-fold. Based on the five fold concentration of milk, the CSIRO Dairy Research Laboratory, Australia and APV Ltd have jointly invented a commercial continuous process for making Cheddar cheese (known as APV Siro Curd Process) (Kosikowski, 1986).

Precheese method is most successful as far as commercial application of UF for cheese making is concerned (Renner and Abd El-Salam, 1991). Skim milk or whole milk is concentrated up to the solids equivalent to that desired in the final cheese. There is usually no production of whey, which enables 100 per cent utilization of whey proteins and casein in the final product. This process is based on completely new cheese technology and new equipment. A true pre-cheese UF process is most successful for the preparation of soft and semi-hard varieties, i.e., those cheeses containing more than 45 per cent moisture, e.g., Cream cheese, Quarg, Ricotta, Feta, Mozzarella, Camembert, etc.

4.2.3 Traditional Dairy Products from UF Retentate: The acid coagulated and fermentation traditional dairy products, namely Paneer, Chhana, Chakka and Shrikhand involve coagulation/fermentation of milk and removal of whey, thereby concentrating proteins and fat in the coagulated mass. The potential use of UF has been reported in the preparation of these products (Sharma and Reuter, 1991, 1992 & Sachdeva et al. 1993). Rasogolla, a chhana based sweet, can also be prepared in dried form employing UF process and spray drying of retentate (Pal et al., 1994). Ultrafiltration in combination with diafiltration helps in producing highly purified food grade proteins with low lactose content, and having highly improved functional properties such as emulsion capacity and foaming stability (El-Shibiny, et al 1996). Such Functional Proteins Are Finding Increasing Applications In Food Industry.

4.2.4 Treatment of Whey by UF Process: The Development of UF process has undoubtedly proved a boon for the cheese makers in the treatment of whey. the use of UF to fractionate and concentrate whey proteins, followed by evaporation and spray drying, is now a well-established commercial process for the manufacture of whey protein concentrate (WPCs) having 35-80 per cent protein. WPC can be further separated into β-lactoglobulin and α-lactalbumin fractions, or be used for the manufacture of casein macropeptide, a compound that may have pharmatherapeutic value (Pearce, 1996). The main attraction of UF for processing whey include an almost 100 per cent recovery of whey proteins for edible and other useful
applications, with a simultaneous reduction in lactose content. It is possible to concentrate protein up to 20 times with a 95 per cent reduction in volume; further increases in protein and reductions in lactose content are possible employing the diafiltration technique. Whey is one of the most notorious membrane foulants of all the fluid milk products, hence it’s pre-treatment and adoption of remedial steps to alleviate fouling problem are very important aspects during UF membrane processing (Pal and Cheryan, 1987c).

4.2.5 Biotechnology Oriented Applications: Most recently, membrane technology has been applied to the bio-technological processes. Ultrafiltration can be used to harvest microorganisms from fermentation broths with much higher recoveries than traditional filtration and centrifugation processes. For example, hollow fibre ultra-filters can give 100 per cent rejection of *Lactobacillus bulgaricus* cells in whey permeate fermentation broth. The fouling problem with UF membranes during cell harvesting is less than with MF process. According to Cheryan (1998), ultra-filtration can be used for numerous applications related to the preparation of enzymatic hydrolysates from milk proteins including; removal of enzyme and unhydrolysed proteins from a reaction mixture, adjustment of molecular weight distribution profile of peptide mixture, compositional adjustments related to salts or free amino acids, removal of bitterness and antigenic molecules from casein and whey protein hydrolysate, and selective concentration of highly functional peptides.

Enzyme extracted from plant and animal tissues can also be purified by UF process (Gauthier and Pouliot, 1996). Ultrafiltration membranes containing immobilized enzymes or whole microbial cells function as continuous bioreactors. These bioreactors have been reported to provide an opportunity for greatly improving the performance and productivity of fermentation (Tejayadi and Cheryan, 1995). Ultrafiltration is also used for the production of high-quality water suitable for pharmaceutical and biotechnology purpose. The most efficient removal of pyrogens, bacteria and particulates at low energy are the main advantages of UF over other water treatment techniques.

4.2. Microfiltration

The development of new ceramic membranes has been a major break through in making MF a commercial separation process in food industry. A wide range of MF membranes are now available with pore sizes varying from 0.1 to 1.4 μm. The potential applications of MF in dairy industry include separation/harvesting of bacteria and spores, fractionation of milk proteins, clarification of whey; and its several applications in food industry.

4.2.1. Bacteria Reduced Milks: Tetra Pak have a patented system called Bactocatch which is capable of producing pasteurised milk with an extended shelf life. The system is a combination of two unit operations i.e. MF and UHT treatment. The process was originally developed to remove spore forming bacteria from milk used in the manufacture of washed curd cheese such as Edam, Gouda and Emmentaler types (Larsen, 1996). The process has been extended to market milk processing. Sachdeva *et al.* (2001) microfiltered skim milk in MF plant having ceramic membrane of 1.4 μm pore size and could achieve very high efficiency (99.999%) of bacterial removal without adversely affecting the organoleptic and nutritional
quality of milk. The shelf life of pasteurised microfiltered milk at 4°C has been reported more than 2 months.

4.2.2. Production of Native Phosphocaseinate: Selective separation of native casein micelles from the whey proteins is another useful application of MF. Whole or skim milk is circulated along a MF membrane with a pore size of 0.1μm. The resultant retentate is an enriched solution of native and micellar phosphocaseinate (PPCN). Diafiltration of PPCN can help in getting up to 90 per cent protein in dry matter and it is further concentrated by vacuum concentration and spray drying (Mambris, 1997) The spray dried retenate can be used in applications where traditionally calcium caseinate has been used. The microfiltrate (permeate) is crystal clear and can be sterile if down stream equipment prevents recontamination.

4.2.3. Fractionation of Caseins: Several methods for the fractionation of caseins have been developed and are based on the association characteristics of caseins (Sachdeva and Buchheim, 1997). These include isolation of β-casein by MF of Ca-caseinate at 5°C, and MF of skim milk at 4°C and pH 4.2-4.6. The application of MF to skim milk in combination with other membrane processes and/or chromatography process opens up possibilities in isolating and purifying caseins and the peptides derived from them that can find application in the pharmaceutical industries. Of special interest in this regard are peptides derived from β-casein that have been shown to exhibit morphine-mimicking, cardiovascular and immuno-stimulating activities.

4.2.4. Clarification of Whey: Microfiltration can be used to remove large particles such as casein fines, microorganisms or microbial spores, fat globules, somatic cells, phospholipoproteins, particles, etc. from whey. Pretreatment of whey by MF has emerged as a necessary step in producing high purity whey protein concentrates. Pearce et al. (1994) reported 30 to 80 per cent residual lipids removal from cheddar cheese whey using an Alfa-Laval MFS-7 fitted with Ceraver ceramic membranes of 1.4 and 0.8 μm porosity, respectively. There is a 1.8 fold increase in the rate of whey proteins in UF processes when the lipids have been removed by MF.

4.3. Nanofiltration

Nanofiltration is relatively a new process and not many applications are attempted so far. In this process monovalent metal ions (Sodium and Potassium) and chloride ions will pass through the membrane, while divalent ions and most other components are rejected to various degrees. True protein is 100 per cent retained. Kelly and Kelly (1985) achieved 64 to 70 per cent reduction in the chloride (on dry matter basis) and overall ash reduction of 25 per cent in whey by employing NF. The application of NF technology to whey (both salt & sweet) to remove the sodium ions and other monovalent ions for further processing can be a viable process (Bird, 1996). Pal et al. (2002) reported that level improved the quality of khoa made there from by obviating the inherent problem of salty taste and sandy texture in khoa could be overcome by NF of cow milk to 1.5 fold nanofiltration of cow milk to 1.5 fold. Dahi prepared from NF cow milk was also found to be superior to that of normal cow milk dahi.

5.0 REFERENCES


1.0. INTRODUCTION

The additives are increasingly being used in food during production, processing, packaging and/or storage without affecting any adverse effects. Additives play a major role in new product development. Food processors modify the food additives to popularize the processed food products. Food additives and their degradation products should be non-toxic at their recommended levels of use and it should show no sign of acute to chronic toxicity, teratogenic and mutagenic effects. Food additives may perform toxicity, teratogenic and metagenic effects. Food additives may perform toxicity in three ways (i) The additives may be extensively metabolized in the gut, thus modifying the toxicity of original compounds (ii) The additives or its metabolites may undergo enterohepatic blood circulation (iii) The additives may induce changes in gut morphology/function and/or microbial population.

There are as many as 2700 chemical additives or mixtures used as food additives. However, new additives are listed every month. It is very important to ensure the food safety at the additives. In view of the above facts, bio additives should be given more priority and efforts should be made to produce bio additives on large scale on reduced cost.

2.0. BIO-PRODUCTION OF FLAVOUR

Flavour plays a very important role on the acceptability of food. Flavour may be defined as a mingled but unitary experience that includes sensation of taste, smell and other coetaneous sensation. Most natural flavours and fragrances are the mixture of chemicals such as terpenes, aldehydes, esters, lactones, higher phenols and other complex molecules that result from secondary metabolites of plants and may be obtained from certain animal sources. Recent advances in microbial genetics, fermentation and enzymatic processes coupled with current demands, for flavour and fragrance chemical, of natural origin have shown interest in adopting new technological tools for flavour production.

2.1. Types of Flavour Production

Plant production method - Most of the flavour compound, in foods, pharmaceutical and cosmetic industry are basically plant derived. Plants or parts of plants used for the production of flavour can be manipulated either by breeding the plant to produce greater amounts of fragrence by applying novel plant breeding method. Plant tissue culture is a technique, that allow, plant call, to be grown in solid or liquid media and has been proved to possess immense potential for production of flavouring compounds on commercial scale. Vanilla flavour compounds and menthol are some of the commercially important products that are worth being synthesized by plant culture techniques (Kumar et al., 1998).
2.2. Genetic Manipulation

Genetic manipulation of microorganisms may be another approach to enhance the flavour biosynthesis. It involves techniques such as non-specific mutation, enhanced gave replication and closing of genetic material into more rapidly growing or stable cell lives. Mutation can induce changes in path ways that cause more accumulation of desirable metabolites or introduce alternate pathways to form products of greater value. Similarly a mutation may decrease the susceptibility of a microbe to end produce inhibitor or catabolic repression which results in increased yield of flavour components.

3.0. BIO COLOUR

The aesthetic quality of food is first judged with first sensory quality colour. Foods are pleasing to exes are more likely to be consumed thereby, contributing varied diets and better nutrition. The natural pigments of fresh fruits and vegetables are very attractive and brilliant. However, there pigments undergo adverse physical and chemical changes during processing that causes partial to significant degradation of natural colour. Therefore, there is a big challenge to food processors to replace the lost colour with natural colour which is acceptable, attractive and safe. (IFT, 1986)

3.1 Annatto extract

Annatto extracts are prepared by beaching the seeds with one or more of approved food grade solvents such as edible vegetable oils and fats and alkaline and alcoholic solutions. Depending on and use, the pigments from alkaline extracts are precipitated with food grade mid, and further purified by recrystallization from approved solvents. The major colouring compound of the oil soluble extract is carotenoid, bixin which shown fairly good light and heat stability but is susceptible to oxidation, which is accelerated by heat and light. Bixin is primarily used in dairy and fat based products such as processed cheese, butter, margarine, cooking oils, saled dressings, desserts, baked good, and snack foods. Bixin can be hydrolyzed by alkaline treatment during or after extraction to produce water soluble diacid norbixin. Norbixin precipitates in an acidic environment. Norbixin is widely used for colouring cheese, smocked fish, sausage casings, flavour confectionery, cereal products etc.

3.2 Anthocynins

Anthocynins are the glycosides of anthocyanidins consisting of 2-phenyl benzo-pyridium structure. The six most common anthocynins are pelargonidin, cyaniding, delphynidin, rehinidin, reonidin and malvidin (Rayer, 1991). Anthocynin pigments are obtained by extraction with acidified water or alcohol followed by concentration under vacuum and/or reverse osmosis. The extract can be spray or vacuum dried to yield powders. Anthocynin appears red in acidic pH and with increase in pH, it turns in blue. Anthocynin exhibits most intensive colour below pH 3.5. However, anthpcynins easily undergoes discolouration in the presence of amino acids and phenolic sugar derivative due to condensation reaction.

3.3 Beet Powder

Beet powder in dark red powder from dehydrating sound mature good quality edible beats. Beet roots contain red pigment, betacyanins and yellow pigments, betaxanthins. The betacyanin content generally for exceed, than that of betaxanthins. The colour extract is prepared by crushing mature, sound clean beet roots. The juice can be concentrated under vacuum to total solids of 40-60 per cent. The powders are prepared by spray drying the
contents. Beet colourants readily dissolve in water and water based products. Beet colour is used at 0.1 to 1.0 per cent level to colour food, such as hard candies, yoghurt, ice creams, saled dressings, cake mixes, powdered drink mixes, gravy mixes, soft drinks and gelative desserts (Marmion, 1984).

3.4 Caramel

Caramel colour is responsible for attractive brown colour of cooked foods and it belongs to the group of melanodin pigment. Liquid corn syrup of 60 Brix or higher reducing sugar content is the most widely used raw material for the manufacture of caramel. Liquid corn syrups are heated at 121°C in the presence of accelerators for several hours or until the proper tinctorial strength is obtained. The product is then rapidly cooled, filtered, blended and standardized. Caramel colour is freely soluble in water and insoluble in most organic solvents.

3.5 Cochineal Extract

Cochineal extract is the concentrated solution obtained after the removal of alcohol from an aqueous alcoholic extract of cochineal. Cochineal is obtained from the dried bodies of the female insects’ coccus cacti. Cochineal extract6 is typically acid (pH 5.0 – 5.3) and has a total solid, content of about 6 per cent. Cochineal extract is used in producing pink shades in candy, confectionery, soft drinks, jams, and jellies at 0.04 – 0.2 per cent level.

3.6 Paprika and Paprika Oleoresin

Paprika is the deep red, sweet pungent powder prepared from ground dried pods of mild capsicum, capsicum annum. Paprika oleoresins are the combination of flavour and colour obtained by extracting paprika with one or more combination or approved solvents. Paprika oleoresins are brown red, slightly viscous, homogenous liquids, pourable at room temperature and containing 2-5 per cent sediment. Paprika oleoresin is used in meat products such as sausages for flavour and colour. It is also used at 0.2 to 100 ppm level or produce orange to bright red shades in seasonings, snack products, soups, cheese, fruit sauces and dippings.

3.7 Lycopene

Lycopene is an important natural pigment present potentially beneficial biological activity beyond their traditional role as vitamin A precursor; lycopene in particular is the one with the most promising implications for human nutrition and health. Consumption of tomatoes and tomato products has been shown to provide nutritional and health benefits. Epidemiological evidence suggests a possible correlation between consumption of tomatoes and tomato products and lowered risk of developing diseases. Lycopene is potent inhibitor of human censer cell proliferation than either α or β-carotene (Levy et al., 1995).

4.0. MICROBIAL METABOLITES AS BIO ADDITIVE

Naturally occurring antimicrobial compound, are derived mostly from lactic acid bacteria. These bacteria are employed in the manufacture alters the sensory characteristics of raw materials often resulting in food, with increased nutritive and economic values. The antimicrobial metabolites of LAB are unique with respect to their role in the bio-preservation of foods.

4.1. Organic Acid

The controlled production of organic acid by micro-organism in-situ is an important form of bio-preservation. Since, it can inhibit the growth of spoilage microflora. The most
active antimicrobial organic acids and acetic, lactic, propionic, sorbic and benzoic acids. The concept of using LAB to prevent botulinal toxigensis though in-situ acid production exploits the inability of clostridium botulinums to grow at pH < 4.8 as a defense suitable for the growth.

**4.2 Carbon Dioxide**

It is a major end-product in fermentation of hexose by heterofermentative LAB. The production of carbon dioxide is responsible for eye formation in many types of cheeses and contributes to the antagonistic activities of LAB.

**4.3 Hydrogen Peroxide**

Lactic acid bacteria produce hydrogen peroxide through electron transport, via flavin enzymes in the presence of oxygen and are reported to be antagonistic towards several other bacteria. Since LAB is catalase negative, the hydrogen peroxide produced is accumulated in growth medium to the extent of auto inhibitory levels.

**4.4 Diacetyl**

Diacetyl, a metabolic end product synthesized from pyruvate aerobically as well as anaerobically, is well recognized for its antimicrobial properties against food borne pathogens, and spoilage microorganisms. Diacetyl is found in red and white wines, brandy, roasted coffee, silage and many other fermented foods apart from giving characteristic flavour in butter, ghee and butter milk.

**4.5 Bacteriocins**

Bacteriocins are small, single or complex protein, or pertinacious substances that exhibit bactericidal activity against a limited range of organisms, usually closely related to the producer. The bacteriocins with a wide inhibitory spectrum are interesting because of their potential to control the growth of spoilage and pathogenic flora. Bacteriocins are directly added to food to inhibit spoilage causing pathogenic microorganism. Bacteriocinogenic cultures can be added to non-fermented food, as starter culture in fermented food, to improve safety and quality.

**4.6 Nisin**

It is added to milk, cheese and several other dairy products, canned foods and infant formulae worldwide. Nisin, the bacteriocin produced by certain strains of LAB, is the only bacteriocin that has been permitted for use in some food, in more than forty five countries including India. Nisin is designated as GRAS, as an antibacterial in some cheese spread, and is used commercially as an antimastitis teat dip. Nisin is less effective at elevated than at refrigeration temperature.

**5.0 CONCLUSION**

Consumers of 21st century are more demanding towards ‘natural food additives’ and the advantages associated with microbial production of flavour are making it a real alternative. Similar change in attitude is focusing towards, the use of focusing towards the use of natural food colours. PFA has also restricted the permissible limit of food colours in processed food products.
6.0 REFERENCES

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1.0 INTRODUCTION

Improvements in the production, manufacture, and/or storage of foods by both the food industry and the consumer have significantly diminished, but not eliminated, the likelihood of food-related illnesses and product spoilage. Several highly publicized and very costly outbreaks and recalls due to contamination of foods with pathogenic bacteria have resulted in greater awareness of food safety for consumers, producers, processors and regulators. Coupled with an enhanced awareness of food safety, more interest and research has also been directed toward developing alternatives to antibiotics in animal feeds, particularly throughout Europe. There remains a critical need to develop, implement, and optimize strategies to better control undesirable microbes in foods both pre and post-harvest, as well as in animals targeted for human consumption.

The production of anti-microbials and the lowering of pH during the transformation of raw foodstuffs into derivatives that are more stable by the lactic acid group of bacteria (LAB) is an approach to food preservation that has been practiced for centuries. The time, temperature and the pH of the fermentation as well as the natural anti-microbials produced by LAB have proven effective for controlling both pathogenic and spoilage microbes in a variety of foods. Several anti-microbials produced by the lactic acid bacteria have been shown to inhibit undesirable bacteria in synthetic media. In recent years, bio-preservatives have also been used effectively to extend shelf life and ensure the safety of foods. However the efficacy of most anti-microbials of LAB origin notably bacteriocins, has not yet been fully explored in foods. Despite the potential negative impact of various food components on biological activity, bio-preservatives have found application in dairy, meat, vegetable, fruit, bakery and fishery products as well as in certain beverages. Strategies for incorporating bio-preservatives into foods include the use of LAB with proven anti-microbial activity as starter cultures or starter adjuncts, the use of a bio-preservative preparation in the form of a previously fermented product, and/or the use semi-purified or purified bacteriocins.

Under the panoply of the term bacteriocin, there are a number of substances produced by the bacteria that are inhibitory to other microbes. The potential for use of bacteriocins in the food industry has spurred research in this area. Bacteriocins have been envisaged as an effective means of aiding in the preservation of foods by controlling fermentation, and by preventing or reducing food spoilage while extending the shelf life and stability of the product with regards to microbial activity. At present, many attempts are being made to incorporate
Jacob et al. (1953) were the first to define the term bacteriocin. Their definition referred to colicin proteins with intra species antagonistic effects. As more substances of similar nature have been found, the term has grown broader. Presently, there are a wide variety of bacteriocins, of which nisin is best characterized with regard to the food industry. General criteria for grouping under the term bacteriocin include: 1) a narrow spectrum of activity against other bacterial strains, 2) An essential, biological moiety for activity, 3) bactericidal activity; 4) adsorption to specific receptors on cells; 5) the genes for production and immunity to the bacteriocins found on plasmids, and 6) lethal biosynthesis.

A recent definition of bacteriocins produced by LAB suggests that they should be regarded as extra-cellular released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity. They should include at least some strains of the same species as the producer bacterium and against which the producer strain has some mechanisms of specific self-protection (Jack et al., 1995). The possibility of exploiting bacteriocins in food fermentations arises where the inhibitory spectrum includes food spoilage and/or pathogenic microorganisms or gives the producing strains a competitive advantage in the food milieu. The target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the LPS of the outer membrane of Gram-negative bacteria, they are generally only active against Gram-positive cells. In the context of fermentation, important targets includes spoiler such as species of clostridium and hetero-fermentative lactobacilli and food borne pathogens including Listeria monocytogenes, Staphylococcus spp., Clostridium, Enterococcus and Bacillus spp. The permeability of Gram-negative bacteria can be increased by sub-lethal injury including that which can occur when using ultra high hydrostatic pressure (UHP) and pulsed electric field (PEF) as non-thermal methods of preservation.

In addition, disruption of the integrity of the outer membrane through the use of food grade chelating agents such as EDTA and citrate which bind magnesium ions in the LPS layer can increase the effectiveness of bacteriocins against Gram-negative bacteria. Many bacteriocins are most active at low pH and there is evidence that bacteriocinogenic strains can be readily isolated from fresh and fermented foods. Strains may naturally produce more than one bacteriocin and heterologous expression of bacteriocins has been demonstrated in constructed strains. Protein engineering has led to the development of nisin derivatives with altered anti-microbial activities or greater solubility at pH 6.0 than the wild type nisin. An advantage of bacteriocins over classical antibiotics is that digestive enzymes destroy them. Bacteriocin producing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality. Bacteriocins of LAB, according to the classification procedure proposed by Klaenhammer (1993) and modified by (Nes et al., 1996) are divided into four major sub classes (table-1). The majority of those produced by bacteria associated with food belong to class II and I.

2.0 ASPECTS TO BE CONSIDERED IN THE USE OF BACTERIOCINS IN FERMENTED FOODS

Sensitivity to bacteriocins is strain dependent and resistance among sensitive cells has been reported with resistance to nisin cited as occurring at a frequency of \(10^{-6}\). Whether that frequency would be valid in the complex back ground of a food is unknown. The use of more
than one bacteriocin or bacteriocin-producing strain in a specific food system must be carefully controlled so that mutants resistant to one anti-microbial will not be cross-resistant to the others. The implications of resistance arising from general mechanisms such as the alteration of membrane fluidity have to be studied in relation to resistance to other anti-microbial agents. Nisin is the only bacteriocin with GRAS status for use in specific foods and this was awarded as a result of a history of 25 years of safe use in many European countries and was further supported by the accumulated data indicating its non-toxic, non-allergic nature. Other bacteriocins without GRAS status, which can be based on documented use prior to 1958, will require pre-marked approval. Therefore, bacteriocinogenic starters particularly if used in bacteriocins in the near future.

Table-1: Classes of Bacteriocin Produced by Lactic Acid Bacteria (LAB)

<table>
<thead>
<tr>
<th>Class</th>
<th>Sub-class</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>Lantibiotics – small heat stable, containing unusual amino acids</td>
</tr>
<tr>
<td>II</td>
<td>II a</td>
<td>Small (30-100 amino acids), heat stable, non-lantibiotics pediocin – like bacteriocins with anti-listerial effects</td>
</tr>
<tr>
<td></td>
<td>II b</td>
<td>two peptide bacteriocins</td>
</tr>
<tr>
<td></td>
<td>II c</td>
<td>sec-dependent secretion of bacteriocins</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>Large (&gt;30 KDa) heat-labile proteins</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>Complex bacteriocins with glyco- and/or lipomoieties.</td>
</tr>
</tbody>
</table>

3.0 MODE OF ACTION

The different charge on different parts of the protein may cause a localized alteration of the cytoplasmic membrane because such proteins possess a weak, detergent activity. The interaction has been said to occur in two stages. The first stage is the adsorption of the bacteriocin to a cell surface receptor. No cellular damage results from the attachment and the adsorption is reversible. After a distinct, the second stage occurs causing lethal alterations in the cell. Data indicates that bacteriocins act as lethal particulates. This is termed “quantal” killing to indicate that one bacteriocin molecule can kill one cell. The mechanism for this effect is unknown. Research has suggested that the adsorption causes an alteration in the cell surface, which is transmitted and/or amplified to other sensitive cellular targets. For Gram-positive microbes, abnormalities are noted in energy production, macromolecule synthesis, membrane transport, and/or membrane permeability. Changes in these processes can induce other cellular anomalies. Trypsin has the ability to inactivate bacteriocins, presumably by enzymatic attack on the essential protein component.

With nisin, the primary target is the phospholipids components in the cytoplasmic membrane. Nisin causes a rapid, non-specific efflux of amino acids and cations and loss of the membrane potential resulting in a situation incompatible with continued cell viability.
According to Morris et al 1984 the cytoplasmic membrane disruption is affected by nisin inactivation of sulfhydryl- groups. Nisin is reactive to cellular nucleophiles acting as electrophilic Michael acceptors. Nisin is only effective against Gram-positive microorganisms. The bacteriocins also often exist in two or more, related but distinct forms. These may be actual variations of structural form, variations based on monomers, or artifacts of preparation treatment.

### 3.0 APPLICATION OF BACTERIOCINS IN FOOD SYSTEMS

While bacteriocin characterization, mode of action, and genetic analysis has proceeded at an extraordinary pace it is conceded that most applications remain in the developmental rather than commercial stage. However, the field is beginning to yield very encouraging results, and it may be confidently expected that bacteriocins will confirm at least some of their much-touted potential in the near future. As ever, nisin is in the vanguard of this exciting research, but the pediocins have also been receiving increased attention in recent years. Nisin is the only one of the LAB bacteriocins that is being commercially produced and used. It is produced by fermentation of milk and nisin producing lactococci and is sold as a commercial preparation under the trade of Nisaplin\textsuperscript{R} by Aplin and Barret Ltd. (UK). Nisin has been approved by the World Health Organization (WHO) for its use in the food since 1988. It has also gained approval from the US Food and Drug Administration (FDA). It has been estimated that nisin is currently approved in over 40 countries worldwide and has found use in many different food systems.

