DETERGENT POTENTIAL OF ENZYMES OF DAIRY MICROFLORA AND THEIR EFFECT ON THE SHELF LIFE OF MILK PRODUCTS

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Thesis submitted in partial fulfilment of the requirement for the degree of

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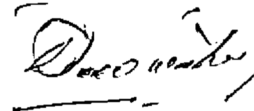

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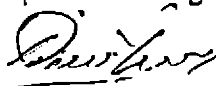

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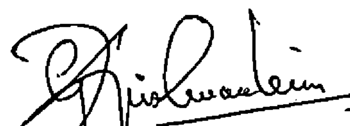

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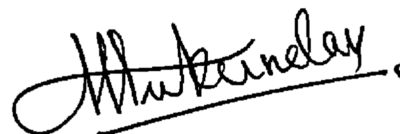

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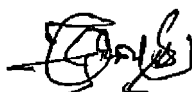

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Dedicated to
my loving parents

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Introduction

1. INTRODUCTION

Assurance of quality of a product is an important mandate in the performance of any industry. Although food industry has undergone tremendous refinement in the processing technology, the sanitation aspect which is of paramount importance has not received the deserved attention. Improvement of hygienic standards during all stages of production is imperative to assure the quality for products. As a part of quality assurance, major emphasis has so far been on 'hygiene' which has eventually resulted in the indiscriminate use of chemicals for sanitation. This practice in the long run will result in residues that act as concealed threats in food chain. The synthetic chemicals used presently for sanitation are to be replaced by eco-friendly alternatives to safeguard public health. The extensive use of chemical based detergents can adversely affect the ecosystem also. With greater thrust being given to environment protection, it is necessary to reduce the technogenic contamination of natural resources.

Enzymes, also known as 'green chemicals' are an ideal choice because of their biodegradability, low toxicity, noncorrosiveness and enhanced cleaning properties. The estimated value of world wide use of industrial enzymes was 1.5 billion dollars in 2000. As per the forecast; global demand for enzymes will rise seven per cent per annum and will reach six billion dollars in 2011. Proteases account for 60 to 65 per cent of global industrial enzyme market and their industrial sales are estimated to be 350 million dollars annually. It is conceived that microbial alkaline proteases having a high level of activity at a broad range of pH will be ideal as cleansing additives in dairy industry. Identification of such enzymes and diligent use of these will be helpful in reducing the use of chemicals without compromising on quality. This could result in substantial savings in terms of water and energy, at the same time improving environmental performance. The milk stones and biofilms that are characterized by impervious protein matrix have been identified as major hurdles that interfere with the efficiency of cleaning regimes in milk processing units. Enzyme based sanitizers are better options in combating these problems.

Safety, stability and shelf life are critical factors for food products in terms of economic feasibility and consumer acceptability. In spite of processing sophistication, microbes still pose a significant threat to the quality of food products. Literature indicate that, whatever practices resorted to for countering the effect of microbes, they evolve their own way to withstand these adverse situations. There is an increasing interest in this emerging microflora with supreme resistance properties, as they can serve as indicator organisms to optimize the processing techniques for economic production of safe foods. Extension of shelf life of products does make a significant contribution to food security too.The quality of dairy products is closely related to the microbial status of raw milk from which they are manufactured. The widespread use of refrigeration in farms and processing units has selectively promoted the growth of psychrotrophs, most of which are thermo-labile but capable of producing thermostable spoilage enzymes. Longer storage prior to processing, adoption of higher processing temperature and prompt adherence of cold chain has singled out psychrotrophic spore formers with potential to produce thermostable enzymes as the major category of spoilage flora in processed products. The quality and shelf life of milk and milk products is often compromised by the functional and flavour defects arising from the activity of heat stable proteases and lipases originating from contaminating bacteria.

Extending the shelf life of fluid milk contributes to the competitiveness of dairy industry in the beverage market. Recent economic and social developments have intensified the interests in Ultra High Temperature (UHT) sterilization of fluid products, as freedom from viable organisms permit their storage at room temperature. Changes in retailing practices and food service establishments have opened up better avenues for sterilized products. However, successful use of UHT treatment may be hindered by thermostable enzymes. The demand for fermented milk products are escalating as they are being widely appreciated as health foods. Milk used for preparation of fermented milk products is often subjected to storage conditions that permit proteolysis. Such proteolytic changes of base milk can affect the final product quality especially texture, an important parameter that determine its acceptability. An understanding of impact of thermostable enzymes on sterilized

2. REVIEW OF LITERATURE

2.1 PSYCHROTROPHS IN MILK

Pseudomonas and *Bacillus* are the two commonly encountered genera of bacteria in milk and milk products. These organisms, which have an optimum temperature of $\leq$15°C, are referred to as psychrotrophs. This also included species that were able to grow at $\leq$7°C (Suhren, 1989).

The psychrotrophic isolates obtained from the raw milk and pasteurized milk from the processing plants in Addis·Ababa mainly belonged to the genera *Bacillus*, *Aeromonas* and *Pseudomonas* (Mahare and Gashe, 1990).

Gram negative bacteria accounted for more than 90 per cent of microbial population in raw milk kept under refrigeration. The Gram negative flora comprised of the psychrotrophic species of *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium* and *Enterobacter* (Sorhaug and Stepaniak, 1997).

Eneroth *et al.* (1998) reported that psychrotrophic bacterial groups in refrigerated pasteurized milk of Norway and Sweden included *Pseudomonas*, *Enterobacteriaceae*, *Aeromonas* and *Bacillus*. Microbial analysis of raw and pasteurized milk at six sites along the production line indicated that recontamination with Gram negative organisms occurred at the filling step. The authors succeeded in isolating Pseudomonads from all the packages, *Enterobacteriaceae* from nine per cent and Aeromonads from three per cent of the packages.

Lira and Neilsen (1998) reported that psychrotrophic bacteria present in raw milk produced thermostable proteases that affected the plasmin system in milk which adversely affected the quality of processed milk and dairy products. The thermostable enzymes mostly attacked K-casein, resulting in destabilization of casein micelle and coagulation of milk in a manner analogous to chymosin.

Burdova *et al.* (2002) reported that storage of pasteurized milk at 10°C permitted psychrotrophs to exhibit remarkable protease and lipase activity. The shelf life was reduced by two third when compared to samples kept at 4°C.

Mc Phee and Griffiths (2002) observed that the average psychrotrophic aerobic bacterial count of silo milk in South West Scotland was 1.3 x 10^5 cfu/ml. Of this 70.2 per cent were Pseudomonads, 7.7 per cent were *Enterobacteriaceae* and 6.9 per cent were Gram positive bacteria. The commonly isolated Pseudomonads included *P. fluorescens biovar* I (32.1 per cent), *P. fragi* (29.6 per cent), *P. lundensis* (19.8 per cent) and *P. fluorescens biovar* III (17.3 per cent).

Pacova *et al.* (2003) recommended monitoring of the frequency of psychrotrophic thermo resistant spore formers as a complementary trait for hygienic quality testing of raw cow milk.

Kumaresan *et al.* (2007) monitored the spoilage initiated by psychrotrophs, when raw milk was kept at 2°C, 4°C and 7°C. They found that storage of raw milk at 2°C significantly lowered the growth, proteolytic and lipolytic activities, thereby assuring better sensory qualities, as compared to samples stored at 4°C and 7°C.

After examining 254 milk samples, Asha (2007) proposed that a positive correlation existed between the total viable count and psychrotrophic count. She also reported a positive and significant correlation between psychrotrophic count and coliform count. More than four log increase in psychrotrophic counts was reported during 10 days of storage under refrigeration.

The average psychrotrophic count of milk samples before pasteurization was 5.42 ± 0.07 $\log_{10}$ cfu/ml and after pasteurization was 4.16 ± 0.09 $\log_{10}$ cfu/ml. The mean reduction in psychrotrophic count by way of pasteurization was 1.26 $\log_{10}$ cfu/ml (Prejit *et al.*, 2007).

Zacharov and Halpern (2007) investigated the diversity dynamics and enzymatic traits of culturable psychrotrophs in raw milk from four farms of Israel over a 10 month period. They observed that *Bacillus* species were predominant

during summer; each dairy having its own unique bacterial profile. Most but not all bacterial isolates had either lipolytic or both lipolytic and proteolytic activities. Only a few isolates showed proteolytic activity alone. The dominant genera *Pseudomonas* and *Acinetobacter* showed mainly lipolytic activity; *Microbacterium* was highly proteolytic and lipolytic whereas lactic acid bacteria (*Lactococcus* and *Leuconostoc)* displayed minor enzymatic ability. They suggested that monitoring the dominant psychrotrophic species responsible for the production of heat stable proteolytic and lipolytic enzymes offered a sensitive and efficient tool for maintaining better milk quality.

2.1.1 Genus *Pseudomonas*

Andersson (1980) monitored the lipase production as a function of temperature. He observed that for *Pseudomonas fluorescens*, lipase production was highest at 8°C, though the organism exhibited maximum growth at 37°C.

Chandler and Mc Meekin (1985) reported that if *Pseudomonas fluorescens* having a generation time of 9.4 h at 4°C enter as a post processing contaminant in milk at a level of one cfu/ml, a count of 3×10^7 cfu/ml (which is a microbial criterion of spoilage) would be reached after 10 days of refrigerated storage.

Anderson *et al.* (1990) reported the survival of *Pseudomonas aeruginosa* within the biofilms associated with polyvinyl chloride piping after seven days of exposure to iodophors and phenolic antimicrobial solutions. They suggested that exopolysacharide structure could act as diffusional barrier to antimicrobial penetration such that organism survived on the interior walls of piping.

Stepaniak (1991) from his trial concluded that the growth and metabolic activity of *Pseudomonas* spp originating from post pasteurization contamination was the single, most detrimental factor determining the keeping quality of pasteurized milk stored at 4 - 7°C. He also reported that even with strict adherence to good manufacturing practices, postpasteurisation contamination (PPC) by *Pseudomonas* spp can occur at a level of 0.001 to 1 cfu/ml in practice.

The *Pseudomonas* genus corresponded to a diverse and ecologically significant group of bacteria found in different types of natural environment (terrestrial, fresh water, marine). Some of them were pathogens and some were responsible for food spoilage. Such a universal distribution resulted from the capacity to get adapted to various conditions and degrade a wide range of substrates including aromatic compounds, halogenated derivatives and organic residues (Palleroni, 1992).

Ternstrom *et al.* (1993) studied the spoilage flora of raw and pasteurized milk samples with special reference to *Pseudomonas* and reported that 51 per cent of the milk samples stored at 7°C were spoiled by Gram negative bacteria which included mainly *Pseudomonas* spp.

Desmasures *et al.* (1997) assessed the microbiological quality of raw milk samples from 27 farms over a period of six months and reported that *Pseudomonas* was one among the most numerous groups, with a mean count of 2000 cfu/ml.

The predominant microorganisms that limited the shelf life of processed fluid milk at 4°C were *Pseudomonas* species. In addition to its ability to grow to high numbers during refrigerated storage, many of these strains produced heat stable extracellular lipases, proteases and lecithinases that further contributed to milk spoilage (Garcia *et al.*, 1989; Sorhaug and Stepaniak, 1997).

Jayarao and Wang (1999) examined bulk tank milk from 131 dairy herds in Eastern South Dakota and Western Minnesota for coliform and noncoliform gram negative rods associated with lowering of milk quality. Gram negative noncoliforms comprising of species belonging to *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Moraxella*, *Acinetobacter* and *Xanthomonas* were observed in 76.3 per cent of bulk tank milk. *Pseudomonas fluorescens* was the most predominant species and accounted for 29.9 per cent of all the isolates examined. Coliforms included *Enterobacter*, *Citrobacter*, *Klebsiella* and *E.coli*.

Dogan and Boor (2003) suggested that, the high level of diversity among *Pseudomonas* isolates from different processing plants could be due to the lack of

long term colonization of Gram negative *Pseudomonas* species in processing environments. In contrast, Gram positive organisms were reported to colonize and have long term existence in food processing environments.

Martins *et al.* (2006) assessed the genetic diversity of 70 isolates of Gram negative proteolytic psychrotrophic bacteria by the random amplified polymorphic DNA fingerprinting technique (RAPD). Cluster analysis suggested that genetic variability among isolates belonging to same species was as high as the variability among different species. They suggested a grouping of *Pseudomonas* isolates based on their ability to ferment glucose.

The bacterial profile of retail milk samples collected from Thrissur district showed that 22.22 per cent of the samples contained *Pseudomonas*. The isolates included seven *Pseudomonas putida*, six *Pseudomonas aeruginosa* and three *Pseudomonas fluorescens* (Asha, 2007).

Nanu *et al.* (2007) reported that out of the 240 raw milk samples collected from the point of production (farmers level) in Palakkad district, 26 (10.80 per cent) revealed the presence of *Pseudomonas aeruginosa.*

Dufour *et al.* (2008) reported that *P. fluorescens* was responsible for the highest depredation of milk because of its ability to synthesise extracellular proteases and lipases that hydrolyse milk protein and fat. Several strains of *P. fluorescens* synthesise an extracellular caseinolytic metalloprotease called AprX Strain differences in the extracellular caseinolytic potential was not resulting from Apr X sequence/structure but from the Apr X level of expression.

2.1.2 Genus *Bacillus*

Shehata *et al.* (1983) monitored the seasonal variation in the population of aerobic spore formers in raw milk. They found that mesophilic aerobic spore formers consisted mainly of *Bacillus subtilis* (42.5 per cent) and *B. megaterium* (34.8 per cent), followed by *B. circulans*, *B. cereus*, *B.pumilus*, *B. polymyxa* and *B.badius*. However they observed that 42.6 per cent of psychrotrophic *Bacillus*

strains belonged to *B. cereus* (42.6per cent). Psychrotrophic strains of *B. pumilus* *B. badius* *B. licheniformis* and *B. firmus* were also reported by them.