#### 3.1. Dairy Industry

In cheese fermentation it is difficult to ensure even distribution of an inhibitor in the final cheese unless it is added while the milk is still liquid. One of the obvious problems in using a broad host range bacteriocin such as nisin at this stage is that, the starter bacteria are as likely to be inhibited as the potential spoilage or pathogenic organism. This can be circumvented by utilizing nisin resistant cultures if nisin is to be added as an exogenous preservative or by using nisin producers with intent immunity, as starters for the fermentation. Notwithstanding this problem, the beneficial effects of including nisin in cheese fermentation have been noted by a number of workers. One of the most striking effects is the prevention of “late gas blowing” due to outgrowth of clostridial spores. This has been reported as a particular problem in a number of hard and semi-hard Dutch and Swiss cheeses, mainly due to the outgrowth of spores of \textit{Clostridium butyricum} and \textit{Clostridium tyrobutyricum}. The addition of exogenous nisin and the use of nisin producing cultures have both proven effective in eliminating this gas defect, but the method has not as yet gained widespread acceptance, probably due to the effect of nisin on the natural flora of the cheese.

An alternative approach, which could be used to control specific pathogens or spoilage organisms in dairy foods, is to utilize bacteriocins with a highly specific activity range. For example enterocin 1146 like many of the pediocin like bacteriocins is extremely active against \textit{Listeria monocytogenes} at levels, which have no effect on lactococcal starters. In dairy foods, which do not require starter bacteria, broad host range bacteriocins are to be preferred, and so inhibitors can be added without regard for the effect on beneficial flora. Nisin has been included in processed cheese and cheese spreads for many years to prevent the outgrowth of clostridial spores. This is a successful commercial operation and nisin has been used to prevent the outgrowth of clostridial spores, including those of \textit{Clostridium botulinum} for over 40 years.
The nisin is added exogenously and allows manufacturers to make a higher moisture product without risk of spoilage or health concerns for the consumers.

### 3.2. Canning Industry

Nisin has been used to good effect in both low acid (pH>4.6) and high acid (pH<4.5) canned foods to prevent the outgrowth of clostridial spores. Nisin used in a number of low acid canned products, including carrot puree, mush-rooms, peas and beans, soups, pasteurized hams, potatoes, sausages, fish, rice spaghetti and macaroni (de Vuyst and Vandamme, 1994 ). In some instances the addition of nisin can allow a more gentle heat process, and thus improve the appearance and taste of the final product.

### 3.3. Meat Industry

Meat has a number of intrinsic properties, which make the use of bacteriocins more problematic than liquid foods. In particular it is difficult to achieve an even distribution, there may be solubility problems, bacteriocins can partition to the fat phase, or simply bind to meat proteins. With the exception of nisin in canned hams mentioned above, bacteriocins have not found many applications in fresh or cooked meats. However, one area in which some progress has been reported is in fermented meats and frankfurters. for example , it has been reported that a combination of nisin and low levels of nitrite inhibited the outgrowth of *Clostridium perfringenes*, spores in frankfurters, where as nitrite alone at the levels normally used , was effective.

There have been a number of reports concerning the ability of pediocins or pediocin producing cultures to control the growth and survival of *L. monocytogenes* in fermented meats such as summer sausage and in frankfurters. One example will serve to illustrate the potential in this area. Luchansky *et al.* (1992) used a pediocin producer and an isogenic non- producer to inoculate sausage batter, which was subsequently spiked with a 4- strain cocktail of *L. monocytogenes*. The number of *Listeriae* remained high (10³ /gram) in the sausage containing the non – producer but fell to less than 10² /gram when the producer strain was used. Significantly, the pediocin produced survived the cooking process at the end of the fermentation and was detectable throughout 60 days storage at 4 C.

Sakacin A, one of the pediocin–like bacteriocins, has also been used to control the growth of *L monocytogenes* in pasteurized ground beef (Schillinger, 1994). The presence of a sakacin A producer prevented growth of a high inoculum of L. monocytogenes at 8C, whereas in the absence of the sakacin a producer levels increased from 10⁵ to 10⁹ /gram with in 10 day. Similar results, including a killing effect against the deliberately added cocktail of *Listeriae* were reported for sakacin a in comminuted pork

### 3.4. Wine and Beer

The malolactate fermentation is an important feature of some wines. This fermentation must be controlled to achieve a high quality product. In many red wines the conversion of dicarboxylic malate to monocarboxylic lactate is a desirable consequence of the growth of LAB such as *Leuconostoc oenos* or *Pediococcus damnosus*. This conversion can lead to a rise of between 0.1 and 0.3 of a pH unit, and also has an effect on the sensory characteristics of the wine. Where the malolactate fermentation is desired the wine can be inoculated with selected strains of *Leuc. oenos*. However, in certain white wines this same fermentation has a negative effect on the wine quality and is undesirable. Nisin can be used to promote the quality of both wine types (Daeschel *et al.*, 1991). The nisin will prevent the growth of all LAB other than the...
deliberately added nisin resistant starter and highly reliable malolactate fermentation in the developing wine. An additional benefit to both instances is that fermentation can be a cause of defects such as ropiness and excess of acetate. The growth of Lactoball and Pediococci in beer is a major cause of spoilage, leading to a beer with too much developed acid and with off flavours due to the production of diacetyl. It has been demonstrated that nisin can be used to control this problem without having an adverse effect on the beer quality.

3.5. Fruits and vegetables

Many canned vegetables and fruit products have been investigated for potential use of nisin. The products range from potatoes, to peas, mushrooms, soups, canned tomatoes, tomato juice, cream style corn and chow-mein. Use of nisin may allow reduced thermal processing of canned fruit thereby preserving color, taste, texture and nutritional value. Nisin at levels of 100 to 200 IU/g product can prevent germination and growth of *C. botulinum* spores. This ability, together with the heat stability of nisin, has allowed for reductions in heat treatments in certain thermally processed foods such as mushrooms.

4.0. FUTURE PROSPECTS OF BACTERIOCINS APPLICATION IN FOOD PRESERVATION

There is currently a large number of research on ‘natural antimicrobials’ for food applications (Goud, 1996), of which bacteriocins comprise one group of compounds that are being studied. Bacteriocin of LAB and other foods grade bacteria have the advantage that the organisms generally have GRAS status with regulatory agencies. Although the purified bacteriocins, except for nisin, have not been licensed for addition to foods, it is clear that bacteriocin residues are currently present in the food supply. Two commercial compounds that have been licensed for addition to foods, Microgard and Alta 2341, are ferment of food grade bacteria that impart antibacterial properties to the foods. It is commonly stated that, except for nisin, applied studies on bacteriocins are lacking. This is understandable because no other bacteriocin have been licensed for addition to foods. Convincing evidence of inhibition of pathogens and spoilage bacteria is required to stimulate commercial interest in bacteriocins as agents for biopreservation. Unfortunately, except for a few bacteriocins, they have a narrow antibacterial spectrum and they are not active against gram negative bacteria. Use of nisin with a chelating agent expands the antibacterial spectrum of nisin to include Gram-negative bacteria.

The range of minimally processed foods available in the marketplace continues to increase. Furthermore, there are an increased number of foods that are packed under anaerobic conditions that rely on refrigeration as the sole line of defense against pathogen growth. The studies included in this review were primarily those in which researchers had studied the effect of LAB and (or) their bacteriocins in food matrices. It is expected that various factors in foods will influence the efficacy of bacteriocins and bacteriocinogenic LAB (Daechael, 1993), such as, adequacy of the food environment for bacteriocin production; loss of bacteriocin-producing capacity; antagonism with other bacteria; inhibition by bacteriophage; development of a bacteriocin-resistant microflora; and inactivation of the bacteriocin by enzymes or binding to fats or proteins. The amount of information on bacteriocins at the basic level and at the applied level for biopreservation of foods is rapidly accumulating although there seem to be many constraints to biopreservation of foods with bacteriocinogenic LAB., especially if genetically modified LAB are used, the time is approaching when the food industry and government can evaluate the value and the acceptability of these techniques.
5.0. REFERENCES

1.0. INTRODUCTION

There are two main objectives in food microbiology; firstly to determine the total load or numbers of microbes in a sample, and secondly to determine the presence or absence of a particular microbial species, usually a pathogen or related type used as their indicators. In contrast to the Quality Control, which is a reactive approach with emphasis on measurement that is statistically relevant and focuses on legal requirements, Quality Assurance is a preventive approach with an emphasis on operational procedures, which must be robust and regularly reviewed and the focus is on consumer. Thus, while the first type of tests for the quality assurance of food products is in accordance with the statutory requirements, the second type of analysis is mainly focused at the public health aspects with regulatory requirements being an integral part. Enumeration of specific groups is gaining major importance in the current times and are directly associated with the safety or assessment of the risk of food poisoning. The results are not always absolute in conventional testing and are often influenced by the method and incubation conditions used. Moreover, the time taken to complete the conventional microbiological assays has changed little as it depends on the time taken for microbes to grow and multiply so as to give a visible colony in the surface of nutrient solid medium, colour change by the metabolism of specific chemicals, or obvious turbidity in the medium.

2.0. BIO-TECHNIQUES: NECESSITY AND IMPORTANCE

Much effort has accordingly been devoted to shortening assay times and to replacing the visible end points with alternative measurements. The procedure used in the conventional assay involve the steps of blending sample with a diluent, serial dilutions and inoculation of liquid or solid growth medium followed by incubation that may vary from 1 to 7 days. The visible colonies that have grown on solid medium are counted or the turbidity of liquid medium is noted. Further biochemical tests are often done especially to confirm the identity of pathogens or organisms indicative of their presence. Thus, it may be seen that much of the routine of food microbiology is laborious, technically demanding and slow to yield results. In recent times, some of the steps have been mechanized, for example use of diluters or spreaders and colony counters, but the overall assay times have not altered much, even with the advent of improved and more selective media.

In addition to the general testing requirements under quality control programmes, there
has been an added element of quality assurance that is being pursued vigorously under the implementation of Quality Management Systems like ‘Hazard Analysis Critical Control Point System’ that is commonly known as HACCP. For the success of these advanced management systems it was increasingly being realized that there was a need to develop certain rapid techniques that are sensitive and accurate.

3.0. BIO-TECHNIQUES IN ASSURING QUALITY AND SAFETY

This has lead to the development of several biotechniques that are now-a-days being employed for assuring the quality and safety of food products. The focus to these methodologies was drawn during the first Symposium held in USA in the year 1973 on ‘Rapid methods in microbiology and immunology’. Since then, many new approaches have been described, some of which have found wider application and are being discussed here. The main techniques are; Electrical methods (conductance or impedance, electrochemical assays), Chemical methods (direct epifluorescent filter (DEFT), bacterial ATP bioluminescence), Cytometry, Biosensors, Agglutination methods (immunological assays) and Nucleic acid technologies (polymerase chain reaction (PCR), riboyping, microarrays)

3.1. Impedance

These assays have been widely accepted for quality assurance programmes. In this case the net metabolic activity of the culture is represented by conductance assays and this correlates well with the microbial load in the material. Impedance includes a capacitance component which can be influenced by microbial and other biomass, whether living or dead. Specific media can be used for a particular organism. The higher the microbial load, the shorter is the assay time. Results can be obtained within 2-4 h. For the detection and counting of specific pathogenic bacteria, substrates used uniquely by the target bacteria of inhibitors to which all but the target bacteria are sensitive are included in the medium to increase the response by the pathogen and minimize the growth and contribution by other microbes. Up to 500 samples can be examined simultaneously on the current machines.

Current commercial instruments are Malthus (IDG, Bury, UK), RABIT (Don Whitney Scientific, Yorkshire, UK), Bactometer (bioerieux Basingstoke, UK) and BacTrac (Sylab, Austria). These instruments operate at frequencies between 2 and 10 kHz, with the capacitance changes with microbial numbers being more pronounced at 2 kHz. All the instruments have similar basic components; an incubator system, a monitoring unit that measures the conductance and/or capacitance and a computer-based data handling system. The detection of microbial growth using electrical systems is based on the measurement of ionic changes occurring in media, caused by the metabolism of microorganisms. The electrical changes are detected at the threshold of detection and the time taken to reach this point is called the detection time.

3.2. ATP Bioluminescence

All living cells contain ATP (adenosine triphosphate), the high energy intermediate that powers most energy-consuming reactions. The test depends on the reaction between ATP and the enzyme luciferase, producing light which is measured photometrically with a claimed sensitivity down to 10^{-16} \text{ mol l}^{-1}. It is a rapid test, taking <1 hour to complete. McElroy in 1947 demonstrated for the first time that the emission of light in the bioluminescent reaction of the firefly, Photinus pyralis, was stimulated by ATP. The light yielding reaction is catalyzed by the
enzyme luciferase, this being the enzyme found in the crude firefly extracts causing luminescence. Luciferase takes part in the following reactions:

1. \[ \text{Luciferase} + \text{Luciferin (LH}_2) + \text{ATP} \Rightarrow (\text{Mg}^{2+})\text{Luciferase}–\text{Luciferin(LH}_2)–\text{AMP} + \text{PP}_i \]

2. \[ \text{Luciferase–Luciferin (LH}_2)–\text{AMP} + \text{O}_2 \Rightarrow \text{Luciferase} + \text{Luciferin(L)} + \text{AMP} + \text{CO}_2 \]

\[ + \text{hv} \rightarrow \text{(light)} \]

The light-yielding reaction is efficient, producing a single photon of light for every luciferin molecule oxidized and thus every ATP molecule used.

The problem with most foods is that they also contain ATP, and that the ATP content of microbial cells is variable depending upon their nature, type of bacterium, yeast, spore etc. and their physiological state. In addition the assay has no specificity. It is possible to destroy the somatic (food) cell ATP, by using detergents to lyse the cells and ATPase to destroy the ATP. However, there is increasing use of ATP measurement as a hygiene test for the cleanliness of food production areas. For this, the total ATP is measured as food residues contain far more ATP than the bacteria and a standard can be set. Also, most foods contain more ADP (adenosine diphosphate) than ATP and this ADP can be converted to ATP enzymatically. Both these developments have increased the sensitivity of the assay. However, one area that ATP bioluminescence has not yet been able to address has been the detection of specific microorganisms. The most promising method in this area is the use of genetically engineered bacteriophages.

3.3. Direct Epifluorescent Test (DEFT)

Automated microscopy methods have been widely applied. Due to the relatively low density and small size of microbes they must be treated to stand out from the background mass. One such example is the DEFT, which depends on the uptake of acridine orange by the cells. Viable cells fluoresce orange under ultraviolet light owing to their ribonucleic acid (RNA) content, whereas dead or non-growing cells fluoresce green due to interactions of the dye with deoxyribonucleic acid (DNA). Samples are concentrated by filtration, stained and viewed microscopically. Image analysis equipment can be used to automate counting. These tests have been employed mainly on products that have not undergone a heat treatment and could give misleading results, and food products that could be easily filtered. The sensitivity is only \( \sim 10^4/\text{ml} \). A variant of the technique is hydrophobic grid membrane technique.

The DEFT is a labour-intensive manual procedure and the first fully automated instrument based on fluorescence microscopy was the Bactoscan (Foss Electric, Denmark). Milk samples placed in the instrument are chemically treated to lyse somatic cells and dissolve casein micelles. Bacteria are then separated by continuous centrifugation in dextran/sucrose gradient and are incubated with a protease to remove residual protein, then stained with acridine orange and applied to a disc rotating under a microscope. The fluorescent light from the microscope image is converted into electrical impulses and recorded. An instrument-based fluorescence counting method (Autotrak), in which samples were spread onto thin plastic tape, was developed for the food industry, but the debris from food samples interfered with the staining and counting and gave significantly higher results than corresponding viable counts.
3.4. Cytometric Techniques

Flow Cytometry based techniques have been reported recently. The sample containing microbes is injected into a stream of fluid which then passes a sensor where each particle is detected. The cells under investigation are inoculated into the centre as a stream of fluid (known as sheath fluid). This constrains them to pass individually past the sensor and enables measurements to be made on each particle in turn, rather than average value for the whole population, the sensing point consists of a beam of light (either UV or laser) that is aimed at sample flow and one or more detectors that measure light scatter or fluorescence as the particles pass under the light beam. Fluorescent probes based on enzyme activity, nucleic acid content, membrane potential and pH have been developed and examined. Use of antibody-conjugated fluorescent dyes confers specificity to the system. Perhaps the most successful application of flow cytometric methods to food products has been the use of Chemnuex Chemflow system to detect contaminating yeast in dairy and fruit products.

The technique is still in its infancy and although good results are being reported for some foods, sensitivity for specific detection of a particular organism in different foods is rather less developed. The success of the system depends on the development and use of suitable staining systems and the protocols for the separation of microorganisms from food debris that would otherwise interfere with the detection system.

A relatively new cytometric technique has been developed by Chemnuex (Maisons-Alfort, France) based on Solid Phase Cytometry. In this procedure samples are passed through a membrane filter which captures contaminating microorganisms. A stain is then applied to the filter to fluorescently mark metabolically active microbial cells. After staining, the membrane is then transferred to a Chemscan RDI instrument, which scans the whole membrane with a laser, counting fluorescing cells. The complete procedure takes about 90 minutes to perform and can detect single cells in filtered samples. It is ideally suited to the analysis of water or other clear filterable fluids, and special labeling techniques could be used to detect particular organism of interest.

3.5. Agglutination Methods

Imunochemical have been applied for the detection of microflora in foods. Microbes are antigenic and thus could simulate the production of antibodies when injected into animals. Antibodies are protein molecules that are produced by animal white blood cells, in response to contact with a substance causing an immune response. The area to which an antibody attaches on a target molecule is known as the antigen. Two types of antibodies can be employed in immunological tests. These are known as monoclonal and polyclonal antibodies. Polyclonal antibodies, which react with a broad range of antigens, and monoclonal antibodies which are highly specific to particular antigenic structures have been used in reagent kits for the detection and identification of specific type of bacteria, their surface structure and toxins. The antibodies are tagged to assist in the measurement of the antigen-antibody complexes. The most sensitive labels are radioactive isotopes but these can not be use in food production environments, hence, fluorescent antibodies labeled with fluorescein or umbelliferones are most common. There have also been reports of the incorporation of antibodies, produced in plants, in packaging films. They show visible changes on reaction with the target microbes.

A number of agglutination reactions have been commercialized by manufacturers and
have been successfully used within the food industry. They offer a relatively fast test time, are easy to use and require no specialist equipment, thus making ideal for quality assurance applications. A good example is the latex agglutination kits for *Salmonella* confirmation; Oxoid *Salmonella* Latex kit, Micro-screen Latex Slide, Wellcolex Colour *Salmonella* Test, Spectate *Salmonella* Test. The last two could even differentiate serogroups. Agglutination kits have been also developed for the detection of microbial toxins.

### 3.5.1. Enzyme Immunoassays:
Enzyme immunoassays have been extensively investigated as rapid detection methods for foodborne microorganisms. They have the advantage of specificity conferred by the use of specific antibody, coupled with coloured or fluorescent endpoints that are easy to detect either visually or with spectrophotometer or fluorimeter. Automation of enzyme immunoassays has taken a number of forms including simply automated standard microplate enzyme-linked immunosorbent assays (ELISAs). The *Vidas* system (biomerieux, Basingstoke) uses a test strip, containing all of the reagents necessary to do an enzyme-linked immunosorbent assay test. *Vidas* ELISA tests are available for a range of organisms including *Salmonella*, *Listeria*, *E.coli O157*, *Campylobacter* and *Staphylococcal enterotoxins*.

### 3.5.2. ELISA:
The *EIAFOSS* (Foss Electric, Denmark) is another fully automated ELISA system. In this case the instrument transfers all of the reagents into sample containing tubes, in which all of the reaction occurs. The *EIAFOSS* procedure is novel as it uses antibody coated magnetic beads as a solid phase. The immuno-magnetic reagents have been so developed for capturing and concentrating pathogenic bacteria and have become essential steps in protocols for some potential pathogens like *Salmonella*, *Listeria* and *E. coli O157* detection. They permit larger volumes of extract and hence of food to be screened and about a 9-fold increase in the sensitivity have been shown by such means.

### 3.5.3. Immunochromatography:
Similarly, *immunochromatography* operates on a dipstick, composed of an absorbent filter material, which contains coloured particles coated with antibodies to a specific organism. The particles are on the base of the dipstick and when dipped into a microbiological enrichment broth, they move up the filter material as the liquid is moved by capillary action. At the defined point along the filter material lays a line of immobilized specific antibodies. In the presence of the target organism, binding of that organism to the coloured particles forms a cell/particle conjugate that moves up the filter dipstick by capillary action until it meets the immobilized antibodies where it sticks. The build up of coloured particles results in a clearly visible coloured line, indicating a possible positive test result. Some of the commercial kits based on this technology are Oxoid *Listeria* Rapid test and the Celsis Lumac Pathstik. The main problem with immunological methods is their low sensitivity. The minimum numbers of organisms required are $10^5$/ml.

### 3.6. Nucleic Acid Hybridization Technologies

The specific characteristics of any organism depend on the particular sequence of the nucleic acid contained in its genome. The sequence of bases of nucleic acids makes different organisms unique. Nucleic acid hybridization technologies have rapidly developed in the recent past for quality assurance purposes. Nucleic acid probes are small segments of single-stranded nucleic acid that can be used to detect specific genetic sequences in test samples. Probes can be developed against DNA or RNA sequences. The attraction for the use of gene probes is that a
probe consisting of only 20 nucleotide sequences is unique and can be used to identify an organism accurately. In order to detect the binding of a nucleic acid probe to DNA or RNA from a target organism, it must be attached to a label of some sort that can be easily detected. These labels could be radioisotopic ($^{32}$P) or more recently non-radiolabelled; avidin-biotin link system.

Probes directed towards cell DNA attach to only a few sites on the chromosome of the target cell. Work on increasing the probe sensitivity has centered on the use of RNA as a target, especially ribosomal RNA (rRNA) that is present in very high copy numbers within cells. The first commercially available nucleic-probe-based assay system for food analysis was introduced by GeneTrak System (Framingham, MA, USA) in 1985, that used Salmonella-specific DNA probes ($^{32}$P labelled) directed against chromosomal DNA to detect Salmonella in enriched foods. Later on, in 1988, they also introduced non-isotopically labeled probes for Salmonella, Listeria and Escherichia coli based on targeting the ribosomal RNA. This type of colorimetric hybridization assays are based on a liquid hybridization reaction between the target rRNA and two separate DNA oligonucleotide probes (the capture probe and reporter probe) that are specific for the organism of interest. The capture probe molecules are extended enzymatically with a polymer of about 100 deoxyadenosine monophosphate residues. The reporter probe molecules are labeled chemically with hapten fluorescein. The other commercially available nucleic acid probes for the confirmation of Campylobacter, Staphylococcus aureus and Listeria are Genprobe (San Diego, USA).

In recent years, several genetic amplification techniques have been developed like Polymerase Chain Reaction (PCR) and its variants including nested PCR, reverse transcriptase (RT) PCR and multiplex PCR, Peptide nucleic acid (PNA) probes, Q Beta Replicase, Ligase Amplification Reaction (LAR), Transcript Amplification System (TAS) also known as Self Sustained Sequence Replication (3SR) or Nucleic Acid Sequence Based Amplification (NASBA). Currently there are at least three manufacturers producing kits based on PCR for the detection of foodborne pathogens; BAX Kits (Qualicon, USA) for Salmonella, Listeria, E.coli O157, Probelia Kits (Sanofi, France) for Salmonella, Listeria, TaqMan System (Perkin Elmer, USA) for Salmonella.

The most promising targets for biosensor based biotechniques in food analysis are free radicals and DNA. Recently, Sci Diagnostics GmbH together with GeneScan Europe AG have commercialized a microarray reader family called BioDetect 645 which can be used for the detection of food pathogens as well as genetically modified foods. The DNA Chips used have an active area of 2cm$^2$ and the indication of DNA hybridization takes place via fluorescence detection by a CCD camera. The sensitivity is <10 fluorophors/µm$^2$. With so called NutriChips lactobacillus lactis, L. brevis, L. plantarum, Salmonella spp., Listeria spp., Campylobacter spp., E. coli and Shigella spp. can be detected. Molecular imprinting approach involving the formation of complex between functional monomers and guest molecule (template) in an appropriate solution, and ‘freezing’ of this complex by polymerization in the presence of cross-linker also holds a great promise as an important future application.

3.7. Sensors and Markers

A sensor for food freshness and shelf-life assessment is a relatively new development involving the application of chemosensors, biosensors and immunosensors. It can offer unique
solutions to the problems of determining the food status with respect to microbial or oxidative spoilage

3.7.1. Electronic Nose: The most significant example is the development of ‘electronic nose’ or ‘application-specific electronic nose’ (ASEN) with sensors and algorithms specialized for a specific application including off-flavour detection due to the microbial deterioration of food. The feature vectors for a population of odour samples is analyzed using pattern recognition technique (PARC) or by artificial neural network (ANN) that can be combined with fuzzy logic to produce neuro-fuzzy logic and applied to the component 7 (evaluation mode) of ASEN to arrive at component 8 (odor identification). CHEMFET microelectronic gas sensors have been reported to detect specific odor development on the spoilage of some food products.

The specific volatiles emanating from cultures of organisms, such as *Staphylococcus aureus* and *E. coli* have been reported and the growth of *E. coli* has been monitored with an electronic nose consisting of hybrid sensor array (eight MeOx (metal oxide) resistors, eight QMB (quartz microbalance acoustic device) volatile sensors and four amphometric gas senspr) and a PCA (principal component analysis) pattern recognition. The system did also distinguish between *E. coli* and *Enterobacter aerogenes* from 3 hours’ culturing time onwards.

A number of electronic nose designs are now available commercially like AromaScan, QMB 6, Nordic electronic nose, MOSES II. In addition to that we also have the commercial presence of Gas Sensor Arrays and Fingerprint Mass Spectra Systems (FMS), based on quadruple mass spectrometer combined with headspace sampler and a computer, like MS-Sensor and 4440B Chemical Sensor, based on PARC.

3.7.2. Marker Approach: Similarly, the marker approach has also proved very useful for the assessment of food condition using diverse indicators like; Glucose profile (for meat freshness), Ethanol (for yeast contamination), Penicillin (mastitis treatment), Trimethylamine (fish freshness), Histamine, Tyramine (past microbial activity in fish, meat, cheese), Cadavarine, Putrescine (advanced microbial spoilage), Hydrogen (microbial spoilage of CAP and vacuum-packed meat).

4.0. CONCLUSIONS

The food industry has the responsibility to produce safe and wholesome food and providing this assurance is ultimately the microbiological goal. What is perhaps needed is a microbiology test that could analyze a batch of food non-destructively, on-line and with the required accuracy, sensitivity and specificity. Although our current technical capabilities fall well short of this ideal situation, the rapidly growing biotechniques have an important place in our armoury against the threats posed by microorganisms in food and would keep on playing a key role in assuring better food quality and safety.

5.0. REFERENCES


APPLICATION OF STATISTICAL TOOLS IN DAIRY PROCESSING BIOTECHNOLOGY

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1.0. INTRODUCTION

"Since the ideal of Science is to achieve a systematic inter-relation of facts, scientific methods must be a pursuit of this ideal by experimentation, observation, logical arguments from accepted postulates and a combination of these three in varying proportions".

The Science of statistics enters into research and/or scientific method through experimentation and observation. That is, experimental and survey investigations are integral parts of statistical techniques. Since statistics, when properly used, makes for more efficient research, it is expected that all researchers become familiar with the basic concepts and techniques of this useful science to draw logical conclusions. Statistics is now playing an important role in almost all the branches viz, Engineering, Agricultural, Animal Sciences (Poultry breeding, animal breeding, animal nutrition), social sciences and basic sciences etc. The biotechnology which is of recent origin has also great scope of application of statistics. The categorization of biotechnology into specific field viz, Plant Biotechnology, Animal Biotechnology and the so called process biotechnology has further widened the scope of application of statistical tools. In the following paras we shall restrict our discussion into the field of Process Biotechnology as applicable to food science and technology.

Food exhibit to a marked degree what is widely called "Biological Variation". Their constitution is heterogeneous and properties of end product are not only affected by the multiplicity of factors influencing their growth but also by the infinite variety of processing and storage conditions. Thus, it is impossible to give a general answer to a question such as "what is the moisture content of paneer, cheese, khoa, butter, etc. produced at a plant or laboratory? Before attempting to answer, some basic answers are pertinent viz, what variety, at what stage, what method of processing, at what temperature and period of storage etc. Having obtained the necessary specifications, the food technologist might be able to quote an average value. Such problems could be observed on certain palatability factors-tenderness, juiciness and fibre cohesiveness - of canned beef as conditions of time and temperature of processing are varied. Evaluating a food product in terms of consumer reaction is also not an easy task. To what extent is the taste panel representative of the entire population of tasters? How to distinguish the variation from sample to sample, judge to judge, particular property of food e.g. odour be separated from another property, such as flavour? To answer these questions is essential to have techniques/statistical tools which can provide valuable assistance to research workers who deal with food products.
2.0. STATICAL TOOLS IN DAIRY BIOTECHNOLOGY

To utilize statistical methods to advantage, a person should:

- Be well versed in the subject matter of the field of specialization.
- Know effective means of presenting data in tabular/graphical/analytical form.
- Be acquainted with a variety of statistical techniques, the limitation and advantages of each, the assumptions upon which they are based, the place each occupies in a logical analysis of the data and the interpretations which can be made from them.

A number of statistical tools are available for use in various fields of research. The processing group mainly deals with the laboratory experiments and most commonly used statistical tools are analysis of variance, missing plot technique, mathematical modelling in the microbiological experiments involving SPC, MBRT, coliform counts, pH and moisture contents etc. in the product, the growth curve and also the bio-assays. Factorial experiments, mathematical modelling coupled with linear and non-linear regression and statistical quality control are also very commonly used tools. Various tests of significance are used for drawing logical conclusion of the study to test underlying hypothesis.

So start with let us define a hypothesis. Any statement about the population from which a given random sample \( x_1, x_2, x_3, \ldots, x_n \) may have been drawn is called a hypothesis or simply "a tentative theory or supposition provisionally adopted to explain certain facts and to guide in the investigation of others". For example,

- The average melting point of butter is 55°C
- The total solids in the milk are 170gm per 100gm
- A given drug cures 90 per cent of the mastitis cases.
- SPC in ice cream sample < 2, 50,000
- Coliforms in khoa < 90/gm
- MBRT in Raw milk > 5 Hours

To investigate statistically, the credibility of a stated hypothesis, there are several factors that must be considered viz;

- The nature of the experiment that will produce the data must be defined.
- The test statistic must be selected. That is, the method of analysing the data should be specified.
- The nature and size of critical region (\( \alpha \)) must be specified.
- The size of the sample (is the number of times the experiment will be performed) must be determined.

2.1. Large Sample Tests

The following tests of significance are valid for large sample sizes (n>30).

2.1.1. Test for a Specified Mean: A random sample of size n gives a sample means \( \bar{X} \). Null hypothesis \( H_0: \mu = \mu_0 \), i.e. the population has a specified mean value. The sample has been drawn from the given large population with mean \( \mu_0 \) and standard deviation

\[ H_1: \mu \neq \mu_0 \text{ - two-tailed} \]
\[ \mu < \mu_0 \text{ - left tail} \]
\[ \mu > \mu_0 \text{ - Right tail} \]

Use the test statistic
\[ Z = \frac{X - \mu_0}{\sigma / \sqrt{n}} \rightarrow N(0,1) \]

**Critical region**

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>5%</th>
<th>1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \neq \mu_0 )</td>
<td>(</td>
<td>Z</td>
</tr>
<tr>
<td>( \mu &gt; \mu_0 )</td>
<td>( Z \geq 1.645)</td>
<td>( Z \geq 2.33)</td>
</tr>
<tr>
<td>( \mu &lt; \mu_0 )</td>
<td>( Z \leq -1.645)</td>
<td>( Z \leq -2.33)</td>
</tr>
</tbody>
</table>

**Decision**

By comparing this calculated value with critical value, we can reject or retain the null hypothesis at 5% or 1% level.

**Example**

A sample of 900 non-descript cows has a mean production per day as 3.4 kg and S.D. of 2.61 kg. Is the sample from a large population with average production of 3.25 kg.

### 2.1.2. Test of Significance for Difference of Means

Suppose we have two independent samples of large sizes \( n_1 \) and \( n_2 \) from two populations with means \( \mu_1 \) and \( \mu_2 \) and sample means are \( X_1 \) and \( X_2 \), respectively.

**Null hypothesis** \( H_0: \mu_1 = \mu_2 \)

**There is no significant difference between sample means:**

\[ H_1: \mu_1 \neq \mu_2 \text{ or } \mu_1 > \mu_2 \text{ or } \mu_1 < \mu_2 \]

**Statistical test:** Under \( H_0 \)

\[ Z = \frac{X_1 - X_2}{\sigma_1^2/n_1 + \sigma_2^2/n_2} \rightarrow N(0,1) \]

If \( \sigma_1^2 \) and \( \sigma_2^2 \) are unknown then estimates provided by the corresponding sample variances \( s_1^2 \) and \( s_2^2 \), respectively are used:

If \( \sigma_1^2 = \sigma_2^2 = \sigma^2 \) then under \( H_0: \)

\[ Z = \frac{X_1 - X_2}{\frac{1}{n_1} + \frac{1}{n_2}} \rightarrow N(0,1) \]

**Example**

In a survey, 400 Sahiwal cows had an average age at first calving as 1,000 days with a
standard deviation of 160 days. For 500 Tharparkar cows, the average age at first calving was 960
days with a standard deviation of 120 days. Test whether the average age at first calving is the
same in both the breeds.

**Example**

The average number of dry days of two large herds of sizes 1000 and 2000 cows were
67.5 and 68.5 days, respectively. Can the herds be regarded to same dry period with a standard
deviation of 2.5 days.

### 2.1.3. Test for a Specified Proportion

A random sample of size n shows that the proportion of number possessing a certain
attribute is 'p'.

\[ H_0: \ p = P_0 \]

Statistical Test: Under \( H_0 \):

\[ Z = \frac{p - P_0}{\sqrt{\frac{PQ}{n}}} \rightarrow N(0,1) \]

For testing significance at 5% level, the population proportion \( P_0 \) is different from \( P_0 \)
reject \( H_0 \) if \( |Z| \geq 1.96 \) (Q = 1 - P)

**Example**

The manufacturers of Foot and Mouth disease vaccine claimed that it was 90% effective
in curing the disease. In a sample of 400 cows, the vaccine provided protection against FM to 320
cows. Determine whether the manufacturer's claim is legitimate (For 5% one tail test \( Z = 1.645 \)).

### 2.1.4. Test for Significance for Difference of Proportions

Let \( p_1 \) and \( p_2 \) be the proportions in two large random samples of sizes \( n_1 \) and \( n_2 \),
respectively from two populations:

\[ H_0: \ p_1 = p_2 = P \) (say)\]

**Statistical Test**

Under \( H_0: \ p_1 = p_2 \)

\[ Z = \frac{p_1 - p_2}{\sqrt{\frac{PQ}{n_1} + \frac{PQ}{n_2}}} \rightarrow N(0,1) \]

\[ P = \frac{n_1p_1 + n_2p_2}{n_1 + n_2}, \quad Q = 1 - P \]
If, $p_1$ and $p_2$ are distinctly different

$$Z = \frac{p_1 - p_2}{\sqrt{\frac{p_1 Q_1}{n_1} + \frac{p_2 Q_2}{n_2}}} \rightarrow N(0,1)$$

**Example**

1000 cows kept under one type of managerial conditions were found to have abortions upto the extent of 4%. While 1500 cows kept under another type of management showed 3% abortions. Test for the significance of the difference between two systems.

2.2. **Small Sample Tests**

2.2.1. **Students' $t'$ tests** (small sample and S.D. unknown) $n \leq 30$

If $X_1, X_2, X_3, \ldots, X_n$ is random sample of size $n$ from a normal population with mean $\mu$ and variance $\sigma^2$, then student’s $'t'$-statistics is defined as:

$$t = \frac{X - \mu}{s / \sqrt{n}}$$

with $n - 1$ d.f.

Where, $X = \frac{\sum X_1}{n}$ is the sample mean $s^2 = \frac{\sum (X_1 - X)^2}{n - 1}$ is unbiased estimate of population variance $\sigma^2$.

2.2.1.1. **Test for Specified Mean**

$H_0: \mu = \mu_0$, i.e., the population mean is $\mu_0$.

There is no significant difference between the sample mean and population mean. The given random sample has been drawn from the normal population with mean $\mu_0$.

Under $H_0$ test statistic:

$$t = \frac{X - \mu}{s / \sqrt{n}}$$

for $n - 1$ d.f.

If the calculated value of $t$ is greater than table value of $t$, for $n - 1$ d.f. at level of significance $H_0$ is rejected.

2.2.1.2. **Test for Difference of means (S.D's unknown)**

Consider two random samples of size $n_1$ and $n_2$, with means $X_1$ and $X_2$ from two normal populations with means $\mu_1$ and $\mu_2$.

$H_0$: $\mu_1 = \mu_2$

Under the assumption that $\sigma_1^2 = \sigma_2^2 = \sigma^2$, under $H_0$

$$t = \frac{X_1 - X_2}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

for $n_1 + n_2 - 2$ d.f.
Where, $S^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$ is an unbiased estimate of the common population variance $\sigma^2$ based on both the samples.

### 2.2.1.3. Paired Observations

If the samples are not independent, some relationship exists between one observation of one sample and some corresponding observation of the other, and the related observation may be paired.

Consider $d_1, d_2, d_3, \ldots, d_n$ and $n$ differences for the paired observation.

$$H_0 : d = 0$$

$$t = \frac{d - 0}{\frac{s}{\sqrt{n}} \sqrt{\sum d_i}}$$

follows a t-distribution with $n - 1$ d.f.

Where, $d = \frac{\sum d_i}{n}$

$$S^2 = \frac{\sum (d_i - d)^2}{n - 1}$$

### SAMPLE SIZE

How large a sample is needed for an experiment $X$? The question is deceptively simple, but the answer is hard to find. To illustrate this, some of the questions that might be asked by the statistician are:

- What is your Hypothesis?
- What are you trying to estimate?
- What confidence level?
- How large a difference do you wish to be reasonably certain of detecting and with what probability?
- Variability / homogeneity of your data?

When answer to these and other questions are provided by the investigator, the statistician can be of help in determining the needed sample size.

### DESIGNING THE EXPERIMENT

The purpose of any experimental design is to provide a maximum amount of information at minimum cost. While planning an experiment statistical efficiency and resource economy both are of prime importance. A number of simple designs such as C.R.D., RBD, L.S.D. and switch over designs are both economical and effective. The basic principles of experiment design viz, replication, randomization and local control need to be considered while planning an experiment. In process Biotechnology very often factorials are commonly used.

### Example

It is suggested that the effects of pH and temperature on the yield of a certain chemical
quality of a milk product are not independent. In this case a design be adopted which utilizes treatment combination viz, pH (H1, H2), Temp. (T1, T2), pH (4.0, 4.4), temp. (30, 40)°C. This will be considered into 2x2 factorial. Similarly 3 levels of pH and 3 levels of temperature would give a 3x3 factorial.

A statistically designed experiment consists of the following steps:

- Statement of the problem.
- Formulation of hypothesis
- Devising of experimental technique and design
- Performance of experiment
- Application of statistical techniques to the experimental results
- Drawing conclusions

Close team work is required between the statisticians and the research Scientists with consequent advantages in the analysis and interpretation.

Now let us consider a few practical situations for experimentation in processing of foods, etc.

1. Consider an experiment to study the effect of storage condition on the moisture content of khoa. Five storage methods were investigated with varying number of experimental units being stored under each condition.

Table: Moisture Contents of Fourteen Samples of Khoa Stored Under Different Conditions (on %)

<table>
<thead>
<tr>
<th>Storage condition A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>5.4</td>
<td>8.1</td>
<td>7.9</td>
<td>7.1</td>
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<tr>
<td>8.3</td>
<td>7.4</td>
<td>6.4</td>
<td>9.5</td>
<td>8.3</td>
</tr>
<tr>
<td>7.6</td>
<td>7.1</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In a food technology experiment involving the storage of frozen straw berries, 10 pints were stored at each of 5 lengths of storage time. When ascorbic acid determinations were made of after storage, two determinations were made on each point:

Example 3  Consider an experiment to investigate the fermentative conversion of sugar to lactic acid. It is desired to compare the abilities of two micro-organisms to carry out this conversion. Quality of substrate is prepared and divided into two unequal portions. Each of the 100 ml inoculated with one or the other of the two micro-organisms. The fermentation is allowed for 24 hours and the amount of unconverted sugar in the substrate is observed in different sample.

Example 4  It is desired for test 10 different baking temperatures when we use a standard cake mix. Fifty samples batches of mix are prepared and 5 are assigned at random to each of the 10 temperatures. Six judges score the cakes. Give the statistical analysis, model used and hypothesis to be tested. These are a few illustrations to generate the data through experimentation.
Now we proceed to discuss a few steps necessary to analyse the basic designs.

- Lay out of the design
- Model used for analysis
- Analysis of variance and drawing of

Various designs along with their layout and analysis are given in all standard books eg. Cochran & Cox., M.N.Dass Federror, etc. However some more illustrations are given below:

Example 5  Twenty-one samples were taken from 6 containers of a particular brand of SMP obtained through six consignments and were tested in four laboratories for moisture content. Analyse the data interpret the results:

<table>
<thead>
<tr>
<th>CONSIGNMENT</th>
<th>Laboratory I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.2</td>
<td>10.5</td>
<td>9.7</td>
<td>10.1</td>
<td>11.3</td>
<td>11.7</td>
</tr>
<tr>
<td>B</td>
<td>12.9</td>
<td>11.3</td>
<td>9.2</td>
<td>11.1</td>
<td>10.7</td>
<td>10.8</td>
</tr>
<tr>
<td>C</td>
<td>11.0</td>
<td>9.3</td>
<td>10.8</td>
<td>12.1</td>
<td>11.6</td>
<td>10.9</td>
</tr>
<tr>
<td>D</td>
<td>12.9</td>
<td>14.1</td>
<td>10.2</td>
<td>16.5</td>
<td>15.1</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Transformations

Very often the data generated through experimentation do not follow a normal distribution which is essential for carrying out the analysis. To overcome this problem the following three transformations are suggested.

- Square root Transformation: Counts of variables, such as weeds per plot or insects caught in traps, tend to be distributed in poission fashion with variance proportional to the mean. A transformation to $\sqrt{X}$ or $\sqrt{X+1}$ (if some counts are small) is often effective.
- Arc Sin Transformation: If the variable consists of the proportion of individuals affected, the distribution tends to be binomial. The transformation used is to the angle whose Sin is the square root of the proportion or percentage.
- Logarithms are used if the effects are proportional instead of additive eg. SPC, Coliform, Bacterial Counts, population, etc.

Very often the data generated through experiment involves dependency or relationship with the variables under study. The commonly used tool such as simple correlation, partial correlation, multiple correlations, Linear and Multiple linear regressions, curvilinear regressions are appropriate eg. In the analysis of milk and milk products, the measurements/observations involved are, fat, sp.gravity, SNF, MBRT, Colliforms, SPC, pH, moisture, acidity etc.

From the foregoing discussion it is pertinent that the role of statistics in processing Biotechnology is then, to function as a tool in designing research, analysing its data and in drawing conclusions therefrom Clearly the science of statistics can not be ignored by any research worker even though he may not have occasion to use applied statistics in all of its detail and ramifications.
1.0. INTRODUCTION

Flavour is unquestionably one of the most important attributes of the food. The acceptability of almost everything that we eat is dependent to some extent on its flavour. Aromatic chemicals that are biosynthesized during normal metabolic processes in plants and animals, and possibly further modified by cooking or processing create flavour of a food. In simple words, we can define flavor as complex sensation of aroma & taste. This intrinsic flavour of food represents the complex impact made by these aromatic components on the senses of odour and taste. As far as food flavours and fragrances are concerned, the debate over the natural versus nature-identical versus synthetic plays an important role. Products of biotechnological processes such as microbes or enzymes might be considered natural or nature-identical compounds. As there is a serious concern in using synthetic products in food flavourings category, role of bioflavours assumes greater significance.

2.0. NEED FOR BIOPRODUCTION OF FLAVOUR COMPOUNDS

Most of the flavour compounds are structurally simple (i.e. ethyl acetate, lactic acid, acetaldehyde etc.) and very amenable to simple chemical synthesis. These are, however, several drawbacks to chemical synthesis. These include:

- Biological routes to many flavour substances are simpler than synthetic ones, because the latter can involve multi-step processes with associated increments in cost per step.

- Another drawback is that chemical synthesis processes result in racemic mixtures, where the need is for specific, optically active stereoisomers. Isolation of desired isomer is a tedious job or even chemically technically impossible. On the other hand, a number of compounds (e.g. y & 5 - Lactones) can be produced in optically active and pure form by relatively simple microbiological reduction.

- Biggest obstacle to chemically synthesized flavour’s acceptance is, there disqualification as being natural.

Clearly the biological production of flavours is driven not only by market demands but also by regulatory influences as well the adoption of such processes also could satisfy simultaneously growing requirements for environmentally friendly "green" products.
3.0. BIOFLAVOUR PRODUCTION

Most natural flavours and fragrances are the mixtures of chemicals such as terpenes, aldehydes, esters, lactones, higher alcohols and other complex molecules that result from secondary metabolisms of plants and may be obtained from certain animal sources. Recent advances in microbial/genetics, fermentation and enzymatic processes, coupled with current demands for flavour and fragrance chemicals for "natural" origin have prompted interest in adopting new technological tools for flavour production. Biological systems involved in flavour production can be grouped into two broad classes:

3.1. Plant Tissue Culture Based

Most of the aroma compounds used in food, pharmaceutical and cosmetic industry, are basically plant derived. Plants or part of plants used for the production of flavour can be manipulated either by breeding the plant to produce greater amounts of fragrance by conventional or traditional means or by applying the novel plant breeding methods. Plant tissue culture (PTC), is a technique that allows plant cells to be grown in solid or liquid media and has been proved to possess immense potential for production of flavouring compounds on commercial level. Vanilla flavour components and menthol are some of the commercially important products that are worth being synthesized by PCT. To induce flavour production by suspension cultures, several manipulation techniques have been employed like manipulation of culture medium composition, environmental factors, addition of exogenous elicitors and induction of cell differentiation (Kumar et al., 1998).

3.2. Microbiologically Mediated System

Microbiological processes are considered to be a better option for flavour production, because of their cost effectiveness and better control over the production parameters. The filamentous fungi, yeasts and bacteria are the major microbial sources. The biosynthetic pathways involved in microbial flavour production can be classified into two groups (Welsh et al., 1989):

- **De novo** biosynthesis of a specific compound by metabolizing cells
- Bioconversion or biotransformation, which involves modification of a chemical substrate by microbial cells.

*De novo* biosynthesis is a common route utilized by microbial cells for flavour production, however relatively low yields make it unattractive and uneconomical. Moreover, bioconversion routes that rely on abundant and relatively inexpensive sources of precursor yield higher levels of product and therefore offer a more attractive industrial process from an economic viewpoint. Culture conditions (media composition, pH, duration of fermentation and temperature) were identified as factors in determining the amount and type of flavour or fragrance compound produced. For example, *Ceratocystis moliniformis*, can produce banana, citrus or peach like aromas and the aroma produced, depends on the carbon and nitrogen sources.

4.0. TECHNICAL CONSIDERATIONS

The success of industrial production of a microbial flavour compound is influenced by the yield of the required product, in a reasonable period of time, from a relatively cheaper precursor. These issues can be addressed by microbial physiology and genetic manipulation approaches.
4.1. Microbial Physiology Approaches

Manipulation of cell physiology may be the easier and quicker approach to increase flavour and aroma productivity. Following points required utmost attention, while applying this approach.

- Large-scale screening of microorganisms for production of the desired aroma or fragrance.
- Quality evaluation of microbiologically derived aroma compounds, with the one that obtained from conventional/traditional source.
- Establishment of physiological conditions to optimize the production of desired components.
- Identification of biochemical pathway involve in the production. It may help in better understanding of the mechanism and also in identifying the rate limiting steps.

Further, enhancement may be obtained through changes in reactor design, cell immobilization, non-aqueous systems and extractive processes coupled with fermentation, experiments have indicated that incorporation of solvents, as component of reaction system, may help in the alleviation of poor water solubility problem of flavouring compounds, hence more yield. (Welsh et al., 1989). For example, in an aqueous system the yeast Pichia pastoris could produce only small amounts of benzaldehyde from benzyl alcohol, but use of hexane as reaction medium caused nine-fold increase in benzaldehyde yield with shortened reaction time.

4.2. Genetic Manipulation Approaches

Genetic manipulation of microorganisms may be another approach, to enhance the flavour biosynthesis. It involves techniques such as non-specific mutation, enhanced gene replication, and the cloning of genetic material into more rapidly growing or stable cell lines. Mutation can induce changes in pathways that cause more accumulation of desirable metabolite or introduce an alternate pathway to form products of greater value. Similarly, a mutation may decrease the susceptibility of a microbe to end produce inhibition or catabolic repression, hence improved yield. Increased copies of a gene, for specific compounds, can increase the output of specific enzymes and result in more product formation. Alternatively genes responsible for desirable microbial trait can be removed from a donor organism, transferred via an appropriate donor, and inserted into host organisms, which is hardier or has more desirable physiological or fermentation properties. In doing so production of desired metabolic can be enhanced.

4.3. Bacteria: As Flavour Producer

Bacterial cultures, since long have been known to be associated with production of characteristic flavour of variety of fermented products, specially the dairy products. Bacteria have potential to produce various flavour metabolites, precursors and enhancers as well as enzymes that can be used to produce flavour compounds (Romero, 1992). A number of review articles are available on microbial generation of flavour chemicals (Belin et al., 1992; Kempler, 1983; Misutani, 1990 & Welsh et al., 1989).

4.3.1. Flavour and Aroma Metabolites: In 1932, Heubylum and co-workers, identified diacetyl, a compound with a butter-like aroma in fermented dairy products, since then a
number of flavour metabolites and their precursors have been reported to be produced by bacteria (Table-1).

**Table-1: Flavour Metabolites of Bacterial Origin**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Organisms</th>
<th>Type of Flavour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyl</td>
<td>Lactic acid bacteria example</td>
<td>Impart the buttery attributes in cultured dairy products</td>
</tr>
<tr>
<td></td>
<td><em>Lactococcus lactis</em> spp. <em>Lactis bivar.</em> <em>Diacelyactis</em></td>
<td></td>
</tr>
<tr>
<td>Alkylpyrazines Teteramethyl pyrazines (TMP) 2- methoxy-3-isopropyl pyrazine</td>
<td><em>Bacillus subtilis,</em> <em>Corynebacterium glutamicum</em> <em>Serratia odorifera</em> <em>Pseudomonas toetlens</em> <em>Pseudomonas parolens</em></td>
<td>Pyrazines provides nutty and roasted flavour</td>
</tr>
<tr>
<td>Terpenoids (through transformation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) 4-Menthol</td>
<td><em>Pseudomonas putida</em> <em>Cellulomonas turbata</em> <em>Bacillus stereothermophilus</em></td>
<td>Mint flavour</td>
</tr>
<tr>
<td>(ii) Peritlyl alcool, 4-terpeneol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aromatic compounds</td>
<td><em>Pseudomonas putida</em> <em>Pseudomonas spp., Serratia, klebsiella, Enterobacter, Pseudomonas acidvorans</em></td>
<td>Biotter almond and cherry flavour Vanilla flavour</td>
</tr>
</tbody>
</table>

4.3.2. **Flavour Potentiators:** Flavour potentiators or enhancers may be defined as the compounds in foods that have little or no flavour or aroma in themselves, but are capable of enhancing or intensifying an existing flavour. Typical examples of such compounds include monosodium glutamate (MSG), and the 5'-mononucleotides like Inosine monophosphate (5'-IMP) and guanosine monophosphate (GMP).