Sharma *et al.* (1984) observed that 36 per cent of the of proteolytic psychrotrophic sporeformers in raw milk, belonged to *Bacillus cereus*. Rest included *B. polymyxa, B. laterosporus* and *B. circulans*, *B. pumilus*, *B. subtilis* and *B. coagulans* in the decreasing order. They reported that 20 per cent of the isolates exhibited a protease activity of more than 300 units/ml.

Phillips and Griffiths (1990) observed that 86 per cent of thermoduric psychrotrophic bacteria isolated from raw milk were *Bacillus* spp. They also reported that thermoresistant mesophilic aerobic spore formers could be detected in commercially sterile milk at levels as high as 10^5 cells/ml.

Meer *et al.* (1991) reported on the occurrence of psychrotolerant strains belonging to *Bacillus cereus* group in fluid milk. *B. weihenstephanensis* has been proposed to accommodate the psychrotolerant *Bacillus cereus* strains.

Vassiliadou, (1992) tested the *Bacillus* species for its ability to produce lactic acid by inoculating milk with a 24 h culture at a level of 0.1 per cent. The mean acid production after incubation at 20°C for seven days by *B. subtilis* (0.18 per cent) and *B. macerans* (0.18 per cent) were higher than for *B. licheniformis* (0.17 per cent) and *B. coagulans* (0.07 per cent).

B. licheniformis and *B. cereus* were the most commonly isolated species of *Bacillus* from milk in all stages of processing. *B. licheniformis* was ubiquitous in farm environment and their count was higher in winter months. *B. cereus* was mainly associated with cattle feed and their number increased during summer months (Creilly *et al.*, 1994).

The interactions of psychrotrophic and mesophilic *Bacillus* species were studied by Sutherland and Murdoch (1994). They observed that mesophilic *B. subtilis* and *B. licheniformis* produced antagonistic factors against the psychrotrophic isolates of *B. cereus* and *B. pumilus*.

Lin *et al.* (1998) opined that spores of *B. cereus* in raw milk were the major source of *B. cereus* in pasteurized milk. They reported that post pasteurization contamination along the milk processing lines was a minor source of *B. cereus* in pasteurized milk.

Matta and Punj (1999) reported that 48 out of 100 raw milk samples contained lipolytic psychrotrophic spore forming bacteria, such as *B. cereus, B.polymyxa, B. licheniformis, B. circulans, B. subtilis, B. laterosporous* and *B.coagulans*. Of these *B. cereus* was the most predominant one.

Bacillus species are widely distributed in the environment and can be introduced into milk and milk products during production handling and processing. They were reported to be capable of producing more diverse proteolytic activities than *Pseudomonas* spp and could produce more than one type of proteinase (Murphy *et al.*, 1999).

Daffonchio *et al.* (2000) studied the genetic relationship within six species of the '*B. cereus* group'. They stated that *B. weihenstephanensis* was closely related *to B. mycoides* and *B. pseudomycoides* by phenotype and genotype. *B.cereus, B. anthracis* and *B. thuringiensis* were clustered in a separate group.The *Bacillus cereus* group known as *Bacillus cereus sensu lato* comprised of *Bacillus cereus sensu stricto,* an opportunistic human pathogen; *Bacillus thuringiensis,* an enteropathogen; *Bacillus anthracis,* the etiological agent of anthrax ; *Bacillus mycoides* and *Bacillus pseudomycoides* both characterized by rhizhoidal growth on agar plates; and the psychrotolerant *Bacillus weihenstephanensis.*

Pacova *et al.* (2003) succeeded in isolating a psychrotolerant species of *Bacillus weihenstephanensis* from raw cow milk. Fifty six mesophilic and psychrotolerant *B. cereus* strains were isolated from bulk milk samples. Of these thirty four mesophilic strains grew at 40 to 43°C but not at at 4 to 7°C. Twenty of the twenty two psychrotolerant strains were able to grow between 10°C and 40°C; but not at 4 and 43°C. Only two cold tolerant strains that were classified as *B. weihenstephanensis* were able to grow at 4 to 7°C.

While assessing the shelf life attributes of pasteurized milk from three processing plants in New York State, Fromm and Boor (2004) found that product shelf life was mainly limited by multiplication of heat resistant psychrotrophic organisms that caused undesirable flavours in milk. The predominant micro organisms identified were Gram positive rods including *Paenibacillus, Bacillus* and *Microbacterium.*

Haque and Russel (2005) classified the strains of *Bacillus cereus* into two groups based on their optimum growth temperature. Group A comprised of members having a broader range of optimum temperature 37^0C to 47^0C and group B included members with the narrow optimum temperature of 47^0C.

Bartoszewickz *et al.* (2008) determined the level of milk contamination by *Bacillus cereus sensu lato.* They succeeded in isolating a total of 680 *B. cereus sensu lato* isolates from forty four samples of milk collected from a dairy farm and two independent dairies in northeastern Poland. They confirmed the occurrence of potentially toxic *B.cereus sensu lato* in both raw and pasteurized milk.

2.2 MICROBIAL ENZYMES

2.2.1 Spoilage Enzymes

Adams *et al.* (1975) reported that microbiological activities of enzymatic origin were a better index of quality of product than the number of micro organisms.

Proteinases generally showed higher heat stability in synthetic milk salt solutions and milk than in buffer solutions, as large amount of proteins in milk prevented autolysis of proteinases (Fox, 1981).

Griffiths *et al.* (1981) opined that lipases were produced concomitantly with proteinase by the same bacterium and were generally more heat stable than the proteases. So lipases are even more likely than proteinases to retain activity in milk powders during prolonged storage.

In processed milk, flavour defects generally occurred when bacterial population of milk increased to $\geq 10^7$ cfu/ml. Off flavours developed in three stages. First, milk lost its freshness and then it became stale. Later they developed rancidity and soapy flavour due to lipolysis and bitter flavour due to proteolysis (Schroder *et al.*, 1982).

Proteolysis occurred during cold storage due to growth of psychrotrophic bacteria (Cousin, 1982). According to Grieve and Kitchen (1985), psychrotrophic proteases hydrolysed K-casein whereas plasmin attacked β-casein primarily.

The proteases from psychrotrophs contributed to overall plasmin activity by acting as plasminogen activators. The extracellular proteinases of *Pseudomonas* species cleaved the peptide bond needed for this conversion (Mitchell *et al.*, 1986).

Renner (1988) reported that both chemical reactions and enzyme activities were responsible for changes in the functionality of milk powders, a comparatively inert dairy product from biological perspective.

Proteinases include a group of proteolytic enzymes that act internally on polypeptide chains rather than cleaving off single amino acids or dipeptides from the ends of polypeptide chains. On the basis of mechanism of action, they are classified into four sub-groups: (i) serine proteases such as subtilisin (EC 3.4.21.62) from *B. licheniformis* or *B. subtilis* (ii) cysteine (or sulphydryl) proteases such as cathepsin B (EC 3.4.22.1) from bovine spleen; (iii) aspartic (or acid) proteases, such as cathepsin D (EC 3.4.23.5) from bovine spleen; and (iv) metalloproteinases such as thermolysin (EC 3.4.24.27) from *B.thermoproteolyticus* subsp *rokko* (Salvesan and Nagase, 1989).

Lipolytic enzymes are defined as carboxyl esterases that hydrolyse acylglycerols. Those that hydrolyse acylglycerols of less than 10 carbon-chain fatty acids are esterases or carboxylases (EC 3.1.1.1); those that hydrolyse to more than 10 carbon-chain fatty acids are lipases or triacylglycerol acyl hydrolases (EC 3.1.1.3). Esterases are more active in aqueous solutions. Lipases are more active at lipid water interface (Jaeger *et al.*, 1994).

Digestion of casein by proteases resulted in bitter flavour and gelation of milk. Lipases hydrolysed tributyrin and milk fat to yield free fatty acids which caused milk to taste rancid, bitter, unclean and soapy. Lecithinase degraded milk fat globule membranes and increased the susceptibility of milk fat to the action of lipases. The hydrolytic products of milk fat and protein decreased the organoleptic quality of fluid milk products (Shah, 1994).

Calcium ions stabilized proteinases against thermal denaturation and autolysis. Milk contains high level of soluble calcium salt (0.38 mg/ml). Hence loss or survival of proteinase activity after heat treatments in milk cannot be predicted by their performance/properties in buffers or synthetic milk salt solutions (Daniel *et al.*, 1995).

Muir (1996) opined that many proteinases present in milk were unlikely to be destroyed by the heat treatments applied during milk processing and would remain active in the final product.

Proteinases from psychrotrophs preferentially attacked casein over whey proteins. Beta-casein and K-casein were more susceptible than αS_2 casein (Triantafyllidou and Roussis, 1999).

Chen (2000) found that the number of bacteria in processed products was comparatively less relevant than the level of enzymes. Failure to recover viable bacteria did not necessarily indicate that their enzyme compliment was such that the product functionality was compromised.

Freshly drawn milk contained indigenous enzymes including proteases and lipases. During handling and processing, milk acquired contaminating bacteria that produced proteases and lipases which added to the enzyme load of milk. The heat treatments used during the preparation of products were not sufficient to inactivate all the enzymes. The activity of proteinases and lipases that survived heat treatment caused changes in functionality and flavour of milk products including milk powders during storage (Chen *et al.*, 2002).

2.2.1.1 Spoilage Enzymes – *Pseudomonas*

White and Marshall (1973) assessed the reduction in shelf life of dairy products under the influence of protease from *Pseudomonas fluorescens* P26. The criticisms encountered mainly were bitterness and unclean flavour. They linked both these off flavours in dairy products to proteolysis.

Adams *et al.* (1975) reported that proteinases produced by ten different *Pseudomonas* isolates survived a heat treatment of 149°C for 10s in buffers at pH 7.5.

Ultra High Temperature (UHT) milk sterilized immediately after addition of pregrown washed cells of *P. fluorescens* did not gel within eight weeks of storage at room temperature whereas UHT milk containing cell free washing from the same strain gelled within seven days, indicating that proteinase activity was extracellular rather than cell bound. Extracellular bacterial proteinases predominantly attacked K-casein with the formation of a material similar to para K-casein, followed by extensive nonspecific hydrolysis. Beta-casein was readily hydrolysed, while αS1 casein was slowly hydrolysed. The crude lipase produced by psychrotrophic *Pseudomonas* species isolated from raw milk retained 55 to 100 per cent activity after heat treatment at 63°C for 30 min (Law *et al.*, 1977).

Proteases from different *Pseudomonas* strains exhibited various degree of heat resistance. Heating at 121°C for 2 minutes destroyed less than 40 per cent of the initial activity of most of these proteases (Egziabher *et al.*, 1980). Patel *et al.* (1983) reported that *P. fluorescens* T 16 isolated from raw milk produced a protease enzyme that retained eight per cent activity after being heated at 120°C for 10 minutes.

Proteolytic enzymes from microorganisms may be intracellular or cell wall associated (periplasmic) or extracellular. *Pseudomonas* spp secreted extracellular alkaline metalloproteases that possessed remarkable heat stability and survived pasteurization and even UHT processing. Proteinases from *Pseudomonas* species isolated from raw milk retained 55 to 65 per cent of initial activity after a heat

treatment of 77°C for 17s and 20 to 40 per cent activity after a heat treatment at 140°C for 53 seconds in buffers at pH 7.0 (Griffiths *et al.*, 1981).

Fitzgerald *et al.* (1982) found that *Pseudomonas* lipase retained 75 to 100 per cent activity after heat treatment at 100°C for 30 sec in skim milk.

Kwan and Skura (1985) reported that proteolytic Pseudomonads isolated from raw milk resided in two clusters; one containing *Pseudomonas fluorescens* and the other containing *Pseudomonas fragi.* The isolates identified with *P. fluorescens* hydrolysed milk proteins and milk fat and produced phospholipase and fluorescent pigment.The *P. fragi* like isolates hydrolysed milk proteins and milk fat but did not produce phospholipase and fluorescent pigment. They also observed that hydrolytic characteristics of isolates were very much dependant on the nature of substrate and conditions under which test was conducted.

Hockney and Cousin (1985) added *Pseudomonas* proteases to cooled pasteurized milk at a level of 0.18, 1.8 and 18 units of protease/ml and stored at 5°C for 18 days. They found that flavour defects, specifically bitterness, became noticeable at the same time tyrosine curve began to rise. The results indicated that inoculation with 50 to 500 cells/ml followed by storage at 5°C for 18 days caused more extensive proteolysis and organoleptic changes than addition of 18 units of protease/ml. Psychrotrophic counts reached 10^6 to 10^7 cells/ml before proteolysis was detected.

Fairbairn and Law (1986) reported that the majority of *Pseudomonas* species produced only one type of proteinase, typically a neutral zinc metalloproteinase with a pH optimum of 6.5 to 8.0. Maximum protease production occurred in the late exponential or stationary phase of growth. They also observed that a typical thermostable metallo proteinase produced by *Pseudomonas* spp isolated from raw milk possessed many properties similar to those of classical heat resistant zinc metalloproteinase thermolysin from *Bacillus thermoproteolyticus rokko.*

Proteinases from psychrotrophs caused bitterness in milk. However enzymes from *P. fluorescens* were reported to yield good results in accelerating cheddar cheese ripening (Tye *et al.*, 1988).

Pseudomonas proteinases lead to bitterness and rancidity in milk owing to proteolysis and lipolysis. Even small variations in pH were found to influence the integrity of casein micelle and their susceptibility to proteolysis (Mc Kellar, 1989).

According to Clements *et al.* (1990), psychrotrophic organisms eventually dominated the microflora of cold stored raw milk. The growth of *Pseudomonas fluorescens* resulted in the production of extracellular proteases and lipases which had a deleterious effect on milk quality. Many of the proteases were unaffected by pasteurization and UHT heat treatment.

The free fatty acids released by Pseudomonads were found to inhibit the growth of lactic acid bacteria (Sorhaug and Stepaniak, 1997; Shah, 1994; Jaspe *et al.*, 1995).

An interesting consequence of enzyme activity from psychrotrophs was the stimulation of growth of starter lactic acid bacteria in milk as they utilized the peptides, amino acids and ammonia that accumulated in milk due to psychrotrophic action (Sorhaug and Stepaniak, 1997).

Heat stable proteases of psychrotrophic microorganisms can acted as plasminogen activators (Lira and Neilsen, 1998).

The lipolytic enzymes from *Pseudomonas* are very diverse and spread over six families while those from *Bacillus* species are more closely related and are only found in two families. However a sequence similarity does not necessarily imply functional similarities (Arpigny and Jaeger, 1999).

While assessing the influence of growth conditions on heat stable phospholipase activity in *Pseudomonas*, Koka and Weimer (2001) observed that both enzyme production and thermostability of enzymes varied significantly with strain, growth medium and incubation period.

Mc Phee and Griffiths (2002) reported that majority of the *Pseudomonas* proteinases were metalloenzymes containing one zinc atom and up to 16 calcium atoms per molecule. The proteases were found to attack K, αS_I and β-casein. Only a low activity was observed on denatured whey proteins. All proteases were found to retain their activity at 4°C. They also found that lipases of *P. fluoroscens* survived commercial pasteurization and UHT processing.

Dogan and Boor (2003) assessed the spoilage potential of *Pseudomonas* spp isolated from fluid milk in terms of its enzyme activity. More than 80per cent of the isolates showed proteolytic activity. Isolates bearing the same genotype had similar extracellular enzymatic profile. Of the tested isolates, 45 per cent had lecithinase and lipase activity, 31 per cent had neither activity, 22 per cent had only lipase activity and two per cent had lecithianse activity without lipase activity.

Proteolytic and lipolytic activities of the psychrotrophs in general and *Pseudomonas* species in particular can be regarded as valuable tools for the detection of spoilage and shelf life prediction of refrigerated foods (Ma *et al.*, 2003).

Rajmohan *et al.* (2002) observed that on subjecting the protease enzyme of *Pseudomonas fluorescens* to a temperature of 100°C for 5 min, the activity increased to 172 per cent. The increase in activity was thought to be due to the release of preformed enzyme from the cells.

Dunstall *et al.* (2005) reported that *Pseudomonas fluorescens* dominated the microflora of refrigerated raw milk and produced thermostable proteases and lipases which survived pasteurization and even UHT treatments. Residual activity was found to degrade casein and fat components of milk, causing a reduction in cheese yield, gelation of UHT milk and off flavours in dairy products. The enzymes produced in the late log or stationary phase contributed to the selective advantage of psychrotrophs and hence had a significant effect on their growth kinetics.

Prakash *et al.* (2007) reported that of the various isolates obtained from raw milk, *M.luteus* showed slow proteolytic activity when compared to *B. cereus*, *P.aeruginosa* and *S. marcescens*. Except *E. coli* all bacterial isolates exhibited lipolytic activity. The putative *Pseudomonas* isolates were characterized by the production of proteases, lipases and lecithinases.

Yagoub *et al.* (2008) studied the spoilage effects of *P.aeruginosa* inoculated into sterilized milk at a level of $10^8 - 10^9$ cfu/ml. They reported a correlaton between number of organisms and reduction in level of protein and fat. Samples with highest bacterial count had lowest percentage of protein and fat.

2.2.1.2 Spoilage Enzymes - Bacillus

Chopra and Mathur (1984) isolated 171 strains of proteolytic thermophilic bacteria from milk and milk products Protease enzyme of five cultures retained 100 per cent protease activily at 65°C for 30 min. Protease of *B. stearothermophilus* RM-67 retained 87.5 per cent of its activity at 70°C for 30 min. The crude proteinase solutions from *B. stearothermophilus* and *B. licheniformis* showed no loss of activity after a heat treatment at 70°C for 10 minutes at pH 7.0.

The lipolytic enzymes of *Bacillus* species often have an alanine residue and a calatytic centre similar in function to that of serine proteases (Arpigny and Jaeger, 1999).

Chen (2000) reported that thermolysin, a thermostable zinc metalloproteinase from *B. thermoproteolyticus,* was characterized by zinc at the active site, enhancement of stability in the presence of calcium, lack of sulphydryl groups and presence of high content of hydrophobic amino acid residues. He also observed that the semipurified proteinases of *B. stearothermophilus* strain AM and *B. licheniformis* strain FG isolated from a milk powder possessed $t^{1/2}$ values of 24 and 19.4 minutes respectively, at 70°C in buffers at pH 7.0.

Lindsay *et al.* (2000) attempted to study the physiology of dairy associated *Bacillus* species over a wide pH range. They reported that *B. subtilis* 115,

B.pumilus 122 and *B. licheniformis* 137 exhibited lipolytic activity at pH 7 and pH 10. But only a very weak lipolysis was observed at pH 4.0.

Bellow *et al.* (2007) monitored the rate of spoilage initiated by *Bacillus subtilis* and *Bacillus cereus* by inoculating them in sterilized milk so as to get 3.1x 10^3 and 3.5 x10^3 cfu/ml. The samples were stored at 7^0C, 12^0C, 21^0C, 37^0C and 45^0C. Significant differences were noticed for protein, fat and acidity during storage. The samples held at 7 ^{0}C and 12^0C showed a shelf life of more than five days whereas those kept at 21^0C, 37^0C and 45 ^{0}C had a shelf life of one to four days.

2.3 IMPACT OF PSYCHROTROPHIC SPOILAGE ENZYMES IN CURD AND STERILIZED MILK

2.3.1 Curd

The Indian Standards (1980) describes dahi as a product obtained by lactic fermentation through the action of single or mixed strains of lactic acid bacteria or by lactic fermentation. Dahi shall be of the following types

a. Plain

b. Flavoured

The following cultures shall be used in preparing dahi.

- *S. lactis, S. diacetylactis, S. cremoris*: single or in combination with or without *Leuconostoc* species, and
- Also as above along with species of *Lactobacillus* such as *L. bulgaricus* and *L. acidophilus,* and *L. casei* and *S. thermophilus*

According to the Indian Standards, dahi should have maximum titratable acidity of one per cent (as lactic acid); yeast and mold count not more than 100/g; coliform count below 10/g; and phosphatase negative.

Hassen *et al.* (1995) identified three stages during acid coagulation of milk: an induction period without any change in viscosity, a flocculation stage with maximum increase in viscosity and a stage characterized by a decrease in viscosity with rearrangement of casein micelle and syneresis of the gel.

Shaker *et al.* (2000) proposed that coagulation of milk by acid was a consequence of removal of calcium bond between casein micelles which caused destabilization of casein that aggregated and formed a curd.

The most important fermentative reaction used in dairy processing is the homofermentative conversion of lactose to lactic acid. Yogurt with an acidity of one per cent is a fairly unfavourable medium for troublesome pathogens like *Salmonella* species and *Listeria monocytogenes* (Tamime and Robinson, 2004).

According to The Prevention of Food Adulteration Act, (2007) dahi or curd is the product obtained from pasteurized or boiled milk, by souring, natural or otherwise, by a harmless lactic acid or other bacterial culture. Dahi may contain additional cane sugar. It could have the same percentage of fat and solids not fat as the milk from which it is prepared. Where dahi or curd is sold or offered for sale without any indication of class of milk, the standards prescribed for dahi prepared from buffalo milk shall apply [Milk solids may also be used in preparation of this product].

2.3.1.1 Titratable Acidity and pH

Gould and Frantz (1945) attempted to study the relationships between pH, titratable acidity and formal titration in milk heated to high temperatures. The heating of milk resulted in a slight but definite increase in formol titration values. Changes in buffering capacity through heat treatment influenced the titration value without a similar influence on pH.

Kroger (1975) reported that final pH of good quality yogurt varied from 4.1 to 4.2. A pH above 4.5 produced a weak coagulum.

Salji and Ismail (1983) found that when samples of yogurt with initial pH 4.89, 4.18 and 3.82 were kept at 4°C for one week, their pH values decreased to 4.27, 4.12 and 3.81 respectively.

Ott *et al.* (2000) opined that important flavour differences observed between different classes of yogurt were due to acidity and not due to the different concentrations of aroma compounds.

Al-Kadamany *et al.* (2002) determined the shelf life of Leben at different storage temperatures. The mean pH of fresh sample was found to be 4.0.The pH dropped to 3.6 on eleventh, seventh and third day when stored at 5°C 15°C and 25^0 C respectively.

Kamruzzaman *et al.* (2002) determined the organoleptic and physicochemical profile of dahi at room and refrigeration temperature. The acidity on zero day was 0.92 ± 0.01 per cent lactic acid. Under refrigeration, the acidity increased to 0.93 ± 0.03 on eighth day and to 0.94 ± 0.01 per cent on twelveth day. The corresponding pH was 4.16 ± 0.07, 4.10 ± 0.04 and 3.91 ± 0.01 respectively.

All the tested samples of dahi, collected from sweet meat shops and milk booths in Hyderabad society had higher acidity than that prescribed by BIS. The mean titratable acidity of samples from sweet meat shops was 1.042 per cent and that from milk booth was 1.089 per cent. The higher titratable acidity could be due to the use of different type of cultures, varied processing techniques and higher storage temperatures (Rao *et al.*, 2002).

Younus *et al.* (2002) compared the physico-chemical quality of 25 samples of dahi and 10 samples each of various brands of yogurt *viz.*, A, B and C collected randomly from the local markets. The study revealed that the dahi samples had a mean titratable acidity of 1.16 ± 0.32 per cent. Yogurt samples from the sources A, B and C had a titratable acidity of 0.89 ± 0.02, 0.87 ± 0.04 and 1.13 ± 0.05 per cent lactic acid respectively.

According to Anema and Li (2003) the pH at which preheating is done, affect the properties of milk proteins. When preheating was done at pH between 6 and 7, different patterns of casein-whey protein aggregation were observed.

The physico-chemical and organoleptic properties of conventionally made dahi from three sources *viz.*, A, B and C and industrial yogurt were compared by Soomro *et al.* (2003). The mean titratable acidity of industrial yogurt was 0.88 ± 0.01 per cent and that of dahi samples from the sources A, B and C were 0.91 ± 0.02, 0.85 ± 0.01 and 0.90 ± 0.02 per cent lactic acid. The corresponding pH for dahi samples were 4.31 ±0.01, 4.31± 0.02 and 4.26 ± 0.02 respectively.

Aly *et al.* (2004) observed that pH of fresh sample of yogurt was 5.15. After storage at 4 ± 2°C for 21 days, pH decreased to 4.15.

According to Salvador and Fiszman (2004) storage temperature (10°C/91days, 20°C/21 days and 30°C/3 days) did not very much affect titratable acidity and pH. After storage pH values barely changed over storage time indicating that yogurt sample did not develop much acidity under any of the storage conditions.

Praseeda (2005) observed that the titratable acidity of fresh samples of curd was 1.85 ± 0.03 per cent lactic acid. Under refrigerated storage, titratable acidity increased to 1.88 ± 0.05 per cent lactic acid on ninth day and then decreased till 21st day of storage probably due to increase in yeast and mold, which might have utilised the lactic acid. The pH also decreased except on the fifth day, and after fifteenth day, values increased gradually. Evaluation of the organoleptic qualities of curd under refrigerated storage showed that the flavour scores deteriorated with storage. Body and texture scores remained more or less same. Color and appearance score also decreased significantly during storage.

2.3.1.2 Syneresis

Cold storage of milk gave rise to an increase in the amount of soluble casein particularly β-casein. As syneresis of milk is highly dependant on the

miscellar content of β-casein, cold storage of milk affected syneresis (Ali *et al.*, 1980).

Syneresis rate increased as the pH lowered, possibly as a result of reduction in net micelle charge and subsequent electrostatic repulsion between micelles. The rate of syneresis accelerated with the temperature; however extend of the effect of temperature varied (Marshall, 1982).

Harwalkar and Kalab (1983) subjected yogurt gel fermented in centrifuge tubes to centrifugal forces ranging between 30 and 2000 g (6°C, 10 min). They observed a sigmoid relationship between the separated whey and g value and suggested, that the g value at the inflection point of curve as a measure of susceptibility to syneresis.

According to Storrey *et al.* (1983), fat concentration of milk directly affected the syneresis time. They proposed that with increased fat content there will be an increased number of interstices within the reticulum occupied by the fat globules, such that there will be increased impedance to whey drainage.

For measuring syneresis, Pearse *et al.* (1984) developed an assay based on the light scattering properties of fat globules. Syneresis was determined by measuring the increase in light scattering properties of clarified whey.

Pearse and Mackinlay (1989) observed that syneresis was sensitive to the concentration of β-casein. Beta-casein was found to affect micelle surface properties and thus had an important role in syneresis. Any alteration in the composition of casein micelle which form the curd network was found to affect coagulation and subsequent syneresis.

Gassem and Frank (1991) studied the physical properties of yogurt made from milk treated with proteolytic enzymes. They found that pH of yogurt ranged from 4.0 to 4.2. Yogurt pH dropped to the range of 3.8 to 3.9 after eight days of storage. Greater proteolysis that occurred in the product might have stimulated lactic acid produotion during storage. However a significant increase in acidity

was not confirmed by analysis of titratable acidity. Enzymes which were actively involved in proteolysis of milk (pH-6.3) may not be that active in yogurt (pH-4).Yogurt made from milk treated with proteolytic enzymes had lower water holding capacity and protein hydration and so higher firmness, syneresis and apparent viscosity than untreated controls.