MSG is commercially produced through fermentation using strains of *Corynebacterium glutamicum*, which has a defective TCA cycle that cause accumulations of excess a-ketoglutarate and these compounds upon reductive animation yield L-glutamate. *Bacillus stibtilis* and *Bacillus ammoniagenesis* have been shown to produce 5'-nucleotides and application of rDNA technique improved the production of guanosine by a strain of *B. subtilis* (Miyagawa et al., 1986).

4.3.3. **Sweeteners and Organic Acids:** Bacterial cultures and enzymes have potential to produce a number of alternative sweeteners, their precursors or modified derivatives. Organic acids (acetic, butyric, propionic acid etc.) are produced since long through bacterial fermentation. Acetic acid is produced by *Acetobactor acetii* and *Glucobacter oxydans*. Propionic acid can also be derived in relatively high amounts from fermentation processes utilizing genus *Propionibacterium*. Bacterial isomerases are used in the production of fructose from glucose. Recently, a new enzyme, D-glucose isomerase was characterized from *Bifidobacteria adolescentis* that may circumvent the need for expensive enzymes. Artificial
sweetener aspartame's precursors i.e. aspartic acid and phenylalanine, can be produced through bacterial fermentation. Attempts were also made to isolate and clone cDNA of thaumatin (sweet tasting protein) into the *E. coli* to study the mechanism of production and its regulation.

### 4.4. Yeast: As a Source of Flavour Metabolites

Yeast is an important contributor to flavour development in fermented products and the compounds produced during fermentation are varied and many. Yeast is one such organism, which provides a mean of imparting rich savoury flavours to food products. The term savory covers a wide range of perceived flavour experiences. Yeast extract, usually produced from brewer's yeast or baker's yeast impart rich meaty flavour and is considered a relatively inexpensive alternative to meat extracts. Manufacture of yeast extracts and their properties have been reviewed by Nagodawithana (1992). Yeasts being their high RNA content may serve as a starting material for the production of nucleotides, having potential to enhance the flavour of the food product. Procedure to manufacture 5' nucleotide rich yeast extracts is given here (Fig. 1).

![Fig.1: Manufacturing of 5’ Nucleotide Rich Yeast Extracts](image)

Yeast addition leads to the production of three major flavouring compounds in fermented beverages, namely CO$_2$, alcohol and glycerol. Beside these three other major flavour compounds include, higher alcohols, vicinal diketones, aldehydes, sulfur compounds, esters and fatty acids (Russel and Stewart, 1992). A recent innovation in immobilized cell technology has offered to develop diacetyl free beer and diacetyl formation in beer is known to impart other flavour defects.

### 4.5. Filamentous Fungi: As a source of Flavour Production

Filamentous fungi; possess the ability to modify or entrance the flavour of many foods (Bigelis, 1992). Flavour of the food production is influenced by the addition of additives, of fungal origin, or by the production of flavour metabolites and flavour modifying enzymes during the course of fermentation or secretion of enzymes that can generate or remove
compounds, which have role in flavour development. All these three approaches are not of recent origin, the application of gene technology may improve the flavour properties, in economic way (Berka and Barnelt, 1989). Food grade acidulants like citric acid, gluconic acid and Kojic acid are produced on commercial level using filamentous fungi kojic acid is used as a precursor for the chemical synthesis of maltol and ethylmaltol, two food additives, that can enhance or modify the flavour and aroma of foods: and beverages. A number of enzymes are isolated from filamentous fungi and used to make flavour compounds or to modify the flavour of foods. (Table-2). Role of filamentous fungi in the development of characterize flavour of cheese, oriental fermented foods and cured meat is well known.

### Table 2: Food Enzymes Derived from Filamentous Fungi

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Microorganisms</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbohydrates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Amylase and glucoamylase</td>
<td>Aspergillus spp. Rhizopus spp. Aspergillus niger Klyuseromyces</td>
<td>Production of glucose and maltose syrups Transform the cheese whey into a sweet syrup</td>
</tr>
<tr>
<td>(ii) Lactase (β-galactosidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nucleolytic enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) 5'- Phosphodiesterase</td>
<td>Penicillium Aspergillus oryzae</td>
<td>Hydrolysis of RNA to produce IMP, GMP (flavour enhancers) convert 5'- Adenine mono phosphate to IMP</td>
</tr>
<tr>
<td>(ii) Adenylic deaminase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipases and Proteinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhizopus spp. Rhizomucor meihei Pencillium spp Aspergillus spp</td>
<td>Acceleration of cheese ripening and development of characteristic cheese flavours.</td>
</tr>
<tr>
<td><strong>Narnginase</strong></td>
<td>Aspergillus niger</td>
<td>Remove bitter taste from citrus juices by reducing the level of naringinin</td>
</tr>
<tr>
<td><strong>Pectinases, cellulase, hemicellulase</strong></td>
<td>Rhizopus, Aspergillus, Pencillium, trichoderma</td>
<td>Increase the extraction rate of flavouring compounds</td>
</tr>
</tbody>
</table>

5.0. CONCLUSION

Preference of consumers for "natural" food additives, and the advantages associated with microbial production of flavours is making it a real alternative. Development of novel and cheap production processes such as solid-state fermentation may help to overcome some of the current limitations of microbial flavour production. Application of genetic engineering to improve the yield and functional attributes of these compounds looks promising. Areas like safely aspects and stability of these compounds need thorough investigations.
6.0. REFERENCES


1.0. INTRODUCTION

The demand for cheese flavours has increased due to consumer demand for a wider choice of convenience and low-fat products that possess cheese flavour. Cheese flavours can be produced by procedures such as synthesis or partial synthesis of individual components, isolation of individual components as flavour complexes from natural raw materials or the formation of flavour complexes by physical/chemical synthesis (Lucas and Kunz, 1992). However, the identification of all the compounds present in a given cheese flavour has not been determined, and the cost of synthetically producing those which have been identified as being important is probably cost-prohibitive. Therefore, enhancement of the major flavour pathways that occur in natural cheese presently provides the most economic route to the production of intense cheese flavours. This is best achieved by enzymatic-modification of cheese. The product made using the enzyme(s) and fresh cheese curd and/or young cheese is popularly known as enzyme-modified cheese (EMC).

2.0. ENZYME MODIFIED CHEESE TECHNOLOGY

EMC technology has evolved from curd slurry model system. The curd slurry technique is a very useful technique for assessing the capability of specific enzymes and starter cultures to generate good cheese flavours in an environment similar to that used during EMC production. Initially, curd slurries were developed by Kristoffersen et al. (1967) as a technique for accelerating cheese ripening. A liquid cheese product with characteristic Cheddar, Brick or Romano flavour could be produced from fresh curd in 4 or 5 day, indicating the possibility of generating a range of intense cheese flavours in a short time from a base substrate by modifying various process parameters.

Essentially, the technology used to produce EMC involves incubating cheese/curd with exogenous enzymes (proteinases, peptidases, lipases and esterases) in a slurry system under controlled conditions until the required flavour is attained. The flavour profile of enzyme-modified cheese can be up to 30 times the intensity of natural cheese. A series of cheese flavours can be produced by using EMC technology.

2.1. Process of EMC Production

The principal difference between a mature natural cheese and EMC is that body and texture are not factors in the quality of the latter (Moskowitz and Noelck, 1987; West, 1996). The process of EMC production involves incubating mature or immature cheese with specific exogenous enzymes and/or micro-organisms, terminating the process by pasteurization.
and standardizing the final product to a desired flavour intensity and composition. In general, most EMC types are produced from cheese pastes made from an immature cheese of the same type to give the most authentic flavour. Other components, notably butter fat and cream, are included as a source of extra flavour compounds. Other flavour enhancers, such as monosodium glutamate, yeast extract, diacetyl and other compounds associated with specific cheese flavours may be added, although some may have to be declared on the labeling of the final product into which the EMC is added (Anon, 1993; West, 1996). A schematic of EMC production is given in Fig. 1.

**Fig. 1: Schematic Representation of Enzyme Modified Cheese Manufacture**

Consistency and quality of the initial substrate is critical for flavour development as variations will affect the final flavour (West, 1996). A number of different processes are used, depending on the EMC type, manufacturer’s preference, product application and appearance of the end product. During the production of EMC, spoilage by bacteria can be a major problem, as optimum conditions exist for their growth. Equipment must be sterile and all obvious measures must be taken to prevent contamination. Dulley (1976) demonstrated that the inclusion of potassium sorbate had a major effect on controlling levels of contaminants, such as yeast and coliforms. Other bacterial inhibitors such as nitrates, sorbic acid and nisin are commonly used (Mann, 1981). Microbial spoilage is a bigger problem in system in which a lipase is not used, as high levels of short chain FFA can have a bacteriostatic effect (West, 1996).
The control of process parameters during EMC production is critical in obtaining a consistent product. Vafiadis (1996) commented on the importance of time and temperature control in the manufacture of EMCs. The heat treatment used to inactivate the enzyme is critical as care must be taken not to destroy the developed flavour by over-cooking. It is also crucial that all enzymes are inactivated as excess activity will lead to off-flavours and problems in the final product. Therefore, it is necessary to monitor batches of EMC for residual proteinase activity (generally 72h after manufacture).

2.2. Exogenous Enzymes for EMC Production

Since proteolysis, lipolysis and lactose hydrolysis are the main biochemical events during maturation, proteinases, peptidases, lipases and β-galactosidase were evaluated for their potential use (IDF, 1990). The enzymes were either from a non-cheese-related source, or extracted from cheese-related microorganisms (Visser, 1993). Neutrase is probably the most commonly used; either alone or with peptidases. A list of commercial enzymes available for EMC production is given:

- Neutrase (B. Subtilis)
- Naturage (Protease + Peptidase+ culture)
- Flavourage (Protease + Lipase)
- Accelase (Protease + Peptidase)
- Lipomods (Lipase + Esterase)
- Promod (Protease + Peptidase)

Addition of proteinases leads to an increase in protein degradation but very often results in the development of bitter flavour and reduced cheese yield. Law and Wigmore (1983) showed that combining proteinase with Lactococcus peptidases gave an acceptable cheese. Their experiments led to the development of a commercial product known as Accelase. Ramasamy et al. (1996) used Accelase AHC 50 for successfully accelerating the cheddar cheese flavour development in cheese slurry. The use of lipases to reduce the maturation time of cheese and to give varieties like Provolone, Cacciocavallo or Ras their characteristic piquant flavour has also been evaluated (Laila et al., 1989).

Since the use of a single enzyme very often disturbs the equilibrium of the flavour components in cheese and causes flavour defects, enzyme manufacturers have developed ‘enzyme cocktails’. Several workers, therefore, advocated the addition of enzyme in proper ratio for balanced flavour development (Fox, 1989). Several workers extracted enzymes from the following cheese-related microorganisms: Lactobacillus, Pediococcus, Leuconostoc, Propionibacterium, Brevibacterium, Micrococcus and Pseudomonas. The enzymes have been added as crude cell-free extracts or in a partially purified form in cheese or cheese slurry for acceleration of flavour development (Law, 1984; Fox, 1989; Hayashi et al., 1990; El Soda and Pandian, 1991 & Lee and Joo, 1993).

One of the recent developments in the area of enzyme addition is the use of plasmin as an agent to accelerate maturation. However, the cost of plasmin presented an obstacle for its industrial application.

3.0 USES OF EMC

EMCs are produced not for use in cheeses as such but to be applied in other foods that conventionally contain natural cheese (Lucas and Kunz, 1992). High-intensity cheese flavour
concentrates, such as enzyme-modified cheese (EMCs), cheese powders and cheese flavours form major alternative to the use of natural cheese in processed consumer foods requiring a cheese flavour (Missel, 1996). Cheese is traditionally added to products as a spray dried preparation for flavour, appearance and texture enhancement. The amount of cheese used varies, and the overall flavour and quality of the product depends on the type of cheese used. (Anon, 1990, 1993; Hermann, 1993; Freund, 1995; Missel, 1996 and West, 1996).

EMCs are used in food recipes to fulfill several roles, e.g. as the sole source of cheese flavour in a product, to intensify an existing cheesy taste or to give a specific cheese character to a more bland-tasting cheese product (Anon, 1996). They have approximately 15-30 times the flavour intensity of natural cheese and are available as pastes or spray-dried powders (Moskowitz and Noelck, 1987; Freund, 1995). The addition of intense cheese flavours to these products creates the desired flavour without an increase in fat content, as same can be added at levels of 0.1 per cent (w/w) and contribute less than 0.07 per cent fat (2.28 calories) per 100 g (Buhler, 1996).

EMCs are ideal in frozen cheese type products as the casein from natural cheese tend to coagulate and produce a grainy texture; since the casein in EMCs have been hydrolyzed to more soluble peptides and amino acids, these problems are overcome (Missel, 1996). Other advantages are reduced production costs (by as much as 40-80%), increased production capacity, enhanced product stability, improved consistency, improved functionality, batch to batch consistency, reduced storage space and ease of handling. Intense cheese flavours also allow better control in the development of new products and refining of existing products (Christau, 1992).

EMC flavours available include Cheddar, Mozzarella, Romano, Provolone, Feta, Parmesan, Blue, Gouda, Swiss, Emmental, Gruyere, Colby and Brick. These cheese flavours have a wide range of applications in salad dressings, dips, soups, sauces, snacks, crisps, pasta products, cheese analogues, frozen foods, microwave meals, ready-made meals, canned foods, crackers, cake mixes, biscuits, fillings, cheese spreads, low-fat and no-fat cheese products and cheese substitutes/imitations. These products are generally added to foods at levels of 0.1-2.0 per cent (w/w), although they can be used at levels of up to 5.0 per cent (w/w) (Moskowitz and Noelck, 1987; Freund, 1993, 1995; Buhler, 1996; West, 1996). Talbott and McCord (1981) described a guide to determine the dosage of EMC for replacement of natural cheese. To determine the dosage the percentage of natural cheese in the product is multiplied by the desired replacement percentage and divided by the flavour intensity of the EMC. Certain high-intensity products can replace up to 50 per cent of the cheese used in some applications (Buhler, 1996).

Regulatory considerations regarding the use of EMC products differ in Europe from the US. In Europe, a framework directive on flavourings has been established, which lists a number of categories of flavourings, flavouring substances, flavouring preparations, process flavouring, smoke flavouring and mixtures thereof (Freund, 1995). EMCs are categorized as flavouring preparations which are defined as substances obtained by physical, microbiological or enzymatic means from material of vegetable or animal origin (EU, 1988). In the US, EMCs are Generally Regarded As Safe (GRAS) approved and as such can be added to specified categories of pasteurized process cheese, non-standard identity cheese, non-traditional reduced-fat and fat-free cheeses and various prepared foods (Freund, 1995).
4.0 CONCLUSION

There are number of critical factors involved in EMC production, which can be summarized as follows: the type of cheese flavour required, which is directly related to the type and composition of starting material, type and specificity of enzymes or cultures used, their concentration, processing parameters (pH, temperature, agitation, aeration and incubation time) and use of processing aids (emulsifiers, bacteriocins, flavour compounds and precursors). The dosage of enzyme and/or starter culture used is dependent on the intensity of flavour required, processing time and temperature and the quality of the initial substrate. To produce a consistent EMC product it is necessary to have a highly controlled process; therefore, a detailed knowledge of the enzymatic reactions under the conditions used must be fully understood before any attempt is undertaken to produce EMCs. Various commercial enzyme preparations are available for the production of EMC flavours. Similarly, starter culture extract are currently receiving much attention in EMC production. Because these extracts have been successful in producing high quality natural cheese and obviously contain the necessary pool of enzymes required for good cheese flavour.

Currently, the consumer demand for novel, nutritious and convenience foods with a cheese flavour is driving the development of customized cheese flavour products, which has highlighted the requirement for further research into cheese flavour pathways and the identification of specific cheese flavour compounds. Development in this area is ongoing and already different EMC flavours have been produced from the same initial substrate and from blends of dairy and non-dairy ingredients leading to the development of novel, cost-effective cheese flavour products.

5.0 REFERENCES


1.0 INTRODUCTION

The dairy wastes are quite rich in degradable organic matter and exert a high oxygen demand. About 2.5 million tonnes of milk is being processed in an organized manner in nearly 200 dairy plants every year, in India. The processing of one litre of milk yields about 8-10 litres of waste water depending on the type of products manufactured. The dairy effluent is peculiar as compared to other industrial wastes, because of relatively high concentrated effluent, particularly whey and butter washings. The strength of dairy wastes that is BOD varies from 300 to 2000 mg/litre.

Whey, a by-product of cheese, paneer and channa industry contains more than half the solids present in the whole milk. On an average, the manufacture of one ton of cheese results in the production of eight tonnes of whey. In India, it is estimated that about 1 800 million kg of whey is annually derived as a by-product which I causes substantial loss of whey solids. Much more important is its high BOD of 35,000-45,000 mg/litre and 100 litres of whey has a polluting strength equivalent to the sewage produced by 45 people. In the treatment of both liquid and solid wastes, there are significant opportunities for the use of bio- technology. Microorganisms are the major tools of biotechnology for controlling pollution. Now, additional techniques are applied to use genetically manipulated microorganisms or their products, which have greater potential for disposing off many of the persistent toxic chemicals from the environment.

The application of biotechnology could be mainly in:
- Methane production from dairy waste, which has an important utility
- Ethanol production from whey
- Single cell protein production
- Single cell oil production
- Metal recovery from waste

2.0. METHANE PRODUCTION

Microbiological formation of methane has been occurring naturally over the ages, in marshes, deep Ocean trenches, hot springs, etc. In the last few decades interest in anaerobic biotechnology has grown considerably, both in harnessing the process for dairy waste, water treatment and in the bioconversion of biomass to methane.

2.1. Basic Principle

Complex organic compounds decompose mostly into methane and carbon dioxide in
anaerobic conditions under the influence of bacterial fermentation. 90-95 per cent of the organic matter can be decomposed to produce biogas. The remaining 5-10 per cent is converted into bacterial cells.

2.1.1. Anaerobic Fermentation: Decomposition of organic material involves the activity of many anaerobic species of bacteria. In principle, there are three phases in methane fermentation. In the first phase, organic compounds like polysaccharides, proteins and lipids are hydrolyzed and simpler organic compounds like sugars, amino acids and fatty acids are formed. In the second phase, these are broken down further by the assistance of acid forming bacteria into propionic and butyric acids, which are decomposed into hydrogen, CO₂ and acetic acid by acetogenic bacteria. In the last phase, methane is produced from acetate, CO₂ and hydrogen influenced by the activity

Methane production can be characterized by reactions 1 to 3 given below

\[
\text{CO}_2 + 4\text{H}_2 \quad \xrightarrow{\text{Methane bacteria}} \quad \text{CH}_4 + 2\text{H}_2\text{O} \quad \ldots \ldots \ldots (1)
\]

Acetate is first formed from CO₂ and hydrogen.

\[
2\text{CO}_2 + 4\text{H}_2 \quad \xrightarrow{\text{Acetogenic bacteria}} \quad \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \quad \ldots \ldots \ldots (2)
\]

The methane bacteria breakdown the acetate according to the following reaction:

\[
\text{CH}_3\text{COOH} \quad \xrightarrow{\text{Methane bacteria}} \quad \text{CH}_4 + \text{CO}_2
\]

It is reported that municipal sludge about 30 per cent of the methane produced is formed by reaction (1) and about 70 per cent by reaction (2) and (3).

Research on lactose-bearing wastes and more particularly whey has led to a quite specific view of the several different organisms involved. The following diagram shows a schematic of a laboratory study of a linear metabolic pathway for the degradation of lactose

2.1.2. Process and Equipment Design: The digester/reactor design governs the rate at which one can process the wastewater. It also affects the amount of gas produced, gas production rate, ratio of CH₄ to CO₂, amount of sludge, etc. There are mainly five principal types of reactors.

(a) Conventional Anaerobic Reactor

Conventional low loaded and completely mixed reactions are not very well suited to dairy waste water treatment. This kind of reactor is more suitable for sludge digestion.
(b) Contact Reactor

A contact reactor resembles a conventional activated sludge process. The process uses a fully mixed reactor and a sedimentation basin. The settled sludge is returned to the reactor enabling the sludge retention time and sludge content to be regulated.

(c) Fluidized Bed Reactor

In the fluidized bed reactor; the carrier medium, for example plastic granules, is kept in suspension by the flow and the biomass adheres to it. The process includes separation and recirculation equipment for carrier medium and biomass.

(d) Anaerobic Fixed-Film Reactor

The reactor contains carrier medium, usually plastic pieces or elements, which have a large surface area, with the biomass growing on it. The direction of flow may be upward or downward.

(e) Up flow Anaerobic Sludge Blanket (UASB)

The waste water is fed to the bottom of the reactor through piping designed to distribute the waste water evenly across the base area. The waste water then passes upwards through the sludge blanket. The process relies on the formation of sludge granules having good settling properties. The formation of such granules is dependent on the composition of the effluent treated. In the upper layer of the reactor, separation of liquid, gas and suspended solids takes place. The purified effluent flows through overflow flutes at the top of reactor.

3.0. ETHANOL PRODUCTION FROM WHEY

As the demand for the limited global supply of non-renewable energy resources increases, the prices of oil and natural gas also keep increasing. The production of ethanol from renewable carbohydrate raw materials for use as an alternative liquid fuel has been attracting a worldwide interest. Among the many carbohydrate materials used for the production of ethanol, cheese whey deserves special. Recently, efforts to produce ethanol from whey permeate lactose have been concentrated on the use of immobilized cells. Moulin et al reported that among the lactose fermenting yeasts, only strains of the genera Kluyveromyces and Candida appear to have a high efficiency in ethanol production.

Zayed and Harper have reported ethanol production from salt whey. Salt whey permeate, containing up to 8 per cent can be employed successfully as an alternative substrate for alcohol production by using agar immobilization system. Salt whey permeate was used as a substrate for ethanol production by salt-tolerant yeast in conventional, rapid and immobilized cell batch systems. In the cell suspension system, Kluyveromyces fragias was selected as the most efficient strain of the 5 yeast strains under test. Entrapment of stationary phase of K. fragias in 1.5 per cent agar substantially improved the activity of the cells. Immobilized K. fragias produced 2.5-2.6 per cent alcohol from whey permeate salted to 8 per cent salt after 60 h. of fermentation.

Roukes et al. studied ethanol production from deproteinised whey. The production of ethanol from deproteinised, acid prehydrolyzed cheese whey by immobilized Saccharomyces cerevisiae cells in Ca-alginate, K-carageenan agar and polyacrylamide gels as well as the effects of substrate concentration and cell recycling on ethanol production was investigated. Maximum ethanol concentration (16.7 g/litre) was observed with immobilized S. cerevisiae cells in Calcium-alginate beads. In the case of deproteinized whey concentrates, maximum ethanol concentration (61g/litre) was obtained at a substrate concentration of 150g/litre after 12
h. of fermentation. In repeated cell recycles, immobilized cells maintained constant fermentation efficiency for 5 days.

4.0. SINGLE CELL PROTEIN (SCP)

The recovery of biomass and particularly protein is one of the best documented areas of bio-technology and by-product recovery. The nutritional value of microorganisms is similar to other forms of biomass but their growth rate is much higher than other sources of protein. The attraction from waste treatment point of view is also that the micro-organisms will grow readily on a wide variety of substrates, including dairy wastes. If the foodstuff is entirely microbial, then a well defined consistent product is likely to be required. This usually means pure culture of fermentation of a standard substrate. Under such circumstances, whey acts as a substrate for the production of microbial Single Cell Protein. Table-1 gives the protein content of various microorganisms.

Table 1: Protein Content of Various Microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Protein per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>45-55</td>
</tr>
<tr>
<td>Algae</td>
<td>47-63</td>
</tr>
<tr>
<td>Bacteria</td>
<td>50-83</td>
</tr>
<tr>
<td>Fungi</td>
<td>31-55</td>
</tr>
</tbody>
</table>

5.0. SINGLE CELL OIL

One can call the oil from microorganisms as “Single Cell Oil” or SCO. The microorganisms, which can accumulate 25 per cent or more of their bio-mass as lipid, are called oleaginous micro-organisms. Table-2 gives some of the potential oleaginous microorganisms.

Oleaginous organisms are chiefly confined to eukaryotes, algae, yeasts and fungi. The potential use of ‘SCO’ is in the production of special oils like gamma-linoleic, which have a much higher value because of their potential use in health care. It is found that gamma-linoleic acid reduces the blood cholesterol level considerably. In this respect microbially derived oils grown on waste materials may become much more important. Domestic and animal wastes are unlikely to be suitable for ‘SCO’ production, because of potential pathogens and toxins in the original waste. Therefore, whey could be the best substrate for ‘SCO’ production.

Table-2: Potential Oleaginous Microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Oil Content ( % w/w)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>30</td>
<td>Lake water</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>72</td>
<td>Lake water</td>
</tr>
<tr>
<td>Botryococcus braunii</td>
<td>53</td>
<td>Lake water</td>
</tr>
<tr>
<td>Yeasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida curvata</td>
<td>58</td>
<td>Whey</td>
</tr>
<tr>
<td>Lipomyces sp</td>
<td>60</td>
<td>Molasses</td>
</tr>
<tr>
<td>Trichosporon</td>
<td>45</td>
<td>Whey</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium</td>
<td>40</td>
<td>Whey</td>
</tr>
<tr>
<td>Geotricum candidum</td>
<td>45</td>
<td>Various wastes</td>
</tr>
</tbody>
</table>
6.0. METAL RECOVERY

Probably, the second most advanced microbial recovery system after protein is the extraction of metals from wastes. Restrictions on the discharge of metals to the environment have become stricter as more evidence about accumulation and toxicity has been reported.

Many bacteria can precipitate metals from solution and historically, *Sphaerotilus*, *Gallionella* and *Leptothrix* have been most studied. Anaerobic bacterial cultures cause the precipitation of metals in the form of insoluble sulphides and a large number of aerobic bacteria can oxidize metals. Micro-organisms play an important part in the precipitation of ocean metallic nodules, corrosion and metal encrustation.