Low pH favored casein fusion. Increase in G^1 was observed only until 14th day. Rearrangements together with particle fusion produced stress such that with time it became thinner. Spontaneous syneresis resulted from the breakage of strands. Rearrangements of network led to the expulsion of whey (Lucey *et al.*, 1998).

The stability of set yogurt towards syneresis or whey drainage can be evaluated either under the influence of regular gravity or by applying additional gravitational forces by centrifugation. This can also be assessed based on gel shrinkage after wetting (Jaros and Rohm, 2003).

Amatayakul *et al.* (2006) reported that for samples with low total solids, centrifugation and drainage methods were not suitable for measuring syneresis as they were likely to cause damage to the structure of yogurt. Hence they made some modifications to the Lucey's siphon method for measuring syneresis. In this technique, the set yogurt was weighed and then kept at an angle of 45° to allow whey on the surface to collect on the side of the cup. Liquid whey was siphoned out and then reweighed. Syneresis was expressed as the percentage weight of whey over the initial weight of yogurt sample.

Castillo *et al.* (2006) studied the effect of temperature and rate of inoculum on gel microstructure and syneresis kinetics in cottage cheese type gels. They found that increasing inoculum concentration decreased rate of syneresis. They opined that syneresis parameters were highly correlated with the rates of acidification network formation and loss tangent.

2.3.1.3 Texture Analysis of Curd

Cousin and Marth (1977) reported a decrease in manufacturing time and an increase in firmness for yogurt made from milk pre cultured with psychrotrophic bacteria. These effects were attributed to the proteolysis associated with the growth of psychrotrophs.

Modler *et al.* (1983) reported that with increasing protein content in yogurt gel firmness increased with a general decrease in syneresis. Sensory firmness also increased with increased protein content. However there was no significant correlation between the measured gel firmness and syneresis.

The firmness of yogurt and viscosity of just stirred gel were found to be influenced by the amount of heat treatment. Heating unfolded the globular whey proteins and exposed sulphydryl groups, which reacted with other sulphydryl groups and formed disulphides that induced linkage and formation of protein-casein aggregates. Viscosity as a function of aggregate size, was influenced by covalent (SH/SS) interactions arising from denaturation of globular whey proteins. Heating at higher temperatures produced intermolecular SS cross links which had a great effect on the strength of curd (Kinsella, 1984).

Labropoulos *et al.* (1981) suggested that differences in protein structure could influence structural firmness of yogurt.

Parnell-clunies *et al.* (1988) reported a high correlation between whey protein denaturation, gel firmness and viscosity. However Savella and Dargan (1997) concluded that degree of protein denaturation were not that significant in determining the physical properties of yogurt, as they observed that UHT and conventional heat treatments that produced same level of whey protein denaturation resulted in yogurt with different physical properties.

Gassem and Frank (1991) evaluated the physical properties of yogurt made from milk treated with proleolytic enzymes.They observed that physical properties and stability of yogurt were influenced by the extend to which milk used in the

preparation of yogurt was proteolysed. Variations in syneresis and texture originated from variations in protein quality of milk. They also observed that though fermentation was rapid in enzyme treated milk, proteolysis did not have a consistent effect on starter culture. The physical properties of yogurt prepared from milk treated with psychrotrophic proteolytic enzymes showed increased firmness, syneresis and viscosity whereas yogurt prepared from milk treated with plasmin showed decreased firmness and viscosity when compared to control. The differences noticed reflect the different specificities of casein hydrolysis of the two enzyme preparations.

Muir and Hunter (1992) opined that texture criteria which included sensory attributes such as firmness, viscosity and extent of syneresis were very important criteria in deciding consumer acceptability of fermented milks.

Bell (1995) suggested that culture material (biomass and extra cellular polysaccharides) might interfere with protein interactions during yogurt fermentation. This might produce weaker gel structures, possibly by a modified gelation mechanism.

Tamime and Marshall (1997) observed that in the acid gels of fermented milks, proteinases originating from microflora had a role as they contributed to the denatured protein matrix which in turn was relevant to the gel properties of fermented milk products.

Degree of whey protein denaturation was found to be an important parameter affecting rheological behavior of gels, as denatured whey protein associated with casein act as a bridging material interacting with other denatured proteins. This cross linking by denatured whey protein led to an increase in the rigidity of network contributing to the increase of G (Lucey *et al.*, 1998).

Assessment of curd rheology in terms of syneresis by Grundelins *et al.* (2000) revealed that grain size was the most important factor that influenced shrinkage of grains in the initial stage of syneresis. But in later stages, pH was the dominating factor with shrinkage being more pronounced at a lower pH.

Rheological properties of fermented dairy products are important in the design of flow processes, quality control, storage and processing and also in predicting the texture of foods. Texture of curd, an important characteristic of plain yogurt determined the identity and acceptability of the product (Shaker *et al.*, 2000).

Mellema (2000) observed that surface dehydration during long storage influenced gel strength. The partial micelle particle fusion and inter particle rearrangements happening during cold storage resulted in more junctions and more bonds per junction.

In back extrusion viscometry, also termed as annular pumping, the sample was placed in a container at the base of universal testing machine. The rod or platen attached to Universal Testing Machine crosshead was driven down into the container and the change in force over time was recorded to assess the textural properties (Bourne, 2002).

Protein gelation has been found to be important to obtain desirable sensory and textural structures in food. Gelation phenomenon required a driving force to unfold the native protein structure, followed by an aggregation retaining a certain degree of order in the matrix formed by association between protein strands. Protein gelation could be achieved by heat, high pressure, acidification and use of salts which could cause modification in protein-protein and protein medium interactions. The characteristics of each gel were different and were dependent on factors like protein concentrations and degree of denaturation (Totosaus *et al.*, 2002).

Enhanced acidification to pH values below 4 could lead to body and texture defects such as gel shrinkage and syneresis (Jaros and Rohm, 2003).

Vasbender *et al.* (2003) proposed that disulphide cross linking was highly relevant for textural properties of acid milk products like yogurt. They also observed that sulfhydryl group disulphide bond interchange reactions took place at ambient temperature and under acidic conditions.

Krasaekoopt *et al.* (2004) compared the texture of yogurt made from conventionally treated milk and UHT milk fortified with low heat skim milk powder. They reported that viscosities of yogurt decreased and firmness increased over the four week period of storage. They also opined that yogurt prepared from milk with high (50 per cent) protein denaturation had lower viscosity. Gel strength of undisturbed yogurt showed an increasing trend under storage. They proposed that increased syneresis would have caused the leveling out of gel strength.

Syneresis and firmness of yogurt increased/varied greatest during the first days of storage and subsequently tended to level out. During long term storage over acidification and increase in hydration of casein were the main causes for this (Sodini *et al.*, 2004).

Yogurt sensory firmness versus instrumental firmness had a nonsignificant correlation, probably due to greater difficulty in evaluating the firmness of this type of yogurt which was softer. Sensory maintenance of shape provided a better texture attribute. They also noticed that increase in syneresis was more noticeable in first few days of storage (Salvador and Fiszman, 2004).

Amatayakul *et al.* (2006) found that firmness and syneresis of set yogurt decreased when casein to whey protein ratio was reduced from 4:1 to 1:1. They suggested that physical characteristics of set and stirred yogurt could be improved by varying casein to whey protein ratio and by the use of exopolysaccharide producing cultures.

Firmness value hardly increased during cold storage probably because test applied was not sensitive enough to effect the rearrangements that occured at the particle-particle level (Serra *et al.*, 2009).

2.3.1.4 Shelf-life of Dahi

Iyengar *et al.* (1967) concluded that fermented milk products could be stored at 5°C for a period of one week without any deterioration in quality.

Vargas *et al.* (1989) focused their study on the shelf life prediction of soy-whey yogurt. They found that flavour and tyrosine value could be successfully used to determine the end point of storage.

Al-Kadamany *et al.* (2002) reported that shelf life of concentrated yogurt, Leben ranged from 8.5 to 10.5 days at a storage temperature of 5°C. They observed that end of shelf life of leben was accompanied by an increase in free whey by 10 to 15 per cent. Acidity increased by 0.5 to 0.6 per cent lactic acid and pH showed a drop of 0.3 to 0.4 units.

Evaluation of shelf life of dahi by Kamruzzaman *et al.* (2002) showed that product remained acceptable for 3 days and 12 days at room temperature and refrigerated storage respectively.

Yadav *et al.* (2007) subjected low fat probiotic dahi to storage studies. Under refrigerated condition, total viable count increased for two days and then decreased. Dahi samples did not develop much acidity under storage conditions. After eight days of storage, samples were disliked by the panel due to the bitterness resulting from proteolysis. They also reported that αS_1 casein degraded more than β-casein.

2.3.1.5 Sensory Evaluation

Amerine *et al.* (1965) considered sensory evaluation as the measurement of a product's quality based on the information received from the five senses. The signals generated at the nerve endings of the sense organs transmitted via the Central Nervous System to the brain where they were integrated with the past experience, expectations and other conceptual factors before the opinion of response was summarized.

Sreenivasan and Ranganathan (1972) reported that dahi samples examined after 24 h of storage exhibited thick consistency and a mild pleasant flavour. Those samples examined after 72h of storage had an unpleasant smell accompanied by slight gassiness and bitterness.

Rangappa and Achaya (1973) reported that initial acidity and physical properties of milk had a considerable effect on the texture of dahi and yogurt prepared from it. Milk stored for too long before seeding gave rise to broken curd with poor taste.

Discrimination test is a type of sensory test that is used to determine whether samples are detectably different from one another. This is a small panel test used in a laboratory environment. Descriptive tests were designed to describe sensory properties of products and measure the perceived intensity of those properties and express the attributes quantitatively (Sidel *et al.*, 1981).

Alkadamany *et al.* (2002) reported that in fermented milk products flavour changes occurred much earlier than texture defects. They suggested that flavour changes can be considered as a criterion to determine shelf life.

Praseeda (2005) evaluated the organoleptic qualities of curd under refrigerated storage for 21 days. The flavour score deteriorated with storage whereas body and texture scores remained more or less same. The color and appearance score also decreased significantly during storage.

2.3.2 High Temperature Processed Milk

UHT processing involves heating milk at a high temperature for a short time in order to obtain a product with a long shelf life at room temperature. During the process, most bacteria are inactivated but heat stable enzymes of native or bacterial origin can survive and cause serious defects during storage of milk (Valero *et al.*, 2001).

The term sterilization when used in association with milk means heating milk in a sealed container continuously to a temperature of either 115°C for 15 minutes or at least 130°C for a period of one second or more in a continuous flow and then packed under aseptic conditions in hermetically sealed containers to ensure preservation at room temperature for not less than 15 days from the date of manufacture (Prevention of Food Adulteration Act, Second amendment, 2007).

2.3.2.1 Acidity and pH

According to Samel *et al.* (1970), reduction in pH during storage of UHT milk was linked to the hydrolytic dephosphorylation of casein, changes in the calcium phosphate equilibrium and interaction between lactose and milk proteins.

Andrews *et al.* (1977) specifically ascribed the drop in pH during the Maillard reaction to the loss of positive charge on the protein molecules caused by the loss of free ЄH2 group of lysine.

Whitelaw and Weaver (1988) reported that only unprotonated amines could combine with sugars. An increase in protein concentration was found to cause an increase in heat induced acidity.

Maillard reactions resulted in the formation of organic acids that caused a fall in pH (Adhikari and Singhal, 1991).

According to Gothwal and Bhavadasan (1992) higher initial pH and higher protein level caused greater browning in milk.

Celestino *et al.* (1997) suggested that higher concentration of protein and possible contributory effect of unprotonated amino groups provided for more lactose-protein interactions which resulted in a greater increase in acidity with storage along with a concomitant decrease in pH.

Kelly and Foley (1997) reported a significant decrease in pH of high heat processed milk during storage. The degradation of lactose with formation of formic acid explained the decrease in pH.

The reduction in pH of UHT milk on storage was caused by precipitation of calcium phosphate, dephosphorylation of casein, breakdown of lactose and proteolysis (Gaucher *et al.*, 2008).

2.3.2.2 Proteolysis

Changes in the milk proteolytic system associated with severe thermal processes included whey protein denaturation, its complexation with caseins and inhibitor inactivation (Grufferty and Fox, 1986).

The shelf life of UHT milk sterilized was mainly limited by the action of proteinases or lipases or both from psychrotrophic bacteria. The indigenous milk proteinase and the physicochemical changes also affected shelf life (Mc Kellar, 1989).

According to Daniel *et al.* (1995) heat inactivation of proteinases was found to be due to conformational unfolding of proteinases (denaturation), self digestion (autoproteolysis) and irreversible non enzymatic covalent modification (deamidation).

Enright and Kelly (1999) concluded that severe heat treatment compatible with milk processing was not sufficient enough to eliminate proteinase activity. Heat treatments severe enough to eliminate proteolytic activity were detrimental to milk protein functionality.

Deeth *et al.* (2002) while assessing the spoilage patterns of skim milk and whole milk observed that skim milk exhibited predominantly bitter flavours while whole milk showed mostly sour flavours. They proposed that different spoilage behaviors could be because of the greater proteolysis in skim milk .This was due to the higher production of protease and greater susceptibility of protein to protease attack. Lipolysis in whole milk contributed to the different off flavour in these types of milk.