7.0. REFERENCES


1.0. INTRODUCTION

Living longer and better is on the minds of most people; especially in wealthy countries and diet is one of the most important facets of a healthy life. Diet-health link is now an integral part of healthy life style. The role of diet and specific foods for the prevention and treatment of disease and improvement of body functions is being investigated around the world. Present day consumers prefer foods that promote good health and prevent disease. Such foods need to fit into current life styles providing convenience of use, good taste and acceptable price-value ratio. Therefore, it seems very likely that the concept of functional foods, which enables the consumers to exercise a level of “self-health maintenance”, significantly influences the food and drink manufacturers worldwide.

Since nowadays, consumers are more diverted towards the health driven foods, hence recent advances in research on bioactive peptides show much promise in new product development using these biomolecules to derive multifarious health benefits. Bioactive foods enriched with health giving bioactive peptide holds a very promising future because researchers are realizing the importance of the importance of the poly functional roles of bioactive peptides. The in situ production of bioactive peptides in fermented dairy products has now been conceptualized as a novel approach to improve their health value of the products. While cheese has long been associated with a high quality nutritional image, more recently research efforts devoted to the development of bioactive cheese with demonstrated health promoting properties of bioactive peptides have been made to push cheese into the “functional foods” category.

2.0. BIOACTIVE FOODS: BEYOND NUTRITION

Functional foods, in addition to their basic nutritive value and natural being, will contain the proper balance of ingredients, which will help us to function better and more effectively in many aspects of our lives, including helping us directly in the prevention and treatment of illness and disease ultimately leading to a healthy and happier life. Functional foods serve to promote health or help to prevent disease, and in general the term is used to indicate a food that contains some health-promoting components beyond traditional nutrients (Berner and O’Donnell, 1998). Functional foods have been variously termed as nutraceuticals, designed foods, medicinal foods, therapeutic foods, superfoods, foodiceuticals, and medifoods. In most cases, the term refers to a food that has been modified in some way to become ‘functional’. Modifications can be achieved by incorporation of phytochemicals, bioactive peptide, ω-3 PUFA and probiotics and/or prebiotics. Milk naturally replete with
bioactive peptides, protective enzymes and other biologically active curative components, is argued to be a “functional food”. It is clear that the market for functional foods is at present, very buoyant and is predicted to expand substantially in the coming decade. Table-1 depicts some broad categories of functional foods.

**Table-1: Categories of Functional Foods**

<table>
<thead>
<tr>
<th>Food Modifications</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of phytochemicals (as plant ingredients</td>
<td>Antioxidant, lower risk of CHD, Cancer, and lower blood pressure.</td>
</tr>
<tr>
<td>or extracts)</td>
<td></td>
</tr>
<tr>
<td>Addition of probiotics</td>
<td>Improved gastrointestinal function, enhanced immune system, lower risk of</td>
</tr>
<tr>
<td></td>
<td>colon cancer, lower risk of food allergy.</td>
</tr>
<tr>
<td>Additional of prebiotics</td>
<td>Improved gastrointestinal function, lower risk of cancer, Enhanced immune</td>
</tr>
<tr>
<td></td>
<td>system.</td>
</tr>
<tr>
<td>Addition of bioactive proteins or peptides</td>
<td>Enhanced immune function, enhanced bioavailability of minerals function,</td>
</tr>
<tr>
<td></td>
<td>hypertensive.</td>
</tr>
<tr>
<td>Addition of dietary fibers</td>
<td>Prevention of constipation, lower risk of colon cancer and lowering of blood</td>
</tr>
<tr>
<td></td>
<td>cholesterol</td>
</tr>
<tr>
<td>Addition of ω-3 PUFA</td>
<td>Lower risk of heart attack, lower risk of some cancers, enhanced immune system</td>
</tr>
<tr>
<td>Removal of allergens</td>
<td>Reduce or eliminate allergy to specific foods</td>
</tr>
</tbody>
</table>

Source: Berner and O’ Donnell (1998)

Bioactive food is an important category of functional foods, which contains health promoting bioactive peptides, serving to regulate a particular body process, such as enhancement of the biological defense mechanisms, prevention and recovery of a specific disease.

### 3.0. DEVELOPMENT OF BIOACTIVE CHEESE: A HOLISTIC BIOTECHNOLOGICAL APPROACH

Dietary patterns of today’s consumers have been changed and they are going for foods having polyfunctional health benefits. Hence, the important allure of the modern health conscious consumer is to consume functional foods to derive health-giving and curative functions, naturally imparted to foods. In an endeavour to expand the range of bioactive dairy products, a small number of researchers and companies have attempted to manufacture cheeses, which contain a considerable amount of these wonder molecules viz bioactive peptides. Cheese will have a number of advantages over the non-fermented products as a delivery system for bioactive peptides. Given the increasingly competitive scenery of the European market in food products, the cheese industries in Ireland and throughout Europe are trying to derive benefit from a marketing advantage, such as added-value probiotics and bioactive peptides containing cheese, which would afford a competitive edge over existing products. The development of bioactive cheese could thus lead to a major economic advantage.
Growing public awareness of diet-related health issues has also fuelled the claim for probiotic foods, which are currently restricted predominantly to fermented milk drinks and yoghurt harbouring beneficial probiotic cultures, such as bifidobacteria and lactobacilli associated with a plethora of health benefits. While cheese has long been associated with a high quality nutritional image, more recently research efforts devoted to the development of bioactive cheese with demonstrated health benefits of bioactive peptides have been made to make cheese more promising as functional foods. Generally, incorporation of probiotic bacteria into milk-based food systems, including cheese still remains at the Biblical age in terms of maintaining viability and probiotic functionality during manufacture and shelf life. Therefore, there is a good potential to incorporate some health attributes in cheese by in situ synthesis of bioactive peptides to derive some health benefits beyond its basic nutrients. This approach of enriching cheese with bioactive peptides seems to be more attractive in terms of its stability and maintaining functionality.

4.0. BIOACTIVE PEPTIDES: THE WONDER BIOMOLICULES

Proteins possess biological and physico-chemical properties. For example, milk is known to contain a wide range of proteins, which either provide protection against enteropathogens or are essential for the manufacture and characteristics of certain dairy products. Research carried out during the last 10 years has shown that both major milk protein groups, caseins and whey proteins, can also be an important source of biologically active peptides. Functional or bioactive peptides are described as defined sequences of amino acids which are inactive within the native protein, but which display specific properties once they have been released by enzymatic activity. These peptides are in an inactive state inside the protein molecule and can be released during enzymatic digestion in vitro or in vivo. Bioactive peptides usually contain 3 to 20 amino acid residues per molecule, except for glycomacropeptides (GMP) which consists of 64 amino acid residues. The sequence of the amino acids in these small molecules is a crucial factor in their activity. The amino acid located in the C-terminal or N-terminal position is often important (Nayak et al., 1999).

The formation and properties of milk protein-derived peptides have been reviewed in many recent articles. Peptides with biological activity can be produced from milk proteins in three ways:

- Enzymatic hydrolysis with digestive enzymes
- Fermentation of milk with proteolytic starter cultures
- Through the action of enzymes derived from proteolytic microorganisms

The bioactivities of peptides encrypted in major milk proteins are latent until released and activated by enzymatic proteolysis, e.g. during gastrointestinal digestion or food processing like during fermentation. The proteolytic system of lactic acid bacteria (LAB) can contribute to the liberation of bioactive peptides. In vitro, the purified cell wall proteinase of Lactococcus lactis was shown to liberate oligopeptides from β- and α-caseins, which contain amino acid sequences present in casomorphins, casokinines and immunopeptides. The further degradation of these peptides by endopeptidases and exopeptidases of LAB could lead to the liberation of bioactive peptides in fermented milk products. Opioid peptides are opioid receptor ligands, which can modulate absorption processes in the intestinal tract, angiotensin-I-converting enzyme (ACE) inhibitory peptides are hemodynamic regulators and exert an antihypertensive effect, immunomodulating casein peptides stimulate the activities of cells of the immune system, antimicrobial peptides kill sensitive microorganisms, antithrombotic
peptides inhibit aggregation of platelets and caseinophosphopeptides (CPP) may function as carriers for different minerals, especially calcium. Bioactive peptides can interact with target sites at the luminal side of the intestinal tract (Meisel and Bocklmann, 1999).

It is now evident that during the fermentation of milk with certain dairy starters, peptides with various bioactivities can be formed and are detected in an active form even in the final products, such as fermented milks and cheese. Furthermore, they can be absorbed and then reach peripheral organs. Food-derived bioactive peptides are claimed to be health enhancing components, which can be used for functional food and pharmaceutical preparations. These peptides are likely to be produced industrially in the future in the form of hydrolysates or peptide mixtures, which can be used as ingredients for various dietary or pharmaceutical products or can be produced by conditioned fermentation in several fermented milk products.

4.1. **Multifarious Health Attributes of Bioactive Peptides**

The multifunctionality of physiologically active peptides derived from milk and their wide distribution among mammals could confer on them a role of messenger molecules. Their contribution to the health of the newborn would be three-fold: an easily assimilated source of organic nitrogen, a good source of essential amino acids and a potential source of bioactive molecules. Several casein-derived peptides may play a significant role in the stimulation of the immune system. They have been found to exert a protective effect against microbial infections and to enhance some functions of the immune system (Migliore-Sammour et al., 1989).

Milk-derived peptides have been shown to exert several beneficial physiological effects. They serve to modulate metabolic processes like digestion, circulation, immunological responsiveness, cell growth and repair, and nutrient intake. These functional peptides display partial resistance to hydrolysis and can exert their effects either locally in the digestive tract or elsewhere in the body. The milk derived peptides with various biological activities, such as opioids, mineral-carriers, antihypertensives, immuno-stimulants, antithrombotics and antigastries. These bioactive peptides are generated in vivo, in vitro and during food processing. Bioactive peptides are largely found in milk, fermented milks and cheeses. Proteolytic enzymes naturally occurring in milk and enzymes from lactic acid bacteria or from exogenous sources contribute to the generation of bioactive peptides. Dairy processing conditions, such as cheese ripening are also relevant (Smacchi and Gobetti, 2000).

In contrast to endogenous bioactive peptides, many milk-derived peptides have multifunctional properties. Regions in the primary structure of caseins contain overlapping peptide sequences, which exert different biological effects. These regions, considered to be strategic zones, are partially protected from proteolytic breakdown. For example, most β-casomorphins and casokinins are both ACE-inhibitory and immunostimulatory and α- and β-lactorphin contain sequences with both opioid and ACE-inhibitory activities (Meisel 1998). It has been reported that physiologically active peptides also have the ability to promote the growth of bifidobacteria in the GIT, thus increasing the number of these beneficial organisms. This is attributed particularly to κ-casein, which can affect bacterial adhesion as a receptor analogue (Lonnerdal, 1998).

Feeding mice with milk fermented by *B. longum, Lactobacillus casei* or *Lactobacillus helveticus* has shown a significant stimulation of phagocytosis by pulmonary macrophages Perdigon et al. (1998) suggest that the increased number of cells secreting IgA (but not IgG) in the large intestine of mice given yoghurt could contribute to limit the inflammatory immune response. Since IgA is considered to be an immune; barrier in colonic neoplasia. The
modulation of mucosal inflammation by IgA is important to prevent the tissue damaging consequences of a permanent inflammatory response, which occurs during the development of tumors and neoplasia. This suggests that the mechanisms by which yoghurt inhibits tumour development could be through the decrease of the inflammatory response. Since the increase of the IgA response might be related to the proteolytic activity of L. helveticus, we are currently investigating the role of individual peptides on the immunostimulation and the regression of mucosal tumours.

They could in the future have interesting dietary and pharmacological applications. Biologically active peptides have been found to have specific activities, such as antihypertensive, antimicrobial, immunomodulatory, opioid or mineral-binding. Many milk-derived peptides reveal multi-functional properties, that is, specific peptide sequences may exert two or more different biological activities. Due to their physiological and physico-chemical versatility, milk-borne bioactive peptides are regarded as highly prominent ingredients for health-promoting functional foods or pharmaceutical preparations. Table-2 exhibits different bioactive milk derived peptides and their biological activities:

Table-2: Bioactive Peptides Derived From Milk Proteins

<table>
<thead>
<tr>
<th>Bioactive Peptides</th>
<th>Protein Precursor</th>
<th>Bioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Casomorphins</td>
<td>α-Casein</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>β-Casomorphins</td>
<td>β-Casein</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>α-Lactorphrin</td>
<td>α-Lactalbumin</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>β-Lactorphrin</td>
<td>β-Lactoglobulin</td>
<td>Opioid agonist</td>
</tr>
<tr>
<td>Lactoferroxins</td>
<td>Lactoferrin</td>
<td>Opioid antagonists</td>
</tr>
<tr>
<td>Casoxins</td>
<td>X-Casein</td>
<td>Opioid antagonists</td>
</tr>
<tr>
<td>Casokinins</td>
<td>α-, β-Casein</td>
<td>ACE-inhibitory</td>
</tr>
<tr>
<td>Casoplastelins</td>
<td>X-Casein, Transferrin</td>
<td>Antithrombotic</td>
</tr>
<tr>
<td>Lactoferrinein</td>
<td>Lactoferrin</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Immunopeptides</td>
<td>α-, β-Casein</td>
<td>Immunomodulatory</td>
</tr>
<tr>
<td>Phosphopeptides</td>
<td>α-, β-Casein</td>
<td>Mineral carriers</td>
</tr>
</tbody>
</table>

Source: Meisel and Bockelmann (1999)

5.0. FERMENTED DAIRY PRODUCTS: A VEHICLE OF BIOACTIVE PEPTIDES

Fermented foods can be described as products whose physical, chemical and biological characteristics have been modified by the activity of microorganisms. They are known to contain specific microbial metabolites, such as alcohol, lactic acid, propionic acid, acetic acid, carbon dioxide and exopolysaccharides, as well as bioprocessed molecules derived from the original food material. These derived products, which could be named “tertiary metabolites”, can play a significant role in the biological activities of fermented products. Bioactive peptides are generated during food processing by chemical and physical but especially by enzymatic treatments as naturally happened during manufacturing of fermented dairy products. The enzymes involved occur naturally in foods or derives from exogenous or
microbial sources. The type of starter used is one of the main factors, which influence the synthesis of bioactive peptides in fermented milks; for example, proteolysis by \textit{Lactobacillus helveticus} is related to the production of antihypertensive peptides.

Many industrially used dairy starter cultures are highly proteolytic. This property is traditionally exploited by dairy industry, as the peptides and amino acids degraded from milk proteins during fermentation contribute to the typical flavour, aroma and texture of the products. The proteolytic system of LAB, such as \textit{Lactococcus lactis}, \textit{Lactobacillus helveticus} and \textit{Lactobacillus delbrueckii} \textit{var. bulgaricus}, is already well known. The release by microbial fermentation of various bioactive peptides from both caseins and whey proteins has been reported in many studies. Nakamura \textit{et al.} (1995) reported the presence of two angiotensin converting enzyme (ACE) inhibitory peptides (Val-Pro-Pro, leu-Pro-Pro) in sour milk, which was fermented with a starter culture composed of \textit{L. helveticus} and \textit{Saccharomyces cerevisiae}. This enzyme plays a crucial role in the regulation of blood pressure in mammals. Immunostimulatory peptides have also been detected in milk fermented with a \textit{L. helveticus} strain. Some workers also studied the potential formation of ACE inhibitory peptides from cheese whey and caseins during fermentation with different commercial lactic acid starters used in the manufacture of yoghurt, ropy milk and sour milk. ACE inhibitory peptides were released both from \textit{αs1}– and \textit{β}-caseins and also from the major whey proteins \textit{α}–lactalbumin and \textit{β}-lactoglobulin.

Fermentation of whey with \textit{Kluyveromyces marxianus} \textit{var. marxianus} was reported to release a tetra peptide with antihypertensive properties. Microbial enzymes have been used successfully for production of bioactive peptides from milk proteins during fermentation. Yamamoto \textit{et al.} (1995) reported that casein hydrolyzed by the cell wall-associated proteinase from \textit{L. helveticus} CP790 showed antihypertensive activity in spontaneously. Fermented milk enriched with the opioid \textit{β}-casomorphin has been produced using a mutant strain of \textit{Lb. helveticus} (Matar and Goulet 1996). Some of the probiotic effects attributed to well-known LAB strains are related to the specific synthesis of bioactive peptides. UHT milk fermented by the probiotic \textit{Lactobacillus CG} strain and subsequently digested by pepsin and trypsin enzymes has been reported to contain bioactive peptides, which correspond fragments of \textit{αs1}– and \textit{β}-CN, and of \textit{α}–lactalbumin, having different degrees of immunostimulating, opioid and ACE-inhibitory activities. The production of these bioactive peptides could partially explain the probiotic properties attributed to the \textit{Lactobacillus GG} strain.

Yogurt contains peptides in the range of 500-10,000 Da, which \textit{in vitro} reduce the risk of colon cancer. A recent study revealed that when adults ingested 500 ml of milk or yoghurt, certain functional peptides could be identified in their stomach, duodenum and blood, notably caseinoglycopeptides with an antithrombotic sequence. It is interesting to note that the level of these peptides is higher after ingesting yoghurt rather than milk, which suggested the role of LAB in the formation of functional peptides in fermented milk products. Many studies have reported on the formation of presence of various bioactive peptides in fermented milk products. Such peptides have been identified in sour milk upon fermentation with strong proteolytic starter cultures, for example, \textit{L. helveticus} and in ripened cheese varieties. The formation of calcium-binding phosphopeptides in fermented milks and various types of cheese has been observed in many studies. \textit{β}-casomorphins have been identified in several fermented dairy products. Some workers have reported on the release of variety of bioactive peptides by enzyme proteolysis of UHT milk fermented with a probiotic \textit{Lactobacillus casei} \textit{ssp. rhamnosus} strain. Upon fermentation, the product was treated with pepsin and trypsin with the intent to simulate gastrointestinal conditions. In the hydrolysate, many bioactive peptides, for
example, ACE inhibitory, immunomodulatory and opioid peptides, were identified, this suggesting that these bioactive peptides may partially explain the health-promoting properties of milk products containing probiotic bacteria. Tabl1-3 shows a number of bioactive peptides with their functional activities derived from different fermented dairy products.

**Table-3: Bioactive Peptides Identified in Fermented Milk Products**

<table>
<thead>
<tr>
<th>Product</th>
<th>Bioactivity Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sour milk</td>
<td>Phosphopeptides</td>
</tr>
<tr>
<td>Sour milk</td>
<td>ACE inhibitor</td>
</tr>
<tr>
<td>Sour milk</td>
<td>β-casomorphin-4</td>
</tr>
<tr>
<td>Fermented milk (treated with pepsin and trypsin)</td>
<td>ACE inhibitory, Immunomodulatory and Opioid</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>ACE Inhibitory (weak)</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>Immunomodulatory, Antihypertensive, Antiamnesic, Microbicidal and Antithrombotic</td>
</tr>
</tbody>
</table>

Source: IDF Bull. 363

Many studies have reported on the formation of presence of various bioactive peptides in fermented milk products. Further studies are needed on the importance in fermented milk products of added starters and non-starter proteolytic bacteria for the formation and transformation of bioactive peptides.

### 6.0. CHEESE: A NOVEL MILIEU OF BIOACTIVE PEPTIDES

Bioactive peptides can also be produced from milk proteins through fermentation of milk by starters employed in the manufacture of fermented milks and cheese. In particular, antihypertensive and mineral binding peptides have been identified in fermented milk, whey and ripened cheese. A few of these peptides have proved effective also in humans after ingestion of fermented milk products. LAB used in cheese manufacture might be indirectly involved in the modulation of the immune response of the host by contributing, during the fermentation process, to the release of peptides bearing hormone-like activities. During the fermentation of milk by LAB, casein undergoes a slight proteolysis capable of generating potentially bioactive peptides.

### 6.1. Bioactive Cheese: A Novel Bio-Boom of Biotechnology

Bioactive peptides may be liberated during the manufacture of cultured milk products, such as yoghurt, Daahi, cheese, etc. Bacterial starter cultures contain several proteolytic enzymes that are responsible for the breakdown of protein into peptides and amino acids during cheese maturing. During fermentation of milk, various long oligopeptides are liberated by degradation of caseins, which would be precursors of peptides with biological activity when cleaved by other enzymes. In fermented milk products and cheese, intracellular peptidases of LAB will most likely contribute to further degradation after cell lysis. The specificities of known peptidases suggest that all peptide bonds in caseins may be cleaved partially. Various
studies have reported on the casomorphins, ACE-inhibitory peptides and phosphopeptides found in several fermented milk products including cheese. Cheese contains phosphopeptides as natural constituents and extensive proteolysis during cheese ripening leads to the formation of other bioactive peptides, such as ACE-inhibitory peptides and precursors of β-casomorphins having several healths attributes.

The starter culture applied in the manufacture of “Festivo” cheese, a novel bioactive cheese, is a mixture of commercial starter cultures containing 12 different strains of the following genera or species” *Lactococcus* sp. and *Leuconostoc* sp. (BD-type culture), *Propionibacterium* sp., *Lactobacillus* sp. as well as *Lactobacillus acidophilus* and *Bifidobacterium* sp. It has been reported that the ACE-inhibitory activity increases during “Festivo” cheese ripening and decreases when proteolysis exceeded a certain level during the storage period. These results would suggest that ACE-inhibitory peptides and probably other biologically active peptides as well, are naturally formed in cheese. This called for the development of a totally new type of cheese with the health attributes of milk derived bioactive peptides. The new ‘Festivo’ cheese based on an innovative concept in the production of healthy foods has been commercially developed (Ryhanen et al., 2001). This is the first time that a probiotic cheese along with the health benefits of bioactive peptides is being produced on an industrial scale. ‘Festivo’ is now manufactured commercially in Finland and has attracted growing interest among health-aware consumers. The new type of cheese, ‘Festivo’, developed for production on an industrial scale, has showed good results in organoleptic analysis. On the basis of this study, it is suggested that the new cheese may possessed multifunctional health effects, since it contain several beneficial components such as probiotics, bioactive peptides, conjugated linolein acid, and calcium.

Several bioactive peptides have been found in cheeses and may be due to the intense proteolysis during cheese ripening. Ripening conditions and the type of starters used affect bioactive peptides synthesis in cheese. LAB, used as starter microorganisms in dairy products or endogenous to milk microflora, have a proteolytic system, which include cell bound proteinases (CEP) and intracellular peptidases. CEP release many different oligopeptides into shorter fragments and amino acids, which contribute directly or as precursors to flavour. Some of these peptides are bioactive. The Festivo cheese was produced by using cheese starter cultures in combination with *Lactobacillus acidophilus* and bifidobacteria. The ripened cheese was found to contain bioactive peptides with potential antihypertensive effects (Ryhanen et al., 2001).

Meisel et al. (1997) reported the presence of low-molecular weight ACE inhibitory peptides in several ripened cheeses. Proteolysis increases ACE inhibitory activity but only to a certain level after which the ACE inhibition index decreases. The ACE inhibitory activity in medium aged Gouda was about double that of the long ripened Gouda. A αs1-casein derived antihypertensive peptide, isolated from 6 month-ripened Parmesan cheese (Addeo et al., 1992), was not detectable after 15 months of ripening. Products having a low level of proteolysis (Quarg) have a low ACE-inhibition index. These results indicate that the bioactive peptides liberated by proteolytic enzymes from LAB during cheese ripening are degraded further to inactive fragments as a result of further proteolysis (Meisel et al., 1997). The following table (Table-4) presents different bioactive peptides detected and identified in a number of cheeses by different workers.
### Table 4: Bioactive Peptides Identified in Several Cheese Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Bioactivity observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quarg</td>
<td>ACE inhibitor</td>
</tr>
<tr>
<td>Parmesan Reggiano</td>
<td>β-casomorphin precursors</td>
</tr>
<tr>
<td>Comte’</td>
<td>Phosphopeptides</td>
</tr>
<tr>
<td>Cheddar</td>
<td>Phosphopeptides</td>
</tr>
<tr>
<td>Edam, Emmental, Gouda, Roquefort, Tilsit</td>
<td>ACE inhibitory</td>
</tr>
<tr>
<td>Mozzarella, Italico, Crescenza, Gorgonzola (Italian varieties)</td>
<td>ACE inhibitory</td>
</tr>
<tr>
<td>Edam, Emmental, Turunmaa, Cheddar, Festivo (Finnish varieties)</td>
<td>ACE inhibitory</td>
</tr>
<tr>
<td>Cheddar, Edam, Swiss, Feta, Camembert, Blue vein (Australian varieties)</td>
<td>Immunomodulatory, Antihypertensive</td>
</tr>
<tr>
<td>Gouda, Havarti, Emmental</td>
<td>ACE inhibitory</td>
</tr>
</tbody>
</table>

Source: IDF Bulletin 363

ACE inhibiting peptides have been isolated from several Italian cheeses characterized by short (Crescenza and Italico) and medium (Gorgonzola) ripening period (Smacchi and Gobbetti 1998). Another bioactive peptide containing the β-casomorphin-7 sequence, which has shown an antihypertensive activity, has been isolated from Crescenza and Cheddar cheeses. Proteolytic enzymes of LAB produce caseinophosphopeptides during ripening of cooked curd cheeses like Comte’ or Grana Padano. Peptides derived from a cheese slurry prepared using *Lactococcus lactis* subsp. *lactis* as starter culture have anticarcinogenic effect (Kim et al., 1995).

### 6.2. Bioactive Cheese: The Pragmatic Vision

Microbial starters, such as LAB in dairy products contribute to the synthesis of bioactive peptides. An increasing number of *in vitro* and *in vivo* studies reveal that biologically active peptides are released from bovine milk proteins upon microbial fermentation and hydrolysis by digestive enzymes. Peptides with different bioactivities can be found in fermented milks and cheese varieties, but the specificity and amount of peptides formed is regulated by starter cultures used for cheese ripening and the rate of proteolysis, which needs further extensive studies on the controlled maturation of cheese. Highly proteolytic starter cultures, for example, *Lactobacillus helveticus*, would seem to produce short peptides with various bioactivities in several cheeses. Food researchers are presently considering bio-active peptides as health-enhancing nutraceuticals for use in functional foods. Besides supplementation of food, production of desirable bioactive peptides during food processing, e.g. by use of specific enzymes or genetically transformed microorganisms is also a target area that seems to hold the interest of these workers. Presently, the development of bioactive cheese with the *in situ* production of bioactive peptides gets immense scientific interest. May in the forthcoming days, cheese with the enriched level of bioactive peptides would occupy place in market shelves. It is in this context that cheeses, a fermented milk product which is natural...
sources of peptides with their physiologically significance have a potential for future food research.