Topcu *et al.* (2006) monitored the proteolysis and the corresponding storage stability of UHT milk produced. During the 180 days storage study, proteinase and plasmin activity were not detected in the UHT milk samples as the spectrophotometric assays were not sensitive enough to detect low levels of activity. Total solids, protein and fat content were almost constant during storage

period. From their observations they concluded that the effects of raw milk quality, storage period and heating temperature had no significant effect on chemical composition of milk.

2.3.2.2.1 Measurement of Proteolysis

Law *et al.* (1977) observed that Hull method (1947) can be used successfully to show a correlation between proteolysis and off flavour in milk; but was unable to detect proteolysis in unspoiled milk. A similar trend was observed when proteolysis was measured as an increase in non protein nitrogen.

Mottar (1981) noticed a significant increase in NPN content during storage of UHT milk. The increase in NPN was attributed to the decomposition of proteins by reactivated proteolytic enzymes that may be indigenous, or of bacterial origin.

Mc Kellar (1981) found that off flavour development in milk due to proteolysis could be measured in terms of TCA soluble free amino groups that are being released. He used trinitrobenzene sulfonic acid (TNBS) successfully, to detect proteolysis in milk before development of off-flavour and suggested this method to be used as an indicator of shelf life.

Church and Swaisgood (1983) developed a rapid sensitive and convenient spectrophotometric assay for measurement of proteolysis of milk proteins in buffered solutions and in milk. α-amino groups released by proteolysis/hydrolysis reacted with O-phthaldialdehide and β-mercaptoethanol to form an adduct that absorbed strongly at 340 nm. Inclusion of SDS in the assay provided a convenient way to terminate proteolysis and ensured full exposure and complete reaction of amino groups.

Mistry and Kosikowsky (1983) tried a dialysis technique for measuring proteolysis in milk. In this method, the dialysis bag was used as a reaction chamber for the simultaneous separation and measurement of end products of enzymatic activity. The substrate along with the enzyme was incubated in a dialysis bag at 37°C. The tyrosine content of outside water was determined using Folins reagent.

Mitchell and Ewings (1985) reported that UHT milk that exhibited a bitter taste showed increase in NPN content from 0.03per cent to 0.06 per cent.

Lopez *et al.* (1993) reported that proteolytic indices like free amino groups, PAGE of casein and reverse phase HPLC of fractions soluble at pH 4.6 can be used to monitor proteolytic degradation. Proteolysis in skim milk was more when compared to whole milk. They also observed an increased activity of native milk proteinase and bacterial proteinase in UHT skim milks.

For assessing the protease activity, 5 µl of crude extract was spotted on to skim milk agar (one per cent skim milk, 0.02per cent sodium azide and 2per cent agar). After incubation at 37°C for 18 h, zone of clearance was looked into (Chantawannakul *et al.*, 2002). According to Cooper (1963), activity of biological substances (antibiotics and enzymes) can be expressed in terms of square of diameter of clear zone.

2.3.2.2.2 Defects Due to Proteolysis

Heat resistant proteases produced by psychrotrophic bacteria of dairy origin spoiled sterile milk with the development of bitter flavour, clearing or coagulation. The susceptibility of sterile milk to protease increased during storage of milk (Adams *et al.*, 1975).

Adams *et al.* (1976) reported that K- casein and β-casein fractions were not susceptible to the proteases liberated by gram negative psychrotrophs of raw milk. They also observed that milk suffering extensive kappa casein degradation coagulated during UHT treatment.

Age gelation, a main problem associated with the keeping quality of UHT milk has been attributed to products of proteolysis by indigenous and microbial enzymes. Polymerization initiated by Maillard type reactions also contributed to this defect (Andrews, 1977).

Continuation of the maillard reactions during storage of UHT processed milk lead to cross linking of protein chains which eventually caused gelation.

Onset of gelation depended on storage time and temperature. The reaction of lactose with amino groups of micellar proteins especially K-casein was found to have effects on the stability of these proteins during storage (Turner *et al.*, 1978).

Mc Kellar (1981) studied the development of off-flavour in UHT milk and pasteurized milk as a function of proteolysis. He observed that UHT milk was approximately twice as sensitive as pasteurized milk to the action of proteolytic enzymes, but unlike pasteurized milk, it did not coagulate when exposed to high concentration of enzyme. Proteolysis could be detected even before the development of off-flavours.

Janzen *et al.* (1982) confirmed a linear correlation existing between relative protease activity and flavour score in both skim milk and whole milk.

Mc Kellar *et al.* (1984) reported that age gelation was not observed in indirectly heated UHT milk.Viscosity remained constant throughout 30 weeks of storage. Gelation was observed at a viscosity of 100 mPaS between 6 and 10 weeks in directly heated UHT milk.

When purified proteinase preparations from *P. fluorescens* strains were added to UHT milk, Mitchell and Ewings (1985) reported the following sequential stages in flavour development; fresh, lack of freshness, slightly stale, unclean or mild off-flavour, slightly bitter and bitter.

Kocak and Zadow (1985), described gelation as a two stage process where proteolysis was followed by non enzymatic physico-chemical changes. The rate of physico-chemical reactions and enzymatic processes were faster at higher storage temperatures.

During UHT heat treatment of milk, activity of plasmin significantly decreased due to thiol-disulphide interactions between β-lacto globulin and plasmin that occurred during the unfolding and denaturation process at high temperatures (Grufferty and Fox, 1986).They also reported that the proteolysis of

casein caused by plasmin is responsible for gelation and bitterness of UHT milk during storage.

According to Renner (1988), a psychrotrophic population as low as 10^4 cfu/ml in raw milk, kept under refrigeration for more than 72 h can produce thermo resistant proteinases that could significantly limit the shelf life after UHT treatment.

Lemieux and Simard (1992) observed that hydrophobic peptide level was highly related with hydrolysis of β-casein, and the extent of hydrolysis of β-casein was positively correlated to bitterness. Peptides produced by bacterial proteinases were less hydrophobic while that produced by plasmin were more hydrophobic.

Celestino *et al.* (1997) found that it was difficult to determine exactly the proteinase activity at which samples gelled because even prior to gelation, some samples showed no proteolytic activity. Hence they proposed the possibility of a physicochemical effect contributing to gelation. Reactions resulting in structural changes of casein micelles also played a significant role in the gelation phenomena

Celestino *et al.* (1997) observed that a small proportion of reconstituted UHT milk gelled at 4.5 to 6 months of storage at 25 ± 1°C with viscosities ranging from 50 to 60 mPaS. The increase in viscosity with time was indicative of progressive denaturation and unfolding of proteins. Gradual aggregation of protein micelle leads to formation of a coagulum.

Sorhaug and Stepaniak (1997) reported that raw milk psychrotroph population of 6.9 to 7.2 log cfu/ml caused gelation and bitter flavour in UHT milk.

Datta and Deeth (2001) observed that bacterial proteinases hastened the gelation process by facilitating the release of β-lactoglobulin K-casein complex from the casein micelle to form a protein network gel. They opined that during gelation changes in viscosity happened in four stages: a short period during which thinning of product occurred; a longer period where there was little change in viscosity; a sudden change in viscosity with eventual gel formation; final stage

wherein viscosity dropped as gel matrix broke up leaving a serum layer and protein curd. They also found that more severe heat treatment delayed the onset of gelation.

Datta and Deeth (2003) reported that proteolysis in UHT milk caused bitter flavour and age gelation. They found that native milk alkaline protease, plasmin and heat stable extra cellular proteinases of psychrotrophic bacterial contaminants were responsible for the proteolysis.

Topcu *et al.* (2006) reported a high level of proteolysis in UHT milk produced from low quality raw milk with high somatic cell and psychrotrophic counts, when stored at room temperature. Defects like bitterness gelation and sedimentation resulting from proteolsis were also observed.

2.3.2.3 Sensory Evaluation

Driessen (1983) observed that cooked aroma and cooked flavour, the most prominent parameters in UHT milk dissipated with time. By 61 days of storage, bitter flavour and lingering after taste became apparent. He opined that these characteristics may be a consequence of protease and lipase activities that survived UHT conditions.

Off-flavours in heated milk often resulted from formation of organic sulphur compounds arising during the decomposition of reactive protein sulphydryl groups. Concentration of aminoacids like methionine and cysteine, have been correlated to the sensory detection of cooked flavour (Swaisgood *et al.*, 1987).

Traditional dairy judging has been criticized for failure to predict consumer acceptance, lack of objectivity in quality assessments, difficulty in assignment of quantitative scores and lack of utility for combining analytically oriented attribute ratings with quality scores (Claassen and Lawless, 1992).

Proteolysis of milk resulted in accumulation of small peptides that lead to the development of astringency and bitterness (Ma *et al.*, 2000).

Thermal processing of milk affected parameters related to product functionality. Heat treatment caused milk fat globule membrane proteins and whey proteins to unfold such that buried sulphydryl groups that were normally masked in native protein were exposed to outer surfaces. Cooked flavour was attributed to the changes in sulphydryl content and disulphide content of protein fraction (Hoffman and VanMill, 1997).

Chapman *et al.* (2001) conducted quantitative descriptive analysis (QDA) and principal component analysis for sensory characterization of ultrapasteurized milk. They observed that perception of bitterness had the most dramatic effect on overall quality perception. QDA was based on the ability to train panelists to measure specific attributes of a product in a reproducible manner to yield a comprehensive quantitative product description amenable to statistical analysis. QDA required replicate judgment to monitor subject performance quantitatively throughout the course of test.

2.4 INDUSTRIAL PROTEASES/ALKALINE PROTEASES

In all living organisms proteolytic enzymes were ubiquitous in occurrence, as they were essential for cell growth and differentiation. Microorganisms elaborated a large array of proteases both intracellular and/or extracellular. Intracellular proteases were important for various cellular and metabolic processes, such as sporulation and differentiation, protein turn over, maturation of enzymes and hormones and maintenance of cellular protein pool. Extracellular proteases hydrolysed protein in cell free environment and enabled the cell to absorb and utilize hydrolytic products (Kalisz, 1988).

Proteinases can be differentiated using selected inhibitors. Diisopropyl fluorophosphates (DFP) or Phenyl methyl sulfonyl fluoride (PMSF) inhibited serine proteases; E-64 (L-3carboxyl-2,3-trans-epoxypropionyl-leucylamido-4 guanidobutane) inhibited cysteine proteinases; Pepstatin inhibited aspartic proteinases; and 1,10-Phenanthroline or Ethylene diamine tetra acetic acid (EDTA) inhibited metalloproteinases (Salvesan and Nagase, 1989).

Cell immobilization for *B. firmus* 44 (Landau *et al.*, 1997), aqueous two phase system (ATPase) composed of polyethelene glycol (PEG) – (4000, 6000, 9000) and potassium phosphate for *B. thuringiensis*, ATPase system composed of PEG-6000 and dextran T500 using *B. licheniformis* (Lee and Chang, 1990) and solid state fermentations (Kaur *et al.*, 2001) were used for improving protease production.

Microbial enzymes were often more useful than enzymes derived from plants and animals because of their great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes were more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Cheetham, 1995; Wiseman, 1995).

Microbial proteases were classified into various groups depending on whether they are active under acidic neutral or alkaline conditions and based on the characteristics of the active site group. Accordingly, they could be metallo – (EC3.4.24), aspartic – (EC.3.4.23), cysteine or Sulphydryl- (EC.3.4.22) and serine (EC.3.4.21) (Rao *et al.*, 1998).

Subtilisin like serine proteases was generally bacterial in origin although there were reports in favor of other organisms also. They were generally secreted extracellularly for the purpose of scavenging nutrients. Subtilases were found to be specific for aromatic or hydrophobic residue (at position P1) such as tyrosine, phenylalanine and leucine. They were highly sensitive to phenylmethyl sulphonyl fluoride and diisopropyl-fluorophosphate. With a molecular weight in the range 15 to 30 kDa, they were most active around pH 10 (Graycar, 1999).

Proteases were essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. They could be cultured in large quantities in a relatively short time by established methods of fermentation. Microorganisms

accounted for a two-third share of commercial protease production in the world (Kumar and Takagi, 1999).

2.4.1 Environmental Conditions Affecting Protease Production

Frankeena *et al.* (1986) observed that there was a link between enzyme synthesis and energy metabolism in *Bacillus*. They also found that metabolism in *Bacillus* was controlled by temperature and oxygen uptake.

Moon and Parulekar (1991) proposed that for maximum enzyme production, organisms should be provided with optimal growth conditions. They reported that lowering the aeration rate from 7.0 l/min to 0.1 l/min, during incubation resulted in a drastic reduction in the protease yield of *Bacillus firmus*. They concluded that, the culture conditions that promoted the production of enzyme were different from that which promoted cell growth.

Shimogaki *et al.* (1991) succeeded in isolating an alkaline protease from *Bacillus* species. They grew the organism in a medium containing 50 g $MgSO_4$ $7H_2O$ and 10 g Na_2CO_3 per litre. Enzyme was harvested after incubation at 35°C for 3 days.

Sinha and Satyanarayana (1991), found no significant variations in the protease yield of *Bacillus* species at an inoculum density of 0.5 to 8 per cent. At higher levels of inoculation, a slight decline in activity was observed. The age of inoculum did not have a marked effect on the secretion of protease. The maximum protease production from *Bacillus licheniformis* strain N3 was at pH 7.0 to 8.0.

When inoculation was done at a level of three per cent, *Streptomyces diastaticus* under shake flask conditions gave maximum protease yield of 280 U/ml after 72 h of incubation at 30°C (Chaphalker and Dey, 1994).

Darah and Ibrahim (1996) reported that excessive aeration during fermentation lead to cell lysis and increased cell permeability due to abrasion by shear forces.