Bioactive peptides or related sequences Proteolytic enzymes from LAB produce bioactive peptides in several dairy products, but, once liberated, bioactive peptides also influence the biochemical activity of the microbial communities in cheese. This role is probably been underestimated in dairy processing, which seems to be a challenge to the development of bioactive cheese. Processing conditions should be carefully controlled in order to synthesize specific bioactive peptide during cheese production by selected or genetically-modified micro-enzymatic processes. Given the advantages that enzymatic synthesis has over, chemical and recombinant DNA approaches to synthesize bioactive peptides, enzymes responsible for their generation in food processing may have a potential application. Trypsin, thermolisin, α-chymotrypsin, papain and subtilism are generally used for the preparative synthesis of biologically active peptides, such as angiotensin, caerulein, enkephalin, oxytocin and dynorphin. However, the restricted availability of suitable biocatalysts is still problem. With the modified stability, activity, and specificity via site-directed mutagenesis of well-known proteinases, proteolytic enzymes of LAB, due to their high regiospecificity and proven efficiency, may be assayed for in situ enzymatic synthesis of biopeptides in cheese.

In the selection of microbial starters, the sensitivity to peptides should be carefully considered because, after liberation in cheese ripening, these peptides may accumulate which could retard or block ripening due to enzyme inhibition. The use of specific peptide inhibitors may be helpful in order to understand and the role of different proteolytic enzymes in generating the desired flavour, removing bitterness in cheese and selecting LAB strains for starters or proteolytic enzymes for accelerating cheese ripening (Samacchi and Gobbetti, 1998).

6.0. CONCLUSION

Fermented food products have been consumed through many centuries by millions of human beings long before the existence of microorganism was scientifically demonstrated. Their nutritive value and therapeutic properties have been and still are widely recognized in all parts of the world and allow them to be classified as nutraceuticals. More and more evidence is now suggesting that substrate derived molecules may play a significant role in the reported beneficial effects of some of these products. Several peptides derived from milk proteins, mainly caseins and whey proteins, have already found interesting applications in pharmaceutical preparations or dietary supplements. Bioactive peptides, isolated from fermented dairy products mainly from cheese, have strengthened the interest to develop bioactive cheese with controlled fermentation. Optimization of fermentation on the one hand, and molecular cloning and gene manipulation for bioactive peptide production and immunity via gene cloning and amplification, site-directed mutagenesis, and hybrid gene formation, etc on the other hand may further improve the production of these biologically active molecules.

One might foresee very promising future for the bioactive cheese with the health promoting effects of bioactive peptides in the field of functional foods and nutraceuticals particularly with regard to the prevention or attenuation of several symptoms related to physiological or infectious diseases. The combinations of special strains of bacteria, specific substrates and optimal fermentation/growth conditions are likely to yield synergies that would not be observed in conventional cheese ripening process. Molecular biology, biochemistry, microbiology, chemistry and many other fields of knowledge will certainly contribute to a better understanding of what can be considered as a new level of interaction between foods,
microorganisms and the digestive system of most animal species including man. Development of bioactive cheese might have been successful in prevention of several physiological disorders of human beings. To decipher such a new reservoir of biological messages that is well encrypted in our food is quite a challenge. Fortunately, we can count on probiotics to help us better understand this new language and continue to improve our quality of life.

7.0. REFERENCES


1.0. INTRODUCTION

Most cheese varieties are made by coagulating milk with selected proteinases, like rennet, which is an enzyme preparation extracted from fourth stomach (veils) of young calves. Addition of rennet to milk breaks down the casein micelle stabilizing protein, called kappa casein, at a specific bond i.e. Phe$^{105}$-Met$^{106}$ and destroys its ability to stabilize the casein micelle. Thus, it brings about an enzymatic modification of casein. Consequently, para-kappa casein is formed which aggregates in the presence of ionic calcium at a temperature of about 20 $^\circ$C and forms a gel. In the past few decades due to the short supply of calf veils, production of calf rennet has not been sufficient to meet the demand of growing cheese industry all over the world. The reduced availability of and the consequent increase in the price of calf rennet led to the search of rennet substitutes suitable for cheese making. The suitability of any rennet substitute depends on several factors, like, availability of the source, ease of production, purity, absence of antibiotics, and thermo-stability. The milk-clotting activity (MCA) of rennet substitute should not be much dependent on pH and the ratio of MCA to proteolytic activity (PA) should be high. The rennet substitute must be suitable for use in the manufacture of large variety of cheese without involving many changes in the traditional method of manufacture and affecting the quality and yield of cheese.

2.0. MICROBIAL RENNETS

Several proteinases from animals, plant and microbial sources possess the ability to coagulate milk under suitable conditions but most of them are too proteolytic relative to their clotting ability which causes development of defects in flavour and texture as well as reduction in yield of cheese. However, few microbial sources produce rennet substitutes which meet the suitability requirements and have reached the stage of commercial production (Table-1). Like chymosin these substitutes are acid (aspartyl) proteinase and similar in molecular and catalytic properties. They have a relatively narrow specificity, with a preference for peptide bonds to which a bulky hydrophobic residue supplies the carboxyl group. Although their narrow specificity is desirable in cheese making all the proteinases are not suitable because they are too active even under the prevailing unfavourable conditions in milk and cheese. The specificity of rennet substitutes from fungal sources like Mucor miehei, Mucor pusillus and Endothia parasitica is quite different. Like chymosin, the acid proteinases of Mucor miehei and Mucor pusillus preferentially hydrolyze the same Phe105-Met106 bond of k-casein while that of Endothia parasitica preferentially cleaves the Ser104-Phe 105 bond. However unlike chymosin, fungal proteinases also cleave several other bonds in k-casein.
The MCA of fungal rennet increases with temperatures in the range of 28-36°C. The fungal enzymes from *Mucor miehei*, *Mucor pusillus*, and *Endothia parasitica* lose their activity at 47, 57 and 57°C, respectively. They are less sensitive to pH in the range of 6.2-6.8. The retention of coagulant properties in the whey after heat treatment is unsatisfactory for further usage. Thermal inactivation temperatures for *Mucor miehei*, *Mucor pusillus*, and *Mucor miehei* and calf rennet are 77, 71 and 67°C for 15 sec at pH 6.0 respectively. The thermal stability of *Endothia parasitica* proteinase is less than that of chymosin at pH 6.6. Earlier fungal rennet were considerably more thermo stable than chymosin or pepsin but now the products have been modified by oxidation of methionine residues in the molecule and have thermal stability similar to chymosin. The thermal stability of all rennet increases markedly with decreasing pH obtained from *Endothia parasitica* is less sensitive to calcium than the calf rennet while that from *Mucor miehei* and *Mucor pusillus* are more sensitive.

The protease of *Mucor miehei* coagulants splits peptide bonds at the aromatic amino acid residues, degrades casein fairly rapidly in the pH range of 5.5-7.0. The incidence of bitter cheese using this enzyme is low. The enzyme is very sensitive to temperature in the range of 37-45°C (optimum 42°C) and calcium chloride. The enzyme is active and attacks alpha and beta caseins in the pH range of 5.5-7.5 and is destroyed at 70°C, but the incidence of bitter flavours in cheese curd is low.

*Mucor pusillus* (Lindt) extract is more protelytic than calf rennet or *M. miehei* extract. The clotting ability of the extract is three times that of calf rennet. An increase in calcium ion in milk decreases the clotting time and added calcium chloride is essential. However the activity is not pH dependent as for other coagulants. The enzyme from *M. pusillus* splits the peptide at aromatic amino acid residues, but is not specific as *M. miehei* extract. The enzyme produces large peptides, whereas calf rennet produces a number of smaller peptide chains and for this reason *M. pusillus* extract and calf rennet are sometimes used together.

The enzyme from *M. pusillus* tends to give hard curd because of its higher proteolytic activity and the curds tend to loose fat into whey. Thus, 0.4-0.5 per cent fat in whey is common and the cheese yields are lower than with other coagulants. Thus coagulant may be blended with pepsin for some cheese varieties. *M. pusillus* enzyme alone tends to produce bitter flavours in ripe cheese and has been used for short cure cheese. Newer methods of purification

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Brand Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mucor miehei</em></td>
<td>Hannilase, Rennilase, Fromase, Miki, Marzyme, Modilase</td>
</tr>
<tr>
<td><em>Mucor pusillus</em> (Lindt)</td>
<td>Noury, Meito, Emporase</td>
</tr>
<tr>
<td><em>Endothia parasitica</em></td>
<td>Suparen, Sure curd</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Mikrozyme</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>Chymogen</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Chy- max</td>
</tr>
<tr>
<td><em>Kluyveromyces lactis</em></td>
<td>Maxiren</td>
</tr>
</tbody>
</table>
of all microbial extracts tend to produce more satisfactory milk clotting extracts with less proteolytic activity. M. pusillus extract has optional activity at pH 5.5; which is very acid for normal coagulation of milk, and its activity may persist in whey which is disadvantageous.

The enzyme extracted from Endothia parasitica is an acid protease but in some extracts a number of proteases and lipases may be present if the extract is not well purified. The coagulant is more caseinolytic than Mucor or calf rennet, and tends to produce more bitter flavours in high moisture cheese. The extract should be used with calf rennet to improve the curd yields. E. parasitica extract is destroyed at 60°C in 5 min.

Improved purification procedures to remove unwanted material have made it possible to produce microbial coagulants with superior properties. Purification procedures have included the use of silicates and bentonite as absorbents, or zeolites in the pH range between 3 and 9 to remove those substances and enzyme systems not required in the rennet.

3.0. RECOMBINENT CHYMOSIN

Although microbial rennet is relatively cheap, these have attracted the attention of industrial enzymologists and biotechnologists. The gene for prochymosin has been cloned in E. coli, Saccharomyces cerevisiae, Kluyveromyces marxianus var. lactis, Aspergillus nidulans, Aspergillus niger and Tricoderma reesei. The enzymatic properties of the recombinant enzymes are indistinguishable from those of calf chymosin. The uses of recombinant chymosin have been assessed on many cheese varieties, always with very satisfactory results. Recombinant chymosin has been approved by many countries but not by all. Three recombinant chymosin are now marketed commercially: Maxiren secreted by K. marxianus var. lactis and produced by Gist Brocades; Chymogen secreted by A. niger and produced by Hanse; and chymax secreted by E. coli and developed by Pfizer. The genes for Maxiren and chymogen were isolated from calf abomasums, while that used for chymax was synthesized. Microbial chymosins have taken market share from both calf rennet and especially fungal rennet and now represents 35 per cent of total market.

The recombinant chymosins currently available are identical or nearly so, to calf rennet but there are several published studies on engineered chymosins. At present attention is focused on elucidating the relationship between enzyme structure and function; but this work may lead to rennets with improved MCA or modified general proteolytic activity (i.e., on alpha-s and/or beta casein) so as to accelerate its action on specific bonds of casein during ripening and/or to reduce its activity on others to eliminate undesirable consequence, such as bitterness. To date, the pH optima, thermal stability, K cat and K m of synthetic peptides have been modified through genetic engineering.

The gene for R. miehei proteinase has been cloned in and expressed by A. oryzae. It is claimed that this new rennet (Mazyme GM) is free of other proteinases or peptidases activities that are present in fungal rennet and may reduce cheese yield. Cloning of the gene for R. miehei proteinase has created the possibility for site directed mutagenesis of the enzyme.

4.0. PRODUCTION OF MICROBIAL RENNET

Microbial rennet can be produced by fermentation of the fungus Mucor miehei, Mucor pusillus, and Endothia parasitica or from bacteria like Bacillus subtilis, or Bacillus prodigiosm. Method of production involves the following steps depicted in (Fig. 1).

4.1. Preparation of Medium

Wheat bran → Moistened (50% moisture) → Filled in 400ml flask → Sterilized (121°C/20min)
4.2. Culture Cultivation

Growing mould for seven days on Czeapek Dox agar supplemented with 0.5 % malt

↓

Transfer of moulds to 9 ml sterile saline water

↓

Addition of 1 ml of suspension to each 400 ml flask containing media

↓

Gentle shaking of flasks gently to spread the moist bran in thin layer

↓

Incubation at 22° C for 4 days

↓

Addition of 100 ml distilled water to each flask and freezing at 0°C for 8 hr

↓

Thawing the contents of the flask at room temperature

↓

Filtration through muslin cloth followed by Hyflosupercel

↓

Cooling to 5°C

↓

Centrifugation at 5000 rpm for 30 min in refrigerated centrifuge

↓

Dissolving the precipitate in distilled water and precipitation with 1.5 volume of acetone at 0°C with constant stirring

↓

Keeping the mixture for 2 hr at 0°C followed by centrifugation at 5000 rpm for 30 min

↓

Dissolving the precipitate in water for use / Freeze drying

5.0. APPLICATION IN CHEESE MAKING

Coagulants are compared with respect to cheese quality, yield, loss of fines in the whey and quality of whey for further processing. In general the proteolytic activity of rennet increases in the following order:

Calf rennet < *Mucor miehei* extract < *Mucor pusillus* extract < *Endothia parasitica* extract
The sensory quality of cheese prepared by using different rennets appears to decrease as indicated below:

Calf rennet > Mucor miehei extract > Mucor pusillus extract > Endothia parasitica extract

Cheeses made with microbial rennet are comparable to that made with the commercial grade calf rennet. Different types of cheese, namely, white cheese, Mozzarella, blue cheese have been made. Higher cheese yield and higher concentration of milk protein and fat are observed in cheese made using microbial rennet. Some microbial strains produce rennets for soft cheese, but produce bitter flavours when used to make ripened cheese. Therefore, it is important to recognize that the activity of various microbial rennets varies according to pH and the system. However many of these rennets have been used satisfactorily as a mixture with other enzymes. The higher proteolytic activity of microbial rennet has proved to be a boon for cheese making from buffalo milk. Method has been developed for the production of cheddar cheese from buffalo milk using these rennet substitutes. Use of microbial rennet in making cheese from buffalo milk results in superior products as compared to calf rennet. The rate of glycolysis, proteolysis and lipolysis are relatively faster resulting in acceleration of cheese ripening.

6.0 SUGGESTED REFERENCES


Teuber, M. (1990). Production of chymosin (EC 3.4.23.4) by microorganisms and its use the electrophoretic pattern and textural assessment of aged Cheddar cheese made using various levels of calf rennet or microbial coagulant (Rennilase 46L). Int. Dairy J., 4: 303-327.
1.0. INTRODUCTION

Food packaging materials not only provide barrier and protective functions but when various kinds of active substances are incorporated into the packaging material, they improve its functionality. Such active packaging technologies are designed to extend the shelf life of foods, while maintaining their nutritional quality and safety. Active packaging technologies involve interactions between the food, the packaging material, and the internal gaseous atmosphere (Labuza and Breene, 1988). The extra functions they provide include: oxygen scavenging, antimicrobial activity, moisture scavenging, ethylene scavenging, ethanol emitting, and so on (Rooney, 1981). The most promising active packaging systems are oxygen-scavenging systems, which absorb oxygen gas in the package and prevent rancidity of food.

2.0. ANTIMICROBIAL FOOD PACKAGING

When antimicrobial agents are incorporated into a polymer, the material limits or prevents microbial growth. This application could be used for foods effectively, not only in the form of films but also as containers and utensils. Food packaging materials may obtain antimicrobial activity by common antimicrobial substances, radiation, or gas emission/flushing. Radiation methods may include using radioactive materials, laser-excited materials, UV-exposed films, or far-infrared-emitting ceramic powders. For examples, berries and grapes are stored in produce boxes, palletized, and stretch wrapped, then flushed with sulfite to prevent fungal spoilage. It is easy to use these types of bulk gas flushing and controlled/modified-atmosphere technologies.

3.0. ANTIMICROBIAL SUBSTANCES

A chemical preservative can be incorporated into a packaging material to add antimicrobial activity to it. For example, preservative-releasing films provide antimicrobial activity by releasing the preservative at a controlled rate. Oxygen absorbents also reduce headspace oxygen and partially protect food against aerobic spoilage such as mold growth (Smith et al., 1990). Common antimicrobial chemicals for food products are such preservatives as organic acids and their salts, sulfites, nitrites, antibiotics, and alcohols. Sorbic acid and its potassium salts have been used as preservative for the packaging of cheese products.

3.1. Anhydrides as Antimicrobial Agents

LDPE films into which benzoic anhydride is incorporated exhibits antymycotic activity when in contact with microbiological media and cheese. Benzoic anhydride, which had been added to LDPE film, was hydrolyzed within five hours and detected as benzoic acid in potato dextrose agar (PDA) and cheese after contact with the film. LDPE films, into which 1 per cent benzoic anhydride was incorporated, completely inhibited the growth of Rhizopus stolonifer,
Penicillium sp. and Aspergillus toxicarius. One study showed that LDPE films containing added 0.5 per cent to 2 per cent benzoic anhydride delayed mold growth on cheese.

3.2. Bacteriocins

A group of antimicrobial substances known as bacteriocins are proteins derived from microorganisms and are effective against microorganisms such as Clostridium botulinum. One such compound, nisin, has been accepted by regulatory authorities in some countries including Japan for food application.

3.3. Antimicrobial Enzymes

Antimicrobial enzymes might also be bound to the inner surface of food contact films to produce microbial toxins. Several such enzymes exist, e.g. glucose oxidase, which form hydrogen peroxide, a potential antimicrobial agent.

3.4. Antimicrobial Activity of Metal Ions

Among metallic ions of Na+, K+, Li+, Ag+, the silver ion has the strongest antimicrobial activity. Antimicrobial activity of metals is due to the minute quantity of ions fortified from the metals. Copper ions can destroy microorganisms and viruses.

3.5. Microban™

These antimicrobial agents used in polymer applications (both flexible films and rigid plastics) are sub-micron-sized which penetrate and disrupt the metabolic function of microorganisms. When introduced into the molecular structure of polymers, these additives attack microorganisms, interrupting their ability to function, grow, and reproduce.

3.6. Sorbic Acid

Sorbic acid has been used for years as an antimycotic i.e., mycostatic, agent in foods. A process has been developed for the production of fungicidal paper for long-term storage of bread. Vegetable parchment has been used as the base paper, whereas sorbic acid as the fungicide. The active compound was coated on the surface in the form of an aqueous suspension, using CMC as a binder to prevent the shedding of crystals from the paper surface. Sorbic acid and its derivatives have been accepted by U.S. regulatory authorities as in-package mycostatic agents.

3.7. Natural Antimicrobials

Antimicrobial compounds naturally derived or occurring in nature have been isolated from plant and animal sources. Compounds from plant origin include extracts of spices: cinnamon, allspice, clove, thyme, rosemary, and oregano are a few that have shown antimicrobial activity. Some plant extracts having similar effect on microbe growth are onion, garlic, radish, mustard etc.

3.8. Antibacterials

Compounds frequently used as antibacterials include inorganic acids/salts, organic acid esters, and complex polypeptides. The nitrites and sulfites find use in a broad range of foods from meats to vegetables. Most common of the organic acid esters are the lactates and sorbates. Of the polypeptides, nisin is most commonly used.
3.9. Ethanol

Ethanol is used routinely as a sterilizing agent in medical and pharmaceutical applications, and in vapor form, it has been demonstrated to extend the shelf life of packaged bread and other baked products. Ethanol sachets, containing encapsulated ethanol, release ethanol vapors, which impart a preservative effect in the packaging headspace. The ethanol prevents microbiological spoilage of intermediate moisture foods, cheese products, and sweet bakery goods.

3.10. Antimicrobials in Paper

In India, an effective fungistatic wrapper has reportedly been made economically by coating grease-proof paper with an aqueous dispersion of sorbic acid and an antioxidant in 2 per cent CMC solution. Foods can be preserved for a minimum of 10 days by wrapping them with the sorbic acid-treated paper.

4.0. ANTIMICROBIAL PACKAGING SYSTEMS

Most food packaging systems consist of the packaging material, the food, and the headspace in the package. If the void volume of solid food products is assumed as a kind of headspace, most food packaging systems represent either a package/food system or a package/headspace/food system. A package/food system has a package-contacted food product, or a low viscosity or liquid food without headspace. Individually wrapped cheese and products like aseptic brick packages are good examples. Antimicrobial agents may be incorporated into the packaging materials initially and migrate into the food through diffusion and partitioning (Padgett et al., 1998). Examples of a package/headspace/food system are flexible packages, bottles, cans, cups, and cartons. Control of the release rates and migration amounts of antimicrobial substances from food packaging is very important.

5.0 FACTORS AFFECTING ANTIMICROBIAL FILM OR PACKAGE

- Casting process conditions and residual antimicrobial activity
- Characteristics of antimicrobial substances and foods
- Storage temperature
- Mass transfer coefficients
- Physical properties of packaging materials

6.0. VERIFICATION OF ANTIMICROBIAL ACTIVITY

The antimicrobial activity of the packaging materials could be measured by microbial experiments. Before food samples are packaged in the antimicrobial packaging materials, target microorganisms may be inoculated onto the surface of the food or mixed into the food samples. Measuring the growth by counting the microorganisms with incubation time can provide the characteristic values of growth rate at the exponential growth phase; maximum growth at- the-stationary phase, and initial lag period. These values can be compared to conventional packaging or reference samples without packaging.

7.0. ANTIMICROBIAL EDIBLE COATINGS AND FILMS

Edible coatings and films have a variety of advantages such as biodegradability, edibility, biocompatibility, aesthetic appearance, and barrier properties against oxygen and physical stress. They can also serve as a carrier for edible antimicrobial agents and...
preservatives. For example, whey protein coatings and films can incorporate adequate amounts of edible antimicrobial agents (e.g., lysozyme, nisin, potassium sorbate, EDTA), as well as a plasticizer (e.g., glycerin or sorbitol). These films may have great potential as a microbial hurdle against gram-positive spoilage and pathogenic bacteria.

8.0. CONCLUSION

Antimicrobial packaging is a promising form of active food packaging. Even though most packaged perishable food products are heat sterilized or has a self-protecting immune system, microbial contamination could occur on the surface or damaged area of the food through package defects or re-storage after opening. The antimicrobial substances incorporated into packaging materials can control microbial contamination by reducing the growth rate and maximum growth population and extending the lag period of the target microorganism.

9.0. REFERENCES


1.0. INTRODUCTION

The term “biotechnology” was coined in 1919 by Kari Ereky, and the term meant all the loses of work by which products are produced from raw materials with the aid of living organisms. Biotechnological applications in the area of food processing are not new, selective breeding of plants and animals to increase the yield and utilization of microorganisms to manufacture wide array of food products like beer, wine, cheese and bread, are centuries old practices. “Dahi” (curd) a fermented dairy products, is very frequently quoted in ancient Mythological literatures, as sacred food item, possessing immense nutritional and therapeutic attributes.

The late eighteenth century and the beginning of the nineteenth century witnessed the advent of vaccinations, crop rotation involving leguminous crops and animal drawn machinery. The end of 19th century was a milestone of biological research and with the discovery of microbes, better understanding of inheritance of genetic traits through generations by Mendel’s work, opened a new channel for biotechnological research and development. This brought industry and agriculture together and biotechnological focus moved towards pharmaceuticals (penicillin), agricultural production, in the beginning of 10th century. The fermentation processes being utilized on commercial scale for a number of food and food ingredients agriculture, bioremediation, food processing, health industry, and energy production.

New biotechnological techniques have permitted scientists to manipulate desired traits and recombinant rDNA technology has changed the orientation of biotechnological processes for wider spread horizon. Modern biotechnology has its “roofs” in basic sciences and the explosion in this area has resulted in three major branches of biotechnology: genetic engineering, diagnostic techniques and cell/tissue culture techniques.

2.0. BIOTECHNOLOGICAL RESEARCH TOOLS

Advancement in the area of molecular genetics in mid and late 20th century reflected in increased availability of research methods in the field of biotechnology. These tools are highly specific and have potential to ensure not only the food security but many other aspects of human life. Some of these technologies are listed here.

2.1. Recombinant DNA Technology

Process in which a piece of rDNA is cleared, attested and then inserted into a self-replicating genetic element termed as plasmid, which in turn [produced new combinations of
rDNA. This technology has been widely utilized in the development of transgenic animals or plants having specific traits.

2.2. Tissue Culture

This process involves *in vitro* culturing of protoplasts isolated from plant/animal tissues under aseptic conditions and with appropriate amounts of growth substances, hormones. In the growing medium, cells grow and divide to form a mass of undifferentiated tissues called as callus. Callus, in turn incubated under conditions that induce organ formation or used for initiation of a cell suspension. This technique has been commercialized for the productions of plants, food colours, and many other food ingredients.

2.3. Monoclonal Antibodies

Popular as “Hybridoma”, in this technique immune system cells called antibodies are produced. These antibodies are further used as detection devices and as highly specific therapeutic agents i.e. in removal of diagnostic tools because they can detect substances in miniscule amounts and measure them with great accuracy.

2.4. Gene Transfer

Gene transfer is slop ahead of rDNA technology and in this process a pieces of rDNA (gene) is transferred into the host cell, using particle bombardment technology. Invention of this technique enabled scientists to genetically modify cereal plants.

2.5. Biosensors

Biosensors are composed of a biological component such as a cell or antibody linked to a tiny transducers. They are detecting devices that rely the specificity of cells and molecules to identify and measure substances at low concentrations.

3.0. APPLICATION OF BIOTECHNOLOGY IN FOOD PROCESSING

Biotechnology as technique or as process has numerous application in food processing area to deliver nutritious, wholesome, safe and convenient food products. These applications are summarized in brief here under:

3.1. Improvement of Raw Material Quality

Traditional breeding methods were aimed to improve the quality of plant products for their efficient processing. The modern biotechnological methods have been utilized to manipulate the macromolecular composition of cereal grains, oil seeds, tuberous crops for delivering a excellent quality value added products. The bread making ability of wheat flours have been improved by modifying the gluten content and it is endowed to the transgene responsible for high MW proteins production into wheat varieties. Similar kind of experimentation is in progress to alter the amylose and amylopectin fractions in rice grain to enhance its cooking quality as well as processing characteristics. Barley varieties have been targeted to produce good quality malt for infant foods as well as for production of beer, having novel aroma compounds. Seed proteins confer various functional characteristics like solubility, gelling, foaming, whipping, emulsifications in processed food products. Through protein engineering in conjunction with rDNA technology the attempts are in progress to enrich these seed proteins.
proteins/protein fraction with desired functionality, like wise production of raw fruits and vegetables with high total soluble solids, higher phytochemical contents, enhanced pigments content, and with desired degree of enzymatic activity is also currently in progress to not only improve pre but post harvesting qualities. High starch content in potatoes produce low fat and crisp products like chips, French fries and through gene alternation high starch containing potato plants have been evolved. Rapeseed has also been modified to produce a high temperature frying oil, low in saturated fat. Improvement in processing characteristics is mainly achieved through employing modern biotechnological tools as they are quick and reproducible.

3.2. Enhanced Nutritional and Therapeutic Products

Traditional “bio processes” have been known to improve the nutritional and therapeutic properties of resultant products. Fermented food products are integrated part of daily diet around the world for their easily digestibility and ability to enhance the absorption of nutrients particularly vitamins and minerals. A myriad of such fermented food products have been documented in various scientific literatures. A part from this, fermentative organisms also release certain metabolites with unique “probiotic” attributes. “Probiotic, food products in generals and “probiotic “ organism in particular are in the center of current R & D activities all over the world. “Functional foods” segment that is registering a steady and consistent grown at present, among processed food products, gathered the momentum primarily from the scientific investigations based on “probiotic” food products. Research activities are targeted to identify and develop novel microorganisms with enhanced life promoting attributes.

Exploitation of lactic acid bacteria (LAB) as potential oral vaccines to deliver antigens at mucosal surfaces have been recommended to develop immunity, Probiotic microorganisms have been identified as major weapon against gastrointestinal/disorders, believed to be one of the mortality agent in developing nations. Viability of these probiotic organisms during food processing operations is of great concern and new products in dried “ready-to-deliver” forms have been under investigation. Biotechnology and conventional plant breeding are both needed for successful modification of oilseeds to improve their nutritional quality. Laurical Canola oil was the first commercialized genetically modified vegetable oil introduced in US market in 1995 and though this product was meant for soap and chemical industry, it has triggered a series of research activities mainly to improve the fatty acid profile with enhanced nutritional attributes like high unsaturated fatty acids, low trans fatty acids etc. Presence of antinutritional compounds like oxalates in spinach, β-oxalylamina acid in lathyrus, gassypol in cottonseeds has been viewed as major problem in effective utilization of otherwise nutritious food. Through gene manipulation, it may be possible to remove or bring down the level of these compounds. One of the primarily goal of modern biotechnology is to produce nutritious staple food crop like β-carotene rich golden rice, iron and zinc enriched rice. However, all such novel approaches have to undergo much scrutiny before being commercialization.

3.3. Biopreservation of Food Products

Traditional fermentation processes apart from providing palatable nutritious product also increase their shelf-life. This forms the principle of “Biopreservation, involving microorganism or their metabolite for enhancing the safety and microbial stability during storage. LAB produced a group of compounds referred as “bacteriocin” and these compounds exhibit antagonistic properties.
against many closely related microorganisms, LAB also generate metabolites like acids, hydrogen peroxide, ethanol, acetaldehyde, diacetyl, carbon dioxide that inhibit the growth of undesirable microorganisms particularly enteric bacteria. Bacteriocins are small molecular weight single or complex proteins or proteinaceous substances and are the major thrust area of research for the production of bacteriocins from food grade starter bacteria. Some of the bacteriocins, organism producing them and their antimicrobial spectrum is depicted in Table-1.

**Table 1: Bacteriocin Producing Lactic Acid Bacteria**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Bacteriocin Type</th>
<th>Antibacterial Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium</em></td>
<td>Bifidin</td>
<td><em>E. coli, other pathogens</em></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> spp.</td>
<td>Nisin</td>
<td><em>Micrococcus, Listeria, staphylococcus, Bacillus, Clostridium, etc.</em></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> spp.</td>
<td>Diplococcin</td>
<td>Wide Range of gram positive</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>Lactobacillin/Lactobacrin</td>
<td><em>L. brevis &amp; other related strains of LAB.</em></td>
</tr>
<tr>
<td><em>Lactobacillus plantarium</em></td>
<td>Lactolin/plantericin</td>
<td><em>L. brevis, L. acidophilus, L. viridescens, L. xyloses, E. faecalis, etc.</em></td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>Lactocidin/acidolin.Acidophil/Lactocin</td>
<td>Narrow spectrum, antibiotic-* L. leichmann, E. coli, L. helvaticus, etc.*</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em></td>
<td>Helveticin</td>
<td><em>Lactobacillus</em> ssp. of closely related strains*</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>Mesenterocin 5</td>
<td><em>Enterococcus, Micrococcus, Listeria spp. Staphylococcus, etc.</em></td>
</tr>
<tr>
<td><em>Pediococcus acidolactis</em></td>
<td>Pediocin A/Ac.H</td>
<td><em>B. cereus, Listeria spp. Staph. aureus, Clostridium perfringens. Cl. botulinum, Pediococcus, Leuconostoc, etc.</em></td>
</tr>
<tr>
<td><em>Propionibacterium jenseni</em></td>
<td>Jensenin G</td>
<td>Limited inhibitory effect-<em>Propionibacterium</em> and related strains.*</td>
</tr>
</tbody>
</table>

Inherent qualities like stability over wide processing conditions, longevity and digestibility by proteolytic enzymes conferred them an ideal biopreservatives. Nisin, an antibacterial polypeptide produced from *Lactococcus lactis* and active against gram positive bacteria and is used to inhibit the growth of *Clostridium botulinum*. It is the only food preservatives of bacterial origin permitted by regulatory agencies and have been successfully tested with certain Indian food products like *Kheer* (Table-2).

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Table 2: Effect of Nisin in Food System

<table>
<thead>
<tr>
<th>Product</th>
<th>Nisin Concentration</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>100-1000 RU/gm</td>
<td>Extended the shelf life of pasteurized milk from 6-7 days to 10 days at 6°C.</td>
</tr>
<tr>
<td>Milk</td>
<td>100 mg/ml</td>
<td>Shelf life extension 2-12 days at 25°C</td>
</tr>
<tr>
<td>Sterilized Milk</td>
<td>100 RU/gm</td>
<td>Shelf life extension from 3-5 days to 60 days at 37°C</td>
</tr>
<tr>
<td>Sterilized Milk</td>
<td>500-1000 RU/ml</td>
<td>Complete inhibition of clostridium perfringences</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>2500 IU/ml</td>
<td>Inhibition of Listeria monocytogenes</td>
</tr>
<tr>
<td>Cheese</td>
<td>500 IU/gm</td>
<td>Inhibited the growth of non-starter LAB for one month</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>25 IU/gm</td>
<td>Enhance shelf life of 10 days at 7°C</td>
</tr>
<tr>
<td>Dahi</td>
<td>1000 IU/gm</td>
<td>Effective in controlling lipolysis in Dahi during storage at 25°C</td>
</tr>
<tr>
<td>Dahi</td>
<td>15 RU/gm</td>
<td>Retain desirable characteristics up to 35 days at 15±1°C</td>
</tr>
<tr>
<td>Kheer</td>
<td>200 IU/gm</td>
<td>Lowering of the thermal processing time &amp; enhancing shelf time for six months</td>
</tr>
<tr>
<td>Nisin</td>
<td>Conjunction with potassium sorbate</td>
<td>Shelf life extension</td>
</tr>
</tbody>
</table>

Most of the recent research with Nisin has focused on increasing production and improving the antimicrobial efficiency of the compounds. This has encouraged the prospects of developing designer Nisin molecule and there is need of synergistic studies too.

3.3. Production of Biocatalysts

Enzymes are proteinaceous substances and capable of altering the rate of chemical reactions without being must upon the completion of reactions. More than 3000 enzymes catalyzing a wide array at reactions are known to exist. In food processing area, these enzymes are involved in number of breakdown and biosynthetic reactions with profound effect on product’s sensory, nutritional and therapeutic attributes. These enzymes are obtained from animals, plants and most notable from microorganisms (Table-3). Enzymes have many advantages over their chemical counterparts in their specificity and high catalytic powder. Microorganisms are allowed to grow on various substrates for producing these biocatalysts which is extracted, isolated, purified and sold as commercial preparation. The immobilization also known as pimaricin is produced by streptomycetes natalensis and active against yeasts, molds and actinomycyes. It is nontoxic antibiotic approved by FDA and joint/WHO expert committee as food additive. It is applied to the surface of cuts and slices of cheese to inhibit mold spoilage (21 CFR 172.155).

Propionibacteria produced products have been commercially available as Microgard ® for use in Cottage cheese and Nomold ™ a powdered preparation for baked products. These microorganisms or their metabolite have immense potential in preserving the minimally processed fruits and vegetables as well raw meat. Such biopreservatives may also be used as “hurdle” in
combined preservation methods of a number of raw and processed food products of microbial cells or enzymes by fixing them on some inert and insoluble polymer matrix, for food applications has been generated immense popularity among researchers as well as among processors. However, utilization of immobilization teaching in complex multistep fermentation processes or biosynthetic reactions is still under investigation. Enzymes may also be entrapped or encapsulated to improve their specificity. These are several areas where enzymes have played a very important role. The ripening of cheeses, lactose hydrolysis in whey, in dairy sector had been thoroughly investigated, whereas enzyme added fruit juice extraction, fruit juice clarification, de-bittering of citrus juices are employed on commercial scale in fruits and vegetable processing industries.

Currently available biotechnological techniques may contribute significantly by modifying the structure of an existing enzyme to improve its functionality, stability and for their large scale production. The protein engineering technique has opened the ways for design of new production and recovery strategies. Development of genetically modified organisms (GMO’s) through rDNA technique, with copies for producing desired enzyme(s) is the major area of investigation. First such commercial enzyme ‘chymosin” has been in application in many western countries for cheese making. The GM chymosin is quite similar to ‘rennet’, traditionally obtained from calf stomach. The aspect of GMO’s utilization for enzyme production seems alterative to reduce most, ensure consistent supply and eliminate variability.

4.0. SUGGESTED REFERENCES


1.0. INTRODUCTION

Biotechnology’s long term promise also incorporates elements of humanitarian reasoning. Concerns about global population growth and stress on natural resources means there is a great need to not only improve production on currently cultivated land but also to begin growing food on less attractive cultivated land. Recent advancement in recombinant technology offer the opportunity not only of better controlling existing food processes, but also of developing entirely new approaches to quality control and food product development. The potential of this new technology is enormous and its true economic impact has yet to be realized.

Bioengineering or genetic engineering refers to new methods of breeding plants, animals, or microorganisms, by introducing a copy of gene for a specific trait. The genes can be copied from any organism. The manipulation of genetic traits of animals and plants is not a new concept. Selective breeding has been practiced for centuries, well before the molecular of the procedure was understood. Genetic engineering is different from traditional breeding methods:- in achieving the desired results much more quickly and its predictability, crossing the species barrier and reducing the random nature of classical breeding.

2.0. GENETIC MODIFICATION TECHNIQUES

Genetic engineering involves the manipulation of genes at cellular and molecular levels. Using techniques of genetic engineering it is now possible to isolate genes of interest from one kind of organism incorporating them into other, thus resulting in stable change in the genetic make-up of recipient organism. The resulting organisms are termed as Genetically Modified Organisms (GMOs) or transgenic plants/animals. The genetic modification techniques are also referred as recombinant DNA technology (r DNA), or molecular cloning. Recombinant DNA technology is a process that involve following major steps.

- Isolation of one of the gene responsible for desired traits
- Breakage of DNA into fragments by site-specific enzyme called restriction endonuclease
- Linkage of DNA segment into vector DNA (mainly plasmid) through ligase
- Transformation of recombinant DNA into host plant.
- Multiplication of implanted gene and expression of traits
For creating transgenic organisms, availability of a transgene that confer the required trait is foremost pre-requisite. Transgene are patented or may be synthesized if the sequence information is available. Besides the transgene the gene cassette consists of a ‘promoter’ and ‘terminator’ gene. The ‘promoter’ gene is essential for recognition and regulation of expression of transgene(s) by the host organism. The most commonly used promoter gene in plant cells is cauliflower mosaic virus 35S promoter. Similarly terminator sequence frequently used one is nos3, isolated from nopaline synthase gene from *Agrobacterium tumifaciens*. Selectable marker genes, such as that exhibit antibiotic resistance are often co-transformed with the actual trait gene(s). The use of these antibiotic resistance marker sequences has generated a lot of controversy regarding the biosafety. Hence attempts are in progress to either eliminate them after the development of transgenic organisms or use the selection techniques based on non-toxic compounds such as mannose. The process involve in genetic transformation is illustrated in Fig.1.

**Fig.1: Steps involved in Genetic transformation through rDNA Technology**

Gene transfer is a technique that transfers a piece of foreign DNA into a cell so that it became a permanent part of the genome. The two most used gene-transfer systems in higher plants are DNA-mediated and *Agrobacterium*-mediated.

*Agrobacterium tumifaciens* is a soil bacterium, induce tumor development in wounded plants and cause crown gall disease. The transformation system is based on ‘disarmed’ Ti plasmid with genes responsible for tumor formation being removed. The foreign DNA is confined by the left and right border sequences (25 basepairs each); these are the only elements from *Agrobacterium* transferred together with the T DNA.

Other transformation methods are based on physical and chemical principles. The physical method is based on particle bombardment technique known as “biolistics”. In this method DNA-coated microprojectiles, usually made of tungsten or gold, is directly into plant cells using microparticle gun. The chemical methods make use of polyethylene glycol (PEG) or CaCl$_2$ to facilitate the entrance of foreign DNA through the plant cell wall. Electroporation and sonication
assisted transformation may also be used to transform DNA. The most commonly used method is still *Agrobacterium*-mediated one followed by particle techniques.

### 3.0. PRESENT WORLD SCENARIO OF GENETICALLY MODIFIED FOODS

The use of biotechnology in agriculture and related areas has gained momentum in last few decades owing to multifaceted orientation including production, quality improvement and nutritional improvement. The initial efforts of plant biotechnology were focused on improving the agronomical traits mainly to ensure food security for ever-growing world population and then efforts were soon diverted to improve the pre as well post harvest characteristics, nutritional and processing characteristics. There is also notable success in the field of enzymes, food ingredients, and safety of processed foods through r DNA techniques.

The genetically modified crops were introduced throughout the 1990s in the United States and were rapidly accepted by farmers in US as well as other parts of the worlds, notably Canada and Argentina (Table-1). Already GM crops are cultivated 50 million hectares of land (Ravishankar and Suresh, 2002). Among the Asian countries China has permitted the commercial cultivation of cotton covering area of 1.2 million acres.

#### Table-1: Worldwide Crop Production Area of GM Crops

<table>
<thead>
<tr>
<th>Transgenic crop production area by country</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Country</strong></td>
</tr>
<tr>
<td>USA</td>
</tr>
<tr>
<td>Argentina</td>
</tr>
<tr>
<td>Canada</td>
</tr>
<tr>
<td>China</td>
</tr>
<tr>
<td>South Africa</td>
</tr>
<tr>
<td>Australia</td>
</tr>
</tbody>
</table>

Two major traits i.e. insect tolerance and herbicide resistance have been engineered into 4 major crops i.e. soybean, corn cotton and canola. At present all over the world 51 per cent of soybean, 20 per cent cotton, 12 per cent canola and 9 per cent corn production are obtained from GM crops. In the year 2000 63 per cent soybean, 64 per cent cotton and 24 per cent corn grown in USA were agro biotech varieties, and it has been estimated that 60-70 percent of all food products in the US contain biotech ingredients.

The 69.4 percent GM crops possess the transgene for herbicide resistance and major crop is soybean. Weed control is one of the biggest challenges in crop production, because weed reduces yield as well as quality. However, weed management through herbicide resistance transgenic
plants has many advantages. About 22 percent food or cash crop that was cultivated in the year 2000 contained Bt gene for insect resistance, whereas 7.2 percent crops were resistance for both herbicides and insects. Rests were with transgenes for other attributes mainly for virus resistance in papaya. Among the horticultural crops farmers have grown genetically modified tomato, papaya, potato and squash. Approximately 19 genetically modified enzymes have been already developed and may be used in food processing industry. In USA 40 per cent of the feed crop have been developed through biotechnology. Biotech crops are considered “not significantly different” from the conventional varieties and its feeding to animals was found safe.

4.0. PRESENT STATUS OF GENETICALLY MODIFIED ORGANISMS IN INDIA

Genetically Modified Organisms (GMOs) are under experimental phase in India. Though number of R&D organizations are conducting laboratory investigations to develop transgenic plants and animals. Mahyco an Indian seed company has introduced Bt cotton initially for commercial cultivation. On March 26, 2002 Indian government had granted permission to grow Bt cotton and last year it was grown on approximately 40,000 hectare land in six states. Field trials indicated 80 per cent increase in crop yield with 70 percent reduction in pesticide application. Advancement in the area of transgenic plant is presented in Table-2.

Table-2: Status of Transgenic Developments in India

<table>
<thead>
<tr>
<th>Organization</th>
<th>Material</th>
<th>Transgene</th>
<th>Objective and Progress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delhi University South Campus, New Delhi</td>
<td>Mustard</td>
<td>Bar, Barstar, Barnase</td>
<td>Plant transformation completed and ready for green house studies Transformation completed</td>
</tr>
</tbody>
</table>

Figure 2: Global Scenario of GM Crop Adoption and Production

16th CAS Course on “Application of Biotechnology in Dairy and Food Processing” (4th Nov. to 24th Nov., 2003), NDRI, Karnal-132 001
<table>
<thead>
<tr>
<th>Institution</th>
<th>Crops</th>
<th>Gene Expression</th>
<th>Transformation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNU, New Delhi</td>
<td>Potato</td>
<td>Amaranthus gene <em>Ama-1</em> for enhancement of lysine in seed protein</td>
<td>Transformation completed and transgenics under evaluation.</td>
</tr>
<tr>
<td>IARI, New Delhi</td>
<td>Rice</td>
<td>Bt toxin gene</td>
<td>Lepidopteran resistance and transformation is in progress.</td>
</tr>
<tr>
<td>TNAU, Coimbtore</td>
<td>Rice</td>
<td>Reporter genes like <em>hph</em> or <em>gus A</em> and GNA gene</td>
<td>To study the extent of transformation in greenhouse.</td>
</tr>
<tr>
<td>Mahyco, Mumbai</td>
<td>Cotton, Brinjal</td>
<td>Herbicide resistance gene <strong>Cry 1Ac</strong></td>
<td>Limited field trials in two locations completed. Insect resistance greenhouse trial completed.</td>
</tr>
<tr>
<td>NBRI, Lucknow</td>
<td>Fruits &amp; Vegetables</td>
<td>Antisense gene for ACC synthase</td>
<td>Transformation work is in progress.</td>
</tr>
<tr>
<td>Rallis India Ltd.</td>
<td>Chilli, Tomato</td>
<td>Snow drop lectin gene</td>
<td>Insect resistance. Transformation experiments in progress.</td>
</tr>
</tbody>
</table>

The first experimentation on transgenic plants was started in 1995 when Brassica juncea plant containing *barnase*, *barstar* and *bar* genes were planted at Gurgaon. Transgenic mustard has shown 200 per cent increase in seed and oil yield in comparison to conventional variety. However, the Genetic Approval Committee (GEAC) has held up the decision to grant approval for commercial production.

5.0 COMMERCIALIZED GENETICALLY MODIFIED FOODS

Private industries and academic sectors have been working together along the same lines for number of years. As a result of this a wide variety of products are available on the shelves of developed countries and are at the doorsteps of third world nations. Some of these breakthroughs are discussed here in various fields.

5.1 Genetically Modified Soybean

Soybean is admired all over the world because of its high quality proteins, high unsaturated fats and other essential nutrients. Recent investigations revealed the presence of wide array of phytochemicals most notably isoflavones, with proven disease preventive attributes. All these scientific research made the soybean a leading item in functional food segment. Soybean is used in the preparation of wide variety of traditional and processed food products including soymilk, tofu,
soy sauce, extruded snacks and many other fabricated foods. The soybean has been genetically engineered for herbicide resistance and for producing high oleic acid oil. Herbicide resistance transgene provide resistance against herbicides like Roundup (glyphosphate) and Liberty (glufosinate). These are broad spectrum herbicide, breakdown easily in soil, eliminate residue carry over problems and minimize adverse impacts on environment, hence farmer can use them round the year without affecting the soybean crop. Two commercial varieties Roundup ready (RUR) and Libertylink are available for commercial crop production with herbicide resistance. The enolpyruvinylshikimate-3-phosphate synthase (EPSPS) gene from Agrobactrium sp. StrainCP4 was utilized to modify conventional soybean for resistance against glyphosphate. In 1997, DuPont has introduced modified soybean oil with the trade name Optimum with high oleic acid. The genetic modification of soybean was achieved by sense suppression of the GmFad 2-1 gene, which encodes a delts-12 desaturase enzymes and this particular enzymes desaturate oleic acid at δ-12 positions resulting in high (>76%) oleic acid in oil.

5.2. Genetically Modified Corn

Corn is one of the major staple food crops especially in third world countries and processed as flour, breakfast cereals, starches, corn syrups and oil. Corn had been targeted for many genetically modified events like insect resistance, glyphosphate resistance, male sterility and glufosinate tolerance. Maximizer™ and Natureguard™ are two commercial corn varieties released in 1996 and exhibit insecticidal activity due to presence of Bt gene. The natural toxin produced by Bacillus thuringiensis confers resistance against the attack of many insects’ particularly European corn borer, corn earworm and southwestern corn borer. Bacillus thuringiensis spores contain a crystalline protein (Cry) and in the gut of insect the protein breakdown and release a toxin, known as δ-toxin. This toxin binds with intestinal wall and creates pores in the lining, resulting in ion imbalance, paralysis of the digestive system and ultimately death of insect. A number of Cry genes (Bt gene) have been identified and were found to have lethal effect against different orders of insects.

Table-2: Different Cry Genes and their Toxicity

<table>
<thead>
<tr>
<th>Cry Gene Designation</th>
<th>Potential Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry 1A(a), Cry 1A(b), Cry 1A(c)</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>Cry 1B, Cry 1C, Cry 1D</td>
<td>Lepidoptera</td>
</tr>
<tr>
<td>Cry II</td>
<td>Lepidoptera, Diptera</td>
</tr>
<tr>
<td>Cry III</td>
<td>Coleoptera</td>
</tr>
<tr>
<td>Cry IV</td>
<td>Diptera</td>
</tr>
<tr>
<td>Cry V</td>
<td>Lepidoptera, Coleoptera</td>
</tr>
</tbody>
</table>

Improvements in nutritional status of maize protein have been tried by engineering the lysine-insensitive form of maize dihydrodipicolinate synthase causing accumulation of lysine in cell-culture.
5.3. **Genetically Modified Fruits and Vegetables**

Fruits and vegetables are important constituents of our diet. Fruits and vegetables, which remain fresh on shelves and full of nutrients especially micronutrients fetch premium price in the market. The traits with greatest influence on product throughput and finished product quality are the solid contents, its chemical composition and excellent storability. Seed companies with bioengineering capabilities have already begun the research in this direction. Few products have already hit the market, like Flavr Savr™ tomatoes, New leaf™ potato, Freedom II™ squash.

5.3.1. **Genetically Modified Tomatoes**

The first food product of modern biotechnology introduced in market for sale was Flavr Savr™ tomato, developed by Calgene, Inc. Davis, California in 1994. GM tomatoes stay firm after harvest because through gene manipulation, the formation of polygalacturonase (PG), a pectic enzyme naturally occurs in tomatoes, is suppressed. The activity of PG is suppressed by introducing into the plant a reverse copy called antisense gene of the tomato DNA that produces the PG enzyme. This allows tomatoes to remain on vine for complete development of colour and flavour without any adverse textural changes. The modified tomatoes have been processed into puree that is also available commercially. The GM tomato puree has been found to contain higher pectin, lycopene than conventional ones. Laboratory trials have been reported to successfully modify the tomatoes for insect resistance delayed ripening and improved ripening.

5.3.2. **Genetically Modified Squash**

Freedom II™ squash is an early entry in market with virus resistant transgenes. Yellow crookneck squash had been engineered for resistance against zucchini mosaic virus (ZYMV) and watermelon mosaic virus II (WMVII). The transgenes for resistance against above mentioned viruses are coat protein genes of cucumber mosaic virus, watermelon mosaic virus and zucchini yellow mosaic virus.

5.3.2. **Genetically modified Potato**

Commercially available GM potato is characterized by presence of transgene for insect resistance and Monsanto is selling this GM variety as New Leaf potato. Potato processed into French-fries and chips and starch content is one of major factor known to have influence on the quality attributes of processed products. Thus enhancement of starch synthesis was targeted as a mean to increase the dry matter content. Rate limiting step in starch biosynthesis is catalyzed by enzyme ADP glucophosphorylase and the activity of this enzyme is controlled by the metabolic state of cell. An unregulated ADP glucophosphorylase has been identified in Escherichia coli and it leads to over accumulation of glycogen. Gene glgC16 was isolated and inserted into improved potato variety that on an average contain 24 per cent higher levels of dry matter relative to control. Transgenic potatoes possess rapid reconditioning ability and showed better cold tolerance.

5.4. **GENETICALLY MODIFIED DAIRY PRODUCTS**

Majority of the biotechnological works to date have been confined to improvement of milk production system. Varietal improvement through r DNA technology is widely accepted practice all over the world. Another important biotechnologically derived product called BST hormone has been banned in majority of countries. Recent innovations of genetic engineering in the area of
dairy starter cultures, has made it possible to enhance the sensory, nutritional, and therapeutic attributes of fermented dairy products. Starter cultures used in dairy fermentations typically belong to a family of bacteria collectively known as the lactic acid bacteria (LAB), describes a collection of functionally-related organisms composed of up to 12 different genera. The starter cultures have genetically modified for phase resistance, probiotic attributes and production of secondary metabolites including flavouring compounds.