Chantawannakul *et al.* (2002) attempted to characterize the protease enzyme of *B.subtilis* isolated from soyabeans. For harvesting the enzyme, organism was grown in nutrient broth at 37°C for 24 h. The cell free supernatant collected after refrigerated centrifugation (4°C/10,000 rpm/5 minutes) served as crude enzyme. After studying the cell morphology during the different growth phases they opined that production of protease reached the highest during exponential phase and remained constant when spores were formed.

Lee *et al.* (2002) also observed denaturation of enzymes and subsequent reduction in activity at high agitation rate during incubation.

Generally, proteases produced from microorganisms were constitutive or partially inducible in nature. Under most culture conditions, *Bacillus* species produced extracellular proteases during post exponential and stationary phases. Physical factors like aeration, inoculum density, pH, temperature and incubation period affected the amount of protease produced. The pH of the culture medium was found to strongly affect many enzymatic processes and transportation of compounds across the cell membrane (Puri *et al.*, 2002).

Joo *et al.* (2003) reported that for *B. clausii,* maximum protease activity was observed when it was grown in a baffle flask, in a shaking incubator (250 rpm) at 37°C for 48 h.

Naidu and Devi (2005) reported that a *Bacillus* spp isolated from soil produced maximum alkaline protease after 96 h of incubation at 55°C in a media containing rice bran at pH 9. The rate of inoculum was four per cent.

Olajuyigbe and Ajele (2005) reported that *Bacillus macerans, B.licheniformis* and *B. subtilis* were capable of growing at temperatures upto 65°C within a broad pH range of 5.0 to 10.0. Their optimal growth temperature and pH were 60°C and 8.0. When incubation was done at 60°C, maximum enzyme production was observed at 48 h when grown in a medium with pH 8.0.

Shafee *et al.* (2005) reported that at a rate of four per cent inoculum, maximum activity of 0.923 µg / ml/ min was obtained after 48 h of incubation in a shaking incubator at 37 °C. The increase in protease production using small sizes of inoculum is suggested to be due to the higher surface area to volume ratio. At higher rates of inoculum of 5 per cent and 6per cent, protease activity decreased even though there was luxurious growth of organism. This can be attributed to the reduced dissolved oxygen and increased competition towards nutrients.

Banik and Prakash (2006) harvested a detergent compatible alkaline protease enzyme of *B. cereus* after 72 h of incubation at 30°C in a shaking incubator (200 rpm).The pH of the medium used was 7.5.

Prakasham *et al.* (2005) attempted the optimization of alkaline protease production by *Bacillus* species using Taguchi methodology. The pH of the medium was observed to be the most significant factor among all selected optimization parameters at an individual level. The combinational influence of least significant factors: inoculum level and salt solution concentration resulted to an interacting severity index of 76per cent suggesting their interactive role in the regulation of protease production. They found that protease production can be improved more than 100 per cent with Taguchis optimized conditions of medium composition.

The alkaline protease enzyme of *Bacillus halodurans*, an alkaliphilic bacterium from Egyptian Soda Lake, was obtained after culturing the organism in an alkaline media containing skim milk at 50°C for 48 h (Ibrahim *et al.*, 2007).

2.4.2 Nutritional Factors Affecting Protease Production

2.4.2.1 Nitrogen Sources

Chandrasekharan and Dhar (1983) used tapioca starch hydrolysate and soyabean meal along with other components to formulate a medium for production of alkaline protease. They reported an activity of 800 U/ml at the end of 120 h of incubation.

Cruegar and Cruegar (1984) reported that alkaline protease production was repressed by rapidly metabolisable, nitrogen sources like amino acids or ammonium in the medium. Kole *et al.* (1988) observed that both inorganic and organic forms of nitrogen were metabolized primarily to produce aminoacids, nucleic acids, protein and cell wall components. The alkaline protease comprised of 15.6 per cent nitrogen and its production was mainly dependant on the availability of carbon and nitrogen sources in the medium.

An increase in protease production by the addition of ammonium sulphate and potassium nitrate was observed by Sinha and Satyanarayana (1991).According to Banerjee and Bhattacharya (1992) sodium nitrate stimulated protease production.

Banerjee and Bhattacharya (1992), in their attempt to optimize the culture conditions for the production of an industrially important alkaline protease from *Rhizopus oryzae*, found that organic nitrogen sources gave better yields than inorganic nitrogen sources. However inorganic salts were preferred for industrial production because of the low costs involved. Mc Kay (1992) reported that whey alone can be used for the production of alkaline protease enzyme.

Any change in the pH of medium during fermentation indicated utilization of substrates or production of metabolic products. The direction of change in the pH value mostly corresponded to the consumption of nitrogenous substrates. When ammonium ions were used, medium turned acidic. When organic nitrogen like amino acids or peptides were consumed, media turned alkaline. The pH variations during fermentation can provide kinetic information about protease production (Chu *et al.*, 1992).

Tryptone (two per cent) and casein (one to two per cent) also served as excellent nitrogen sources of protease production (Phadatare *et al.*, 1993).

Kumar (1997) reported that among the various organic nitrogen sources tested, *Bacillus lentis* strain MK5-6 exhibited maximum protease activity when 0.5

per cent tryptone was added to the basal fermentation medium comprising of whey and corn steep liquor.

Extracellular protease production by *Bacillus* spp was strongly influenced by media components: variation in C/N ratio, presence of easily metabolisable sugars, metal ions and rapidly metabolisable nitrogen sources like amino acids (Puri *et al.,* 2002).

According to Naidu and Devi (2005) the best nitrogen source for protease production for *Bacillus* species was beef extract, while yeast extract and tryptone exhibited almost similar effect. Addition of inorganic nitrogen sources in the production medium resulted in low enzyme yield.

While assessing the effect of various nitrogen sources on the production of protease enzyme by *Bacillus* spp, Nascimento and Martins (2004) observed that maximum activity was obtained when ammonium nitrate was used in the medium. Moderate to good level of enzyme activity was obtained when ammonium chloride, ammonium citrate and potassium nitrate were used as nitrogen sources. Organic nitrogen sources were found to repress the enzyme activity although growth was stimulated.

Shafee *et al.* (2005) studied the effects of various nitrogen sources on production of protease by *B. cereus* strain 146. They observed maximum protease activity with beef extract. Organic nitrogen sources like yeast extract peptone and tryptone enhanced bacterial growth but repressed protease production. Ammonia specific repression of protease was also reported by them. They also observed a high production of protease in the presence of lysine and glycine. When ammonium carbonate was used as a nitrogen source, maximum protease activity was obtained at 24 h of incubation which got reduced after 48 to 72 h.

2.4.2.2 Carbon Sources

Frankeena *et al.*. (1986) reported catabolite repression as a phenomenon in which the synthesis of inducible or constitutive enzymes was repressed by glucose

or other rapidly metabolisable carbon sources. They observed catabolite repression by glucose in *Bacillus licheniformis.*

Takii *et al.* (1990) reported that organic acids like citric acid or sodium citrate can be used as the sole source of carbon for production of proteases at alkaline pH. Use of organic acids was interesting in view of their economy and ability to control pH variations.

Malathy and Chakraborthy (1991) and Phadatare *et al.*, (1993) also reported the positive influence of lactose maltose and sucrose on the yield of alkaline protease from *Bacillus* species.

Mehrotra *et al.* (1999) screened the alkalophilic bacterial strains obtained from saline-alkali soil for their ability to produce alkaline protease. The most potent producer was identified as *Bacillus* spp. The organism exhibited maximum enzyme activity in the presence of one per cent glucose and one per cent ammonium chloride at pH 10.5, when incubation was done at 40°C for 20 h.

Mahmood *et al.* (2000) reported that *Bacillus* species possessed high level of protease expression. They suggested that more protease was synthesized when the carbon sources available in the medium were used poorly for growth purposes.

Johnvesly and Nailk (2002) found that citric acid also is a good carbon source for *Bacillus* spp JB-99. According to these authors, culturing the organism in one per cent glucose (w/v) repressed the synthesis of alkaline protease completely.

Nascimento and Martins (2004) observed the growth pattern of a thermophilic *Bacillus* spp SMI-2 along with protease production. They found that *Bacillus* spp. grew very fast and the formation of protease started from 5 hrs of growth and reached a maximum of 1.93 U/mg protein in 9 h and then began to fall. They suggested that production of enzyme could be correlated with the onset of a high rate of protein turnover during sporulation. They also observed that the

best carbon source for protease production were starch and trisodium citrate. One per cent glucose was found to improve protease production moderately.

Naidu and Devi (2005) reported that wheat bran maximally supported the alkaline protease production by a *Bacillus* spp. isolated from the soil of detergent industry. Rice bran gave comparable results along with casein and soya meal. Among the ten carbon sources studied, starch, sucrose and lactose proved to be appreciably good for protease production.

Shafee *et al.* (2005) conducted a trial to find out the effects of carbon sources on protease production by *Bacillus cereus* strain 146. They found that glucose induced highest protease production when compared to other carbon sources at 24 and 48 h of incubation. Maltose, starch and cellobiose poorly supported the production of protease.

2.4.2.3 Salt Supplementation

Potassium phosphate at a concentration of 2 g/l was found to be the ideal choice of phosphate source for protease production, possibly because of their buffering action (Moon and Parulekar, 1991). When phosphate concentration exceeded 4 g/l, precipitation of media occurred during autoclaving.

Phadatare *et al.* (1993) observed that this problem could be overcome by the supplementation of disodium salt of EDTA.

Banerjee and Bhattacharya (1992) reported that salts like calcium, sulphate, phosphate, iron, magnesium, manganese, copper, cobalt, molybdenum, potassium and boron were helpful in enhancing protein yields. A three fold increase in protease activity was reported in *Rhizopus oryzae* by the use of $AgNO_3$ at a concentration of 0.05 mg/100 ml. They also observed that $ZnSO_4$ at a level of 125 mg/100 ml improved protease production. The combined use of $ZnSO_4$ and Tween 80 resulted in 2.5 fold increase in protease production.

Ferrero *et al.* (1996) suggested the use of $MgSO_4$, $CaCl_2$, $MnSO_4$ and $ZnSO_4$ along with trisodium citrate for protease production by *Bacillus licheniformis* MIR 29.

Kumar (1997) investigated the effect of various inorganic salts like. KH_2PO_4, K_2HPO_4, $MgSO_4$, NaCl, $Na_3C_6H_5O_7$, $CaCl_2$, KCl, $FeSO_4$, $FeCl_3$, $CoCl_2$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$ at different levels in a medium containing one per cent whey powder, 0.25 per cent cornsteep liquor, 0.5 per cent tryptone, one per cent glucose and one per cent sodium carbonate (pH-10). Most of the salts showed enzyme values comparable to that of control. Salts like KH_2PO_4, KCl, $CaCl_2$, $FeCl_3$, $MnCl_2$, Na_2HPO_4, and NaH_2PO_4 exhibited lower enzyme production. A combination of sodium citrate and $MgSO_4$ at 0.2 per cent and 0.02 per cent levels respectively resulted in 19 per cent higher enzyme yields than sodium citrate alone.

Adinarayana *et al.* (2003) succeeded in harvesting a thermostable serine alkaline protease from *Bacillus subtilis*- PE-11 using a culture medium containing glucose 0.5 per cent, peptone 0.75 per cent and salt solution 5 per cent ($MgSO_47H_2O$ 0.5 per cent, KH_2PO_4 0.5 per cent and $FeSO_47H_2O$ 0.01 per cent)

For extracting an oxidant and SDS stable alkaline protease from *Bacillus clausii* 1-52, Joo *et al.* (2003) utilized a growth media comprising of K_2HPO_4, 4(g/l); Na_2HPO_4, 1(g/l); $MgSO_47H_2O$, 0.1(g/l); and Na_2CO_3, 6(g/l).

Johnvesly *et al.* (2001) used a chemically defined basal medium consisting of $NaNO_3$ 5.0 (g/l), K_2HPO_4 5.0 (g/l), $MgSO_42H_2O$ 0.2 (g/l) and Na_2CO_3 10 (g/l) for the production of a thermostable alkaline protease from thermoalkalophilic *Bacillus* spp. JB – 99.

Olajuyigbe and Ajele (2005) used a medium comprising of 0.5 per cent glucose, 0.75 per cent peptone, 0.5 per cent $MgSO_4$ 7 H_2O, 0.5 per cent KH_2PO_4 and 0.01 per cent $FeSO_4$ $7H_2O$(w/v).for studying the production dynamics of extra cellular protease from *Bacillus* species.

Supplementation of culture medium with metal ions substantially improved the protease production of *B. cereus* strain 146. Highest level of protease activity (2 μg/ml /min) was observed in the presence of Mn^{2+} at 24 h. As incubation time increased, the activity reduced. Addition of Ca^{2+}, Cu^{2+} and Mg^{2+} resulted in high protease production only at 48 h incubation. The heavy metal ions Cu^{2+} and Li^{+} caused inhibition at 4 h of incubation (Shafee *et al.*, 2005).

2.4.3 Recovery of Enzymes

The removal of cells, solids and colloids from the fermentation broth is the primary step in the recovery of extra cellular enzymes. Anustrup (1980) recommended the use of flocculating agents to effect the formation of larger agglomerates so as to accelerate the solid – liquid separation. This step was found to prevent clogging of filters. He also reported that ammonium sulphate can be used for the inorganic salting out process of proteins when the media is acidic or neutral.

Ultrafiltration was a preferred alterative to evaporation for the recovery of enzymes. This pressure driven separation process offered purification, as a result of molecular separation and concentration. The major disadvantage of this process was the fouling or clogging of membrane due to the precipitates in the final product (Sullivan *et al.*, 1984).

For the recovery and purification of protease enzyme from *Bacillus stearothermophilus* RM-67 Chopra and Mathur (1985) inoculated the organisms at a level of 4 per cent into tryptone yeast extract salt medium. The cell free supernatant was collected after refrigerated centrifugation at 7000 rpm for 30 min. The 40 to 70 per cent precipitate obtained after saturation with solid ammonium sulfate was collected, dissolved in 0.05 M phosphate buffer (pH 7.5) and dialysed against the same buffer for 24 h. The dialysate was subjected to gel filtration by Sephadex G-100 Pooled active fractions recovered were further passed through Diethylaminoethyl (DEAE)) sephadex A-50 column (30 x 2.5 cm) which was equilibrated with .02 M KCl in 0.05 M phosphate buffer (pH 7.5). The enzyme

was eluted subsequently with a linear gradient of 0.02 to 0.15 M KCl. The pooled fractions with an activity of 300 µg/ml were subjected to disc electrophoresis (3.5 mA for 3 h).

Durham *et al.* (1987) succeeded in isolating two alkaline proteases from *Bacillus* spp GX6638 using ammonium sulphate precipitation, desalting and gel filtration chromatography.

Shih *et al.* (1992) suggested the use of sodium sulphate and organic solvents for protein recovery. Despite better precipitating qualities, use of sodium sulphate remained restricted because of its poor solubility.

The extracellular alkaline protease of *B. lentus* strain MK5-6 was extracted with 2.5 volumes of acetone and further purified by Diethylaminoethyl (DEAE) and CM-Sepharose CL-6B ion exchange chromatography, and Fast Protein Liquid Chromatography (FPLC) linked sephacryl S.200 gel filtration chromatography. The four step purification scheme resulted in 3.8 fold purification of AP-1 with an overall recovery of 27.9 per cent and 5.1 fold purification of AP-2 with a recovery of 22.7 per cent. AP-1 and AP-2 were estimated to have molecular weights of 28000 and 29000 daltons respectively (Kumar, 1997).

Son and Kim (2002) used 60 to 80 per cent ammonium sulfate saturate of cell free supernatant for isolating caseinolytic extracellular protease from *B.amyloliquifaciens* S94. The dialysed ammonium sulphate pellet was applied to QAE-Sephadex column to eliminate the contaminating proteins. Fractions with caseinolytic activity were pooled and applied to SP-Sephadex column. The unadsorbed fractions with caseinolytic activity were eluted with a linear gradient of 0 to 0.7 M KCl in 20mM Tris-HCl buffer (pH-7.0). Further fractionation of elute with Sephacryl S-100 resulted in 48 fold purification of protease.

Adinarayana *et al.* (2003) succeeded in isolating a thermostable serine alkaline protease from *Bacillus subtilis* PE-11. After growing the organism in a defined media for 48 h, the cell free supernatant was collected by refrigerated centrifugation (10,000 rpm/ 4°C/30 min). The supernatant was fractionated by

precipitating with ammonium sulphate between 50 per cent and 70 per cent saturation. The precipitate was dissolved in 0.1 M Tris HCl buffer, subjected to Sephadex G-200 chromatography and equilibrated with Tris HCl buffer (pH – 7.8). The column was eluted at a flow rate of 60 ml/hr with a 1:1 volume gradient from 0.1 M to 1 M NaCl in the same buffer. Fractions with high protease activity were pooled, dialyzed and concentrated by lyophilisation.

Banik and Prakash (2006) resorted to extractive fermentation using aqueous two phase system with polyethelene glycol/potassium phosphate for the recovery of protease from *B. cereus*. The fermented broth of extractive fermentation was centrifuged at 10,000 rpm for 30 min at 4°C. Ammonium sulphate precipitation was carried out at 80 per cent saturation and centrifuged at 4°C for 20 min. The precipitated sample was desalted and concentrated on Amicon ultra-15 filter and then subjected to anion exchange chromatography equilibrated with the 25 mM Tris HCl buffer (pH-8.0). The column was eluted with the same buffer containing 150 mM NaCl. Fractions of one ml each were collected at the flow rate of 30 ml/h, active fractions were pooled and purity was assessed by 12.5 per cent SDS PAGE.

Vidyasagar *et al.* (2006) isolated the extracellular protease from the culture filtrate by alcohol precipitation and gel permeation chromatography. Purification of the halophilic protease was performed in the presence of 20 per cent NaCl. The protease was purified 116 fold with a final yield of 18 per cent.

Saxena *et al.* (2007) achieved the partial purification of amylase from a *Bacillus* spp. PN5 by precipitating the cell free supernatant with ammonium sulphate to 85 per cent saturation. The precipitate was further dissolved in 0.05 M glycine NaOH buffer with pH 10.0. It was then dialysed overnight against the same buffer. The dialysate served as the enzyme.

2.4.4 Charcteristics of Alkaline Proteases

Chopra and Mathur (1985) reported that two proteinases were produced by the same isolate of *B. stearothermophilus* from raw milk: RM-67 I proteinase, a

metalloproteinase with a molecular mass of 67.6 kDa and RM-67 II proteinase a serine proteinase with a molecular mass of 20 kDa; both had a pH optimum of 8.0.

Alkaline proteases (EC 3.4. 21-24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center or are of metallo type. Alkaline serine proteases are the most important group of enzymes exploited commercially (Kalisz, 1988).

Many detergents available in the International market contain proteolytic enzymes. Majority of these proteases are reported to be produced by the members of genus *Bacillus*.Subtilisins have been the enzyme of choice in detergent formulations (Samal *et al.*, 1989).

Shimogaki *et al.* (1991) observed that the alkaline protease BYA from the alkalophilic *Bacillus* species Y was stable at a wide pH 6.5 – 13.0 with pH 10.0 – 12.5 as optimum. The enzyme characterized as a serine protease remained stable in the presence of surface active agents like SDS and sodium alkylbenzene sulfonate. The enzyme with a molecular weight of 42,000 retained 50 per cent and 40 per cent activity when subjected to a temperature of 75°C and 80°C for 10 minutes.

Brown *et al.* (1993) proposed that for the proteases to be used as a detergent enzyme, it should possess the properties like: stability at relatively high temperatures (25 to 60°C); stability in the alkaline pH range (pH 9.0 to 11.0); stability in the presence of chelating agents, oxidizing agents, fluorescent dyes and surfactants; and also stability during the normal shelf life of detergent.

The detergent industry has been identified as the single major consumer of several hydrolytic enzymes acting in the alkaline pH range. The use of different enzymes as detergent additives arises from the fact that proteases can hydrolyse proteinaceous stains, amylases are effective against carbohydrate stains and lipases are active against oily or fat based stains. To be used as a detergent additive, the enzyme should possess activity in an alkaline pH and should be compatible with detergents (Anwar and Saleemuddin, 1998). Rao *et al.*, 1998 have reported that

serine proteases are well represented by various species of *Bacillus* like *B. amyloliquifaciens, B. licheniformis and B. subtilis.*

Anwar and Saleemuddin (2000) reported that alkaline protease from *Spilosoma oblique* can successfully be incorporated in biopharmaceutical products like contact lens cleaners and enzymatic debriders. Kim *et al.* (2001) characterized an alkaline protease of molecular weight of 36 kDa from *B. cereus.*

Serine proteases are of industrial significance because of their activity and stability at alkaline pH. They are characterized by a nucleophilic serine residue at the active site and are distinguished by having essential aspartate and histidine residues which along with serine forms the catalytic triad (Gupta *et al.*, 2002).

Fu *et al.* (2005) opined that for an enzyme to be used as detergent additive, it should be stable and active in the presence of typical detergent ingredients like surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids.

Recently the use of alkaline protease has increased significantly in various detergents and also in industrial processes like preparation of feed additives, dehairing, decomposition of gelatin on X-ray films and peptide synthesis. Of these, use as laundry detergent additive is one of the most important industrial application (Gupta *et al.*, 2005). Prakash and Banik (2005) estimated the molecular weight of alkaline protease of *B. cereus* as 28 kDa.

2.4.4.1 pH Stability

Durham *et al.* (1987) succeeded in isolating two alkaline proteases AS and HS from an alkalophilic *Bacillus* spp. strain GX6638 which showed optimal protease activities at a broad pH range of 8 to 12 at a temperature of 65°C. Protease AS exhibited extreme alkaline stability. It retained 88 per cent of initial activity at pH 12 over a 24 h incubation period at 25°C. Protease AS showed higher stability at pH above 9.5 and addition of Ca^{2+} extended the half life of enzyme by 10 fold at 60°C. At pH 9.5, the protease HS had a half life of more

than 200 min at 50°C and 25 min at 60°C. At pH above 9.5, enzyme HS readily lost the enzyme activity even in the presence of exogenously supplied Ca^{2+}.

The alkaline protease purified by Takami *et al.* (1990) from *Bacillus* species showed an optimum pH of 10.5 and 11.0 to 12.0 towards elastin and keratin respectively. The enzyme was maximally active towards casein at pH 12.5. It showed slight elastolytic activity at pH 12.0. The phenomenon was related to the binding function between the enzyme and substrate.

Shimogaki et *al.* (1991) studied the caseinolytic activity of protease BYA at 35°C over a wide range of pH ranging from 4.0 to 13.0. The enzyme showed maximum activity at pH of 10.0 to 12.5. They also found that protease BYA was more stable than subtilisin Carlsberg.

The thermostable alkaline protease from *Bacillus* spp B18 was stable at a wide range of pH 5.0 to 12.0. It retained 60 per cent of the initial activity at 70°C after 60 min incubation at pH 10.0. Another alkaline protease from *Bacillus* spp. No. 221 was reported to be maximally active at pH 11.5 to 12.0 and stable at pH values ranging from 4.0 to 12.0 at 60°C. It had a molecular weight of 30,000 daltons. Calcium ions were effective in stabilizing the enzyme especially at higher temperatures (Fujiwara *et al.*, 1993).

Sookheo *et al.* (2000) reported three proteases, S, N and B from *Bacillus stearothermophilus* TLS33, with optimum pH 8.5, 7.5 and 7.0 respectively. The protease S was very active over a broad range of pH with 60 per cent proteolytic activity detectable at pH 6 to 10 in the presence of 5 mM $Cacl_2$. In contrast, proteases N and B retained little activity above pH 9.0.

Johnvesly and Nailk (2002) studied the pH stability of alkaline protease of *Bacillus* spp. JB-99 by preincubating the enzyme in different buffers of pH 5 to13 for 2 h at 45°C. Enzyme had an optimum pH of 11. More than 84 per cent activity was retained at pH 12.0. They opined that high pH optimum is a distinctive feature of all alkaline proteases.

Lindsay *et al.* (2002) reported that tolerance to alkaline pH is a general trait for *B.cereus*. They observed that cells exhibited more pronounced stress symptoms at pH 10 than at pH above 10 possibly because a better buffering system was induced at pH 10.0 to 12.0.

The protease from *B. amyloliquifaciens* S94 displayed a broad pH activity profile in the neutral to basic range. At pH below 6.0, very little activity was present. A gradual increase in activity was seen above pH 7.0. The purified protease was maximally active at pH 10 against azocasein. Sixty three per cent of the maximal activity was observed at pH 11.0, but there was no activity over pH 12.0 (Son and Kim, 2002).

Protease enzyme from *B. clausii* 1-52 was found to have a pH 11 to be optimum for protease activity. The enzyme was stable over a wide pH, ranging from 5 to12. Eighty per cent of the maximum activity was lost below pH 4.5 and above 12.5 (Joo *et al.*, 2003).

Nascimento and Martins (2004) observed that optimum activity for the protease from a thermophilic *Bacillus* was at pH 8.0. At pH 6.5, 7.0 and 7.5, only 29 per cent, 53 per cent and 59 per cent of the maximum enzyme activity was present. After incubation of crude enzyme at pH 5.5, 8.0 and 9.0, for 24 h, there was a decrease in activity by 51 per cent, 18 per cent and 66 per cent respectively.

Aftab *et al.* (2006) characterized the alkaline protease enzyme of *Bacillus brevis* SSAI isolated from the soil of Karachi. The enzyme was found to be stable in the pH range of 7 to 12. Optimal pH and temperature for enzyme was 8.0 and 60°C respectively. Enzyme remained stable upto 80°C.

2.4.4.2 Temperature Stability

While assessing the effects of temperature on the stability of protease BYA, Shimogaki *et al.* (1991) observed that after exposure to a temperature of 60°C and 70°C for 10 minutes, the enzyme retained 90 per cent and 30 per cent of the native activity respectively.

The alkaline protease from *Bacillus* spp. B.18 was stable at temperatures ranging from 30°C to 70°C. It retained 50per cent of the initial activity at 80°C with complete inactivation at 90°C. Addition of Ca^{2+} enhanced the stability of enzyme at higher temperature (Fujiwara *et al.*, 1993).

Poffe and Mertens (1988) investigated the thermostability of psychrotrophic proteases. They observed that best inactivation was obtained at 55°C for 1 h. They proposed that increased thermoresistance at 80°C, 90°C and 100°C owe to the inactivation of protease inhibitors at high temperatures.

Sookheo *et al.* (2000) purified three thermostable zinc metallo proteases designated as S, N and B from *B. stearothermophilus* strain TLS33 using lysine affinity chromatography. The proteases S, N and B possessed molecular masses of 36, 53 and 71 kDa; exhibited maximum activity at 70°C, 85°C and 90°C respectively. Thermostability of all the enzymes was found to increase in the presence of Ca^{++}.

A protease enzyme from *Bacillus* species JB-99 retained 63 per cent and 25 per cent of the original activity after 1 h treatment at 70°C and 80°C. However in the presence of 10 mM Ca^{++}, the enzyme retained 83 per cent and 74 per cent of the original activity (Johnvesly *et al.,* 2001).