The first genetically modified product approved by FDA in 1990 was ‘chymosin’, an enzymes used in cheese manufacturer to coagulate milk. Natural ‘chymosin’ obtained from calf stomach lining became limited in supply and moreover being of animal origin cheeses made from it is considered as ‘non-vegetarian’. The advent of modern biotechnology has enabled researchers to create unlimited, cheaper and more consistent supply of chymosin through GMO,s. This chymosin is 40-50 percent cheaper than conventional enzyme preparations. Calf chymosin was one of the first mammalian enzymes, which was cloned and expressed in microorganisms. Many different laboratories have cloned the gene for calf chymosin in E. coli and analyzed the structure of the gene as well as properties of the recombinant chymosin. The gene for prochymosin has also been cloned in E. coli K12, Saccharomyces cerevisiae, Klyveromyces marxianus var. lactis, Aspergillus niger ssp. awamori and Trichoderma reesei. Some of the genetic modification events and their potential benefits are summarized in Table-3.

### Table-3: Genetic Modification Events in Dairy Processing

<table>
<thead>
<tr>
<th>Genetic Modification</th>
<th>Altered Gene</th>
<th>Potential Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation of Lactose fermentation and Protease activity</td>
<td>‘Lac’ gene</td>
<td>Shelf stable fermented product without problem of high acidity and bitterness.</td>
</tr>
<tr>
<td>Gene incorporation of extracellular polysaccharides</td>
<td>EPS+ synthesis</td>
<td>Creamier yoghurt and Dahi without syneresis.</td>
</tr>
<tr>
<td>Starter modification for L-Alanine production</td>
<td>L-alanine dehydrogenase gene.</td>
<td>Sweeter fermented dairy products</td>
</tr>
<tr>
<td>Increased diacetyl production</td>
<td>α-Acetolactate decarboxylase gene</td>
<td>Increased accumulation of diacetyl precursor α-Acetolactate</td>
</tr>
<tr>
<td>Modified probiotic cultures</td>
<td>Idh D-lactate dehydrogenase</td>
<td>Prevent formation of D-lactate and hence check acidosis and encephalopathy</td>
</tr>
</tbody>
</table>

### 5.5. Genetic Modification of Fats and Oils

Like, protein, carbohydrates, and water, fats and oils are a major nutrient. Yet, over the years, they have become the number one concern for consumers. It has forced to both, food technologists and plant breeders to do something and that has been with a common goal to modify their fatty acid composition. However, there is fundamental difference in approaches used between food technologists and plant breeders.
Nutritive value, functionality and oxidative stability are the three quality parameters, forming a quality triangle. It is apparent that a conflict exists within the quality triangle and the major challenge is to develop oils that meet a specific nutritional requirement and at the same time possess certain functional characteristics as well as oxidative stability. Biotechnology has been extremely active in developing plants, animals and microbial sources of special fats. Better information about the metabolism of special fats such as omega-3 and –6 lipids if is known, the tools to produce more of these specialties are at hand (Katz, 1996). Through gene manipulation, enzymes involved in fatty acid synthesis were targeted to alter the fatty acid composition. Currently, genetically engineered soy bean, rapeseed, sunflower oils are available in the market. Genetically enhanced soybeans that are lower in saturated fats, higher in oleic acid offer better frying stability without further processing. Soybean and canola oils with more stearic acid content provide desired plasticity to margarine and shortenings, without hydrogenation and make it healthful.

6.0. PROSPECTIVE GENETICALLY MODIFIED EVENTS

Despite apprehension among consumers and regulatory authorities biotechnological research for the betterment of food processing sector is continuously going on. Some of the promising innovations have been submitted for approval from regulatory authorities, while some others are in experimental phase. These are discussed here.

6.1. Golden Rice

A group of scientists at Swiss Federal Institute of Technology, Zurich has genetically engineered a more nutritious type of rice by inserting three genes that produce β-carotene. Popularly termed as “Yellow Rice” this could help in alleviation of vitamin A deficiency disorders, currently affecting 124 million children world-wide. First time researchers were able to introduce a complete biosynthetic pathway through their 8 years efforts. This group also tried to develop iron rich rice, but the project was terminated since rice naturally prevents its absorption in the gut.

6.2. Genetically Modified Wheat for Bread

Bread making quality of wheat flour largely depends on the viscoelastic properties conferred to wheat dough by the gluten proteins. These proteins are of high molecular weight glutenin sub-units and provide elasticity to dough. Wheat varieties differ in amount and composition of gluten protein and it reflects on bread-making ability of wheat flours. Introduction of extra copies of high MW glutenin genes into the popular wheat variety ‘Bob white’ has resulted in more proportion of high MW proteins that provide the extensibility needed for good bread. This GM wheat is also subjected to approval.

6.3. Genetically Modified Organisms (GMO’S) as Food Processing Aid

Fermentation process and the quality of finished products is greatly influenced by the functionality of microorganisms involve in the process. Limited enzymic activity of malted barley and limited fermentative ability of brewing yeast, cause about 20 per cent carbohydrates released into the wort remains in the beer, mostly as dextrins. To produce low carbohydrate beer commercial enzyme preparations comprising glucoamylase are often added to either worts prior to
boiling with hops or to fermentative beer. However, proteinases present in these commercial enzyme preparations have deleterious effects on beer quality particularly its foaming property. Using amylolytic yeasts can solve this problem. Attempts have been made over the years to produce amylolytic yeasts by conventional genetic means. Initial results were not very encouraging, but recombinant DNA techniques have enabled these difficulties to be overcome. The STA2 gene produce an extra cellular glucoamylase was cloned from *Saccharomyces cerevisiae var. diastaticus* and has been inserted into recombinant plasmid Pdvk2. The yeast strain NCYC1234 (pDVK2) is the outcome in this direction. Likewise, baker’s yeast was genetically engineered to utilize maltose, by encoding for an enzyme maltose permease that could speed up the leavening process in breads. Researchers in U.K. produced this transgenic strain by simply rearranging and duplicating certain yeast genes.

### 6.4. Other Prospective GM Foods

A good amount of work has been devoted to improve the nutritional quality of conventional food products through genetic engineering. Reduction in anti-nutritional elements in foods like cotton, peanut, wheat is under investigations. To reap the rich dividend in the functional food segment many phytochemical producing genes have been targeted to enhance the level of these protective agents like glucosinolates in brassica, flavonoids, carotenoids in fruits & vegetables. Transgenic fish (Salmon) has been developed by a US based company called Aqua Bounty Farms and this fish grows faster than natural salmon. Recent studies indicated that major genes for meat quality could offer excellent opportunity not only for increasing the level of meat quality, but also decreasing variability. A gene CALCA was found to be involved in the regulation of calcium levels, a key factor in postmortem tenderization.

### 7.0 SAFETY AND REGULATORY ASPECTS OF GM FOODS

Large ranges of crops, genetically modified organisms, ingredients and enzymes have now been produced using biotechnological tools, although most of these products have yet to reach the stage of commercial development. In future, the GM foods are expected to offer consumers many benefits including improved choice, quality, and flavor and keeping qualities, at a lower price. May countries around the world are keen to benefit from modern biotechnology. At the same time there is concern about the need for appropriate safeguard to protect human health and the environment. Despite the development of GMOs in last decade only few of them have reached to commercial level. The multifactorial reasons may include:

- Lack of any real or perceived consumer benefits from GM foods.
- Doubtful intentions of the multinational biotechnology giants.
- Mistrust of the government.
  - Conflicting messages from the scientific community.
  - Potential lack of choice
  - Fear of unknown risks out weighting potential benefits.

Besides these the potential environmental impact of GM crops on both on conventional crops and on nature and ethical concerns, are responsible for slow acceptability. Some of the risks, which may be associated with GN foods, include:
7.1. Potential Allergenicity

One scientific issue related to the transfer of genetic material between organisms that requires particular attention is the possibility of the introduced protein in the new food could cause allergenic reactions in the sensitive individuals. This is especially true for the genes derived from foods that commonly cause food allergy. These foods include milk, egg, fish, seafood, tree nuts and legumes and these accounts for 90 per cent of food-allergic reactions.

7.2. Altered Nutrient Composition

Though genetic modification there may be possibility of alteration in nutrient and toxicants (i.e. anti-nutritional factors) levels than normal.

7.3. Antibiotic Resistance Marker Genes

Selective marker genes that encode resistance to anti-biotic are frequently used in the development of transgenic plants and microorganisms. It is important to ensure that these marker genes will not spread to pathogenic microorganisms leading to a significant increase in resistance to clinically important antibiotics. Care must also be taken to ensure that antibiotics that inactivate enzymes and that are present in food will not significantly reduce the efficacy of orally administered drugs.

In USA, transgenic plants or other products undergo tight scrutiny before released for commercial purpose. FDA (Food and Drug Administration), EPA (Environmental Protection Agency), FIFRA (Federal Insecticide Fungicide and Rodenticide Act), APHIS (Animal and Plant Health Inspection Service) and TSCA (Toxic Substances Control Act), are the agencies that regulate the GM crops and animals. Similarly in other countries also there is provision in laws to keep strict vigilant for such products.

8.0. SUGGESTED READINGS


www.bio.org
www.foodsafety.gov
www.greennature.com
www.ifis.com

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16th CAS Course on “Application of Biotechnology in Dairy and Food Processing” (4th Nov. to 24th Nov., 2003). NDRI, Karnal-132 001
1.0. INTRODUCTION

Several unit operations employed in dairy/food industry require product contact surfaces to be cleaned after each processing schedule to maintain hygiene and good product quality. Their required application of a specific sequence of chemical agents and sterilants at specified time-temperature combinations. Chemical cleaning agents and sanitizers often used together to attain the twin objective of cleaning and sanitization. Various builders, silicaser, surfactants and other detergent adjunct are the main components of detergent formulations. Complex phosphates particularly, sodium tripolyphosphate (STPP) have remained choice detergents for dairy and food industry for many years. Their only limitation is that they are unstable in hot solutions and in the presence of strong alkalies.

Dairy and food processing equipments employed in thermal processing operations pose most serious challenge for efficient cleaning. One of the most difficult problems is removal of proteinaceous milk stones formed during pasteurization and UHT sterilization of milk. Adoption of new processing operations know-how viz. Ultrafiltration (UF) and reverse osmosis (RO) offer a few dimension to cleaning problems. The membranes employed in these operations are susceptible to fouling leading to blockage of membranes pores. Synthetic detergent formulations are not efficient in handling these specific problems of the dairy and food industry. Furthermore, these detergents are corrosive, toxic and non-biodegradable. Extensive uses of synthetic detergents result in increased BOD levels in dairy food plant effluents and often lead, to formation of slumps in the area adjacent to these plants. They therefore pose serious ecological problems and enzyme-based detergents are fast emerging an alternative to synthetic detergents owing to their biodegradability, low-toxicity, non-corrosiveness, environmental-friendliness, enhanced cleaning properties, increased efficiency and stability in different formulations. They are therefore also being referred as “green chemicals”.

2.0. DEVELOPMENTS IN ENZYME-BASED DETERGENTS

Genesis of enzyme based detergents dates back to 1913, when Otto Rohm obtained a patent for using pancreatic enzymes as washing aids for laundry cleaning. The product was marketed under brand name ‘Burnus’ as a presoak detergent. Several improved pancreatic products were developed and marketed during the middle of 20th century. These products however suffered from the disadvantage of optional activity and instability at neutral pH. A major breakthrough was achieved in mid 1960’s when NOVO Industry introduced an alkaline
proteinase preparation produced by *Bacillus licheniformis*, which was stable and active at an alkaline pH of 8-10. The product was named ‘Alkalase’. Several other proteolytic enzyme based formulations followed in the subsequent years. In the 1980’s, cellulase and lipase based detergents formulations, were introduced in the market. Today, modern biotechnological tools are being applied for genetic and protein engineering to develop new generation enzyme preparations for better functionality and performance. The new biodetergent formulations could be based on one or a combination of enzymes: proteinases, amylases, lipases and cellulases. These hydrolases target homogeneous biopolymers like proteins, carbohydrates, lipids and cellulose. The following table summarizes the specific substrate and mode of action of the above listed enzymes.

### Table-1: Hydrolase Enzymes and their Substrate

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Enzyme classification</th>
<th>Substrate</th>
<th>Natural Source</th>
<th>Reaction Catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Protease</td>
<td>Proteins, polypeptides</td>
<td>Proteinaceous food, milk, meat products and cheese</td>
<td>Hydrolysis of amide or peptide bond</td>
</tr>
<tr>
<td>2.</td>
<td>Lipase</td>
<td>Tri, di, mono glycerides</td>
<td>Milk fat, natural animal &amp; plant fat Margarine, oils</td>
<td>Ester bond hydrolysis</td>
</tr>
<tr>
<td>3.</td>
<td>Amylase</td>
<td>Amylose, Amylopectin</td>
<td>Starch based soils flour, potato and gravy</td>
<td>Hydrolysis of 1, 4 glycosidic bond</td>
</tr>
<tr>
<td>4.</td>
<td>Cellulase</td>
<td>Cellulose</td>
<td>Amorphous cellulose vegetable, fruit and grains or cereals</td>
<td>Hydrolysis of β 1, 4 glycosidic bonds.</td>
</tr>
</tbody>
</table>

#### 2.1. Proteases

These enzymes work by breaking the peptide bond in amino acids chain of the protein molecule. Proteinase can be classified according to the nucleophile or reactive component found at their catalytic sites. Of the four broad categories of proteases: Serine, sulfhydryl, metallo and aspartyl, only the serine proteases are preferred for detergent formulations. The aspartyl proteinases perform poorly or not at all in alkaline pH range of detergent formulations. The Metallo proteases do not survive the levels present to reduce water hardness while the sulfhydryl proteases are too slow and not compatible with oxidants such as bleach. Over the years, several sources starting from pig pancreatic glands have been tried for large-scale enzyme production. However, bacilli among bacteria are considered superior for the following technological advantages

- Short cultivation time reduces cost of production
- Proteases secreted by bacilli in the fermentation broth are relatively pure as small amounts of other enzymes produced are digested by proteases.
- Simple operations such as centrifugation, filtration or filter presses can easily remove cell mass
- Proteases from bacillus are fairly stable in alkaline detergent formulations
- These proteases are resistant to inactivation by surfactants, oxidative bleaching agents and elevated temperatures and are therefore appropriate for cleaning in dairy and food industry.
Table-1: Commercial Alkaline Protease/Subtilisins Based Detergent Formulations

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Source Organism</th>
<th>Optimum pH</th>
<th>Optimum Temperature (°C)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td><em>Bacillus licheniformis</em></td>
<td>8-9</td>
<td>60</td>
<td>Novo Nordisk Bagscaverd, Denmark</td>
</tr>
<tr>
<td>Savinase</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>9-11</td>
<td>55</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Esperase</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>9-11</td>
<td>60</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Maxacal</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>11</td>
<td>60</td>
<td>Gist-brocades, Delft The Netherlands</td>
</tr>
<tr>
<td>Maxatase</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>9.5-10</td>
<td>60</td>
<td>Gist-Brocades, Delft The Netherlands</td>
</tr>
<tr>
<td>Opticlean</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>10-11</td>
<td>50-60</td>
<td>Solvay Enzymes, GmbH Hannover, Germany</td>
</tr>
<tr>
<td>Optimase</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>9-10</td>
<td>60-65</td>
<td>Solvay, Germany</td>
</tr>
<tr>
<td>Protosol</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>10</td>
<td>50</td>
<td>Advanced Biochemicals, Thane, India</td>
</tr>
<tr>
<td>Alkaline protease ‘Wuxi’</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>10-11</td>
<td>40-50</td>
<td>Wuxi Synder Bioproducts Ltd., China</td>
</tr>
<tr>
<td>Proleather</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>10-11</td>
<td>60</td>
<td>Amano Pharmaceuticals Ltd., Nagoya, Japan</td>
</tr>
<tr>
<td>Protease P</td>
<td><em>Aspergillus sp.</em></td>
<td>8</td>
<td>40</td>
<td>Amano, Japan</td>
</tr>
<tr>
<td>Durazym</td>
<td>Protein engineered variant of <em>Savinase</em>™</td>
<td>10-10.5</td>
<td>50</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Maxapem</td>
<td>Bleach resistant Protein engineered variant of <em>Alkalophilic Bacillus sp</em></td>
<td>11-12</td>
<td>60</td>
<td>Solvay, Germany</td>
</tr>
<tr>
<td>Purafect</td>
<td>Recombinant enzyme donar- <em>B. Lentus Expressed in Bacillus sp.</em></td>
<td>10</td>
<td>40-65</td>
<td>Genencor International Inc., Rochester, USA</td>
</tr>
</tbody>
</table>

2.2. Lipases

Lipases hydrolyze water insoluble triglyceride components into more water-soluble products such as monoacid diglycerides, free fatty acids and glycerol. Its activity is negligible towards monomeric water-soluble form and increases when substrate is in aggregated insoluble form. Lipases bind to the surface of lipid at the lipid-water interface and a conformational change takes place making the active site accessible to substrate molecule. Enzyme thus forms a complex with the surface molecule, which results in carbonyl ester bond hydrolysis.

Lipase activity is adversely affected by extremes in temperature, pH and matrix composition. At reduced temperature conditions, target sites are solids and hence less accessible to the enzyme. Many surfactants cause irreversible unfolding, denaturation and inactivation of lipases. Bleaching agents and binders also affect the enzyme activity. In view of these considerations, genetic modifications of the properties of lipases have been attempted. Some examples of genetically engineered enzymes include modification of lipases from *Pseudomonas alcaligenes* by inducing mutation at active sites i.e. replacement of methionine at position 21 by leucine. This led to increased cleaning performance. Cloning has resulted in
improvement in functioning of lipases in terms of increased efficiency at lower operating temperatures, broadening of stain specificity, etc.

Table-2: Commercial Lipase Based Detergent Formulations

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Source Organism</th>
<th>Optimum pH</th>
<th>Optimum Temperature (°C)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipolase</td>
<td>Recombinant enzyme donor- <em>Humicola sp.</em> Expressed in <em>Aspergillus oryzae.</em></td>
<td>10.5-11</td>
<td>40</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Lumafast</td>
<td>Recombinant enzyme donor-<em>Pseudomonas mandoicea</em> Expressed in <em>Bacillus sp.</em></td>
<td>7.5-9</td>
<td>60</td>
<td>Genencor, USA</td>
</tr>
<tr>
<td>Lipofast</td>
<td>Not available</td>
<td>8.5</td>
<td>50</td>
<td>Advanced biochemicals, India</td>
</tr>
</tbody>
</table>

2.3. Amylases

A variety of starch preparations are used in food applications. Their residues need to be effectively removed from the equipment surfaces. The amylases that can degrade starch component in the foods are: α-amylase, isoamylase, pullulanase, glucoamylase etc. Of these α-amylase has been an active ingredient in many biodetergent formulations, although, of late pullulanase and isoamylase derived from *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* have been incorporated in many commercial preparations (Table-3). The bacterial α-amylase is sensitive to pH, temperature and the presence of calcium. A certain level of Ca$^{2+}$ stabilizes the enzyme against denaturation and also attack of contaminant proteases. It is often recommended that amylases are combined with other enzyme preparations to improve detergent properties and application range.

Table-3: Commercial Amylase Based Detergent Formulations

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Source Organism</th>
<th>Optimum pH</th>
<th>Optimum Temperature (°C)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAN</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>6-7</td>
<td>70</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Termamyl</td>
<td>Recombinant enzyme donor- <em>Humicola sp.</em> Expressed in <em>Aspergillus sp.</em></td>
<td>7-8</td>
<td>70-75</td>
<td>Novo Nordisk, Denmark</td>
</tr>
<tr>
<td>Maxamyl</td>
<td>Alkalophilic <em>Bacillus sp.</em></td>
<td>6-8.5</td>
<td>100</td>
<td>Gist-brocades, The Netherlands</td>
</tr>
<tr>
<td>Solvay amylase</td>
<td><em>Thermostable Bacillus licheniformis</em></td>
<td>5-8</td>
<td>75-90</td>
<td>Solvay, Germany</td>
</tr>
</tbody>
</table>

3.0. PRODUCTION OF BIODETERGENTS

The prime consideration in developing enzyme-based detergents is that the enzyme to be incorporated should be effective at low levels (0.4-0.8%) in the detergent solutions. Low use concentration is important because these enzymes added to the detergent formulations are biocatalysts and they are not consumed during cleaning process but a single enzyme triggers
many chemical reactions. It should be compatible with various other detergent components primarily oxidizing and sequestering agents and should possess adequate temperature stability for wide range of cleaning applications. It should also have long shelf life.

In the early phase of developments, enzymes were used in powdered forms, which led to dust problems during the manufacture process. Furthermore, enzymes had reduced stability owing to autolysis and determined effects of other detergent ingredients in the presence of moisture. To overcome these problems, granulation and enzyme prilling, wherein enzymes are encapsulated in inert water soluble waxy substances are now used. In modern day biodetergent formulations, enzymes are mixed with the finished powders as granules or prills. Wax-coated coloured enzyme detergent granules often referred as signal granules are incorporated to symbolise the presence of this attribute and appeal the prospective users.

4.0. STABILIZATION OF ENZYMES

Stabilization of enzyme is important for protecting the commercial value of the detergent formulations. The enzyme in detergent may undergo proteolytic and autolytic degradation or denaturation during harsh operating conditions. Loss of enzyme may also occur during storage and transport. The loss of enzyme activity is brought about by partial unfolding of the polypeptide chain, since the inactivating agent breaks down the delicate balance of non-covalent bonds responsible for maintaining the active confirmation. Depending on the type of enzyme, mechanism of inactivation has to be identified and proper procedural modifications made to prevent the occurrence of inactivation. Calcium salts, sodium formate, borate, polyhydric alcohols and certain protein preparations are some of the stabilizing agents, which are added to protect enzymes against denaturation. Sodium chloride at 18-20 per cent concentration is effective in preventing contamination of the commercial crude preparations during storage. Enzymes can also be stabilized through protein engineering by either modifying an existing protein or creating de novo, a protein of prespecified properties.

5.0. BIODETERGENTS IN DAIRY/FOOD INDUSTRY APPLICATIONS

5.1. Cleaning of Membranes

Membrane processing viz. ultra filtration (UF) and reverse osmosis (RO) are being increasingly used in concentrating, clarifying, fractionating and/or sterilizing liquid foods like milk, whey, fruit juices, wines, beverages etc. As the processing progresses, even small adsorption of constituents cause pore blocking resulting in membrane fouling which ultimately leads to reduction in permeate flux rates and loss of product quality. The major foulants in the membrane processing operations could be protein, fat, inorganic salts, starch etc. Whey proteins, α-la and β-lg form nearly 95 per cent of the proteinaceous deposits during UF of whole milk. Calcium is also the primary foulant during whey concentration. Calcium generally exists as colloidal phosphate. When the concentration of calcium phosphate exceeds its solubility index, it tends crystallize forming deposits as specific membrane foulant.

Common cleaning agents such as nitric acid, phosphoric acid and sodium hydroxide are not very efficient in cleaning of membranes. Strong acids and alkali at higher temperatures can damage the membranes over long run. Several new generation enzyme based detergents have however performed exceedingly well and were able to restore 100 per cent flux after cleaning. The use of proteases and lipases based biodetergent based formulations is getting popular in cleaning of these membranes in the dairy industry.
5.2. **Milk Stone Removal**

Milk stones that form on heat exchanger surfaces essentially comprise of calcium phosphate denatured and precipitated milk proteins and insoluble salts from hard water and washing solutions. Biodetergents containing proteases are therefore efficient in removal of these milk stones.

5.3. **Removal of Biofilms**

The term ‘biofilm’ refers to the biologically active matrix of cells and extra cellular substances in association with a solid surface. On many occasions biofilms are termed as biofouling. Biofilm formation involves accumulation of living microorganisms and their decompositional products as deposits on the surface in contact with the liquid medium i.e. milk. The matrix of biofilm consists largely of water (98-99%) and remainder is an assortment of extra polymers (polysaccharides, glycoproteins etc.), which are referred to as extra cellular polymeric substances (EPS). The microorganisms are initially deposited on the surfaces and actively multiply to form colony of cells. These cells become large enough to interact with organic and inorganic debris, nutrients and other microorganisms leading to the formation of microbial biofilm.

Enzymes have proved very effective in cleaning the extra cellular polymers, which form the biofilm matrix. The specific enzyme requirement mostly varies according to the type of micro flora making the biofilm. The mixture of $\alpha$-amylase, $\beta$-glucanase and proteases were found very effective. Recently an enzymatic preparation comprising of EPS degrading enzymes derived from a streptomycin isolate was reported for the removal and prevention of biofilm formation.

6.0. **CONCLUSION**

In the wake of globalization, dairy and food industry in India need to adopt adequate measures to maintain hygiene and safety to meet global standards of quality. Improperly cleaned food contact surfaces may have food constituents viz. protein, fat, starch etc. deposited on the surface. These may sometimes be difficult to clean using conventional detergent solutions. Furthermore, membrane processes viz. ultrafiltration (UF) and reverse osmosis (RO), which have emerged as efficient, and environment friendly processes are being adopted for liquid food processing. Membrane fouling is a major problem associated with use of these processes and often results in reduced flux rate. These foulants if removed with strong alkali and acid cleaners lead to damage of the membrane in the long run. Biofilm formation which results from attachment of microorganisms to the improperly cleaned equipment surfaces need application of enzymes to degrade and facilitate removal of extra cellular polymers which form the biofilm matrix. Biodetergents, which are also termed as ‘green chemicals’, have emerged as an alternative to conventional synthetic preparations for their better cleaning properties, performance during cleaning operations and reduction in pollution related problems.

7.0. **SUGGESTED REFERENCES**

Kanwajia, S.K. (2001). Application of biodetergents and biocleaners in dairy and food industry. In compendium of the short course on "Advances in Preservation of