Chantawannakul *et al.* (2002) characterised the protease enzyme of *Bacillus subtilis* strain 38 isolated from traditionally fermented soyabean.The optimum pH and temperature for the enzyme activity was found to be 6.5 and 47°C respectively. The interference to the enzyme activity was highest at 60°C.

Son and Kim (2002) reported that protease from *B. amyloliquefaciens* S94 exhibited maximum activity with azocasein as substrate, at 45°C but was lost abruptly at temperatures above 50°C. At temperatures below 20°C, the enzyme maintained over 20 per cent of its maximum activity.

The alkaline protease of *B. clausii* 1-52 exhibited maximum activity at 60 to 65°C. The activity decreased rapidly above 70°C (Joo *et al.*, 2003).

Thermostability of protease from a thermophilic *Bacillus* species was assessed by Nascimento and Martins (2004). Results suggested that the enzyme was stable at 30°C for 2 h. When exposed to 40°C and 80°C for 2h, 14 per cent and 84 per cent of the original activity was lost

Ibrahim *et al.* (2007) succeeded in isolating an alkalophil, capable of producing alkaline protease from Wadi Natrum of northern Egypt. The crude alkaline protease of *B. halodurans* WN-SKS showed reasonable activity at temperatures 65 to 70°C with maximum activity at 70°C. A rapid decrease in enzyme activity was detected above 80°C and enzyme was completely inactivated at 90°C.

2.4.4.3 Stability in the Presence of Metal Ions

In an experiment conducted to find out the effect of various divalent cations on protease BYA activity, Shimogaki *et al.* (1991) observed that protease activity was markedly inhibited by mercuric chloride and copper sulphate (98 per cent and 74 per cent inhibition) and moderately by cadmium chloride (25 per cent inhibition). The activity was not affected by $CoCl_2$, $Nicl_2$, $Bacl_2$, $FeSO_4$, $CaCl_2$, $MgSO_4$, $Mncl_2$ and $Zncl_2$.

The silver and mercury ions hydrolytically degraded disulphide bonds (Torchinsky, 1981). Paliwal *et al.* (1994) reported that the cations Ca^{2+}, Mg^{2+} and Mn^{2+} enhanced the thermostability of *Bacillus* alkaline protease. The cations were known to protect the enzyme against thermal denaturation by playing a vital role in maintaining the active conformation of enzyme at higher temperature.

Kumar (1997) observed that at 1mM concentration Na^{+}, Mg^{++}, Mo^{++} and Zn^{++} enhanced the activity of enzyme AP-2 of *B. lentus* by 127.8 per cent, 126.2 per cent, 133.6 per cent, and 106.3 per cent respectively. Inhibitory effect of ions of mercury and cadmium was proposed to be due to its reaction with protein thiol groups converting them to mercaptides.

The thermostability of alkaline protease from *Bacillus brevis* enhanced in the presence of acetate salts of Ca^{2+} and Na^{+}, as it afforded protection against autolysis. Addition of Ca^{++} (10 mM) and glycine (IM) individually and in combination increased the shelf life of protease. The enzyme retained more than 50 per cent activity after four days at 60°C in the presence of Ca^{++} and glycine (Banerji *et al.*, 1999).

Kumar *et al.* (1999) reported that the metal ions of mercury, cadmium and lead reacted with the protein thiol groups converting them into peptides. They also found that silver and mercury ions degraded disulphide bonds.

Chantawannakul *et al.* (2002) characterised the protease enzyme of *Bacillus subtilis* strain 38 isolated from traditionally fermented soyabean. Out of the inhibitors tested, only 1,10-phenanthrolene specific for metalloprotease, inhibited enzyme activity.

In a trial to find out the effect of metal ions on the protease activity of *Bacillus sp.* JB-99, Johnvesly *et al.* (2001) observed that Ca^{2+}, Mn^{2+} and Cu^{2+} enhanced enzyme activity whereas Hg^{2+} markedly inhibited the activity. They also reported that in the presence of 1M and 5M NaCl at 45°C for 2 h, the protease retained 84 per cent and 41 per cent of activity.

Beg *et al.* (2003), reported that protease activity was stimulated by Mn^{2+} and Ca^{2+}. They suggested that metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of enzyme at higher temperatures. Similar effects of Mn^{2+} on the activity of protease was observed by Manachini *et al.* (1988), and Rahman *et al.* (1994)

Adinarayana *et al.* (2003) investigated the influence of metal ions on the stability of protease enzyme of *Bacillus subtilis* PE-11. They found that Ca^{++}, Mg^{++} and Mn^{++} increased and stabilized the activity possibly because of the enzyme activation by metal ions. They were also found to be capable of maintaining the active conformation of the enzyme at high temperature. Other

metal ions such as Zn^{++}, Cu^{2+}, Hg^{2+}, Na^{+}, Cd^{2+}, Al^{3+} did not show appreciable effect on enzyme activity.

Nascimento and Martins (2004) reported that the activity of protease from a thermophilic *Bacillus* spp. was inhibited in the presence of K^{+}, Cu^{2+} and Zn^{2+}. Mercury ions inhibited the enzyme completely at 1 mM concentrations.

Aftab *et al.* (2006) observed that Ca^{2+} and to a lesser extend Mn^{2+}, Fe^{2+} and Cd^{+2} had a stabilising effect on the alkaline protease enzyme of *B.brevis* SSA1. Zn^{2+} completely inhibited the enzyme whereas Ag^{2+} and Ni^{2+} had a partial inhibitory activity on the enzyme.

Vidyasagar *et al.*, 2006 reported that of the metal ions tested, Ca^{2+} stimulated the protease activity of *Halogeometricum borinquense.* Activity was found to be completely inhibited by Cu^{2+} and Zn^{2+}.

2.4.4.4 Effect of Inhibitors

Protease BYA from Bacillus spp was completely inhibited by active site directed inhibitors of serine protease like PMSF and DFP, indicating that enzyme was a serine protease (Shimogaki *et al.*, 1991).

Freeman *et al.* (1993) proposed that EDTA, a chelator of divalent cations reduced the activity of protease by removing the Ca^{++} that stabilized the tertiary structure. They suggested that loss of activity was due to enzyme denaturation rather than by inhibition.

Kumar (1997) investigated the effect of chemical inhibitors, organic solvents and denaturing agents on protease enzymes of *Bacillus lentus.* He observed that on preincubation with 0.1 mM PMSF, 5 mM β-mercaptoethanol and 10 mM EDTA, the enzyme activity reduced to 98 per cent, 96.7 per cent and 68 per cent of control.

The alkaline protease enzyme produced by *Bacillus* JB-99 was markedly inhibited in the presence of 1mM PMSF. This was suggestive of the enzyme being

a serine protease. Tosyl-L-Phenylalanine chloromethyl ketone (TPCK) at a level of 1mM could inhibit 15 per cent activity. No inhibitory effects were observed with EDTA and 1,10-phenanthrolene suggesting that enzyme was not a metalloprotease (Johnvesly *et al.*, 2001).

Son and Kim (2002) attempted to purify and characterize the caseinolytic extra cellular protease from *Bacillus amyloliquefaciens* S94. The inhibiton studies showed that the enzyme was insensitive to leupeptin and metal chelators like EDTA indicating that enzyme is not a metalloprotease. Pepstatin A, iodoacetamide and E-64 did not significantly affect its activity. The activity was completely abolished by PMSF and TPCK.

The protease enzyme of *B. subtilis* was inhibited by 1,10-phenanthrolene indicating that enzyme is a metalloprotease (Chantawannakul *et al.*, 2002).

Chen *et al.* (2004) reported that *Bacillus* strains produced extra cellular and intra cellular proteases and lipases. Inhibitor studies showed the presence of both serine and metalloproteases for *Bacillus* species. In general lipases were more thermostable than proteases.

Aftab *et al.* (2006) reported that alkaline protease from *B. brevis* SSA1 was stable in the presence of 5 mM SDS and oxidants like hydrogen peroxide, a common ingredient in bleach based detergent formulations.

Banik and Prakash (2006) purified the alkaline protease enzyme of *B. cereus* to homogenity. The detergent compatible alkaline protease of *B. cereus* was strongly inhibited by the serine inhibitors PMSF and pefabloc at 5 mM. Residual activity after 2 h at 37°C was 29 per cent and 5 per cent respectively. The cysteine type inhibitors like para chloromercuribenzoic acid and β-mercaptoethanol showed less inhibitory action. No inhibition was detected when metalloprotease inhibitor EDTA and aspartate protease inhibitor aprotinin was added. The enzyme was found to be a serine protease with a molecular weight of 28 kDa on SDS PAGE. Its maximum protease activity on casein was at a pH 10.5 and temperature 50°C.

Nogueira *et al.* (2006) reported that alkaline protease enzyme of *Teredinobacter turnirae* retained 376 per cent activity while the commercially available *Bacillus* protease (Sigma) retained 150 per cent activity when treated with one per cent SDS at 50°C for 45 minutes.

Inhibitors of sulphydryl protease ($HgCl_2$) and metalloprotease (EDTA) did not affect the activity of protease enzyme of *Halogeometricum borinquense*. Mercaptoethanol did not affect enzyme activity. The enzyme retained 53 per cent of maximum activity in the presence of 0.1 per cent sodiumdodecyl sulphate (Vidyasagar *et al.*, 2006).

Nilegaonkar *et al.* (2007) reported that protease enzyme from *B. cereus* MCM B-326 was inhibited 15 per cent by PMSF and 100 per cent by EDTA. Dithiothreitol (DTT) inhibited 97 per cent of the protease activity suggesting that the enzyme contained an S-S bond as a part of its monomeric structure.

2.4.5 Enzyme Based Cleaning Formulations

The soil accumulated on glass and stainless steel surfaces to a similar degree but had different accumulation characteristics. The rate of soil accumulation was linear on steel, and on glass, the mass of soil changed in early cycles, but remained unchanged thereafter (Moore *et al.*, 1951).

The composition of cold milk soil varied greatly with the surface, cleaning system and site within the equipment. Twomey (1975) reported that milk soil had a typical composition of five per cent water one per cent fat, 60 per cent protein and 32 per cent minerals.

The most widely employed industrial method to evaluate the cleaning and sanitizing efficiency for membrane processes was to determine its volumetric flux and the degree to which it returned to its original value after cleaning (Beaton, 1979). The complete restoration of water flux was used as an index of proper cleaning.

Alagezym and Morgunova, (1981) assessed the composition of accumulated milk solids on tubular and plate parteuriser. The values for protein and fat were eight per cent and five per cent for tubular type and 22 per cent and nine per cent for plate type.

The function of any cleaning system is to maintain the equipment in an optimal condition such that it won't impair the quality of the product at the level of plant operations. Studies on membrane fouling indicated that deposits were mainly proteinaceous in nature. Enzyme based cleaners marketed for cleaning membranes was found to rely on proteases to cleave and solubilise the protein foulant (Harper and Moody, 1981).

Whittaker *et al.* (1984) opined that enzymes could not act as satisfactory cleansing agents and did not fare well when used alone. Enzymes, because of its large molecular weight were unable to penetrate the biofilm effectively without the aid of surfactants and chelating agents. They suggested an enzyme – surfactant – chelating agent combination like Enzyme – EDTA – Triton X to be very ideal.

The suitability of an enzyme preparation for use in detergents mainly depended on its compatibility with the detergents at moderately high temperature. It had to be compatible with various detergent components and also with oxidizing and sequestering agents. Temperature stability was also desirable for the enzyme to be active in a wide range of cleaning temperature (Ward, 1985).

Traditional cleaning practices of high temperature and extreme pH cannot be tolerated by reverse osmosis /ultrafiltration systems made of cellulose acetate. Enzyme based cleaners relied on enzymes to cleave proteins into fractions small enough to be rinsed from the system. For this method of cleaning to be cost effective, the enzymes must be able to hydrolyse proteins at a reasonable rate (Smith and Bradely, 1987).

The stability of protease BYA in the presence of various surface active agents was looked into by Shimogaki *et al.* (1991). Proteases were incubated in 50 mM borax-NaOH buffer (pH 10.5) with SDS, alkylethoxylate and sodium

alkylpolysulfonate at a level of five per cent v/v at 4°C for 4 h. Residual activity was found to be 83 per cent, 91 per cent and 93 per cent respectively.

Composition wise, detergent preparations comprised mainly of an alkaline source (sodium hydroxide, sodium carbonate, sodium bicarbonates, and trisodium phosphate), a surfactant (Teepol, Triton 100 and Tween 80) and a chelating agent (sodium hexametaphosphate, EDTA).The alkali was meant to hydrolyze the proteins and saponify the lipids. The surfactant improved the penetrability of the detergent into the soil and the chelator helped in precipitating out the mineral residues present in soil (Rai, 1995).

The detergent industry has been the single major consumer of several hydrolytic enzymes acting in the alkaline pH range. Proteases hydrolyse proteinaceous stains, amylases were effective against starch and other carbohydrate stains while lipases were active against oily or fat stains. For an enzyme to be used as detergent additive it should be active in alkaline pH range as well as compatible with detergents (Anwar and Saleemuddin, 1998).

The alkaline protease enzyme harvested from the shake culture of *Bacillus brevis* showed compatibility with commercial detergents like Arial,Surf Excel, Surf Ultra and Rin in the presence of Ca^{2+} and glycine. The enzyme was found to improve the cleaning power of various detergents. It could remove blood stains completely when used in detergents in the presence of Ca^{++} and glycine (Banerji *et al.*, 1999).

Son and Kim (2002) monitored the effect of surfactants reducing agents and salts on the activity of protease from *B. amyloliquefaciens* S94. They found that neither SDS nor Triton strongly affected the enzyme activity. Reducing agent like β-mercaptoethanol did not influence the activity of enzyme. In the presence of 0.5 M salts (KCl, NaCl, NH_4Cl, LiCl) the enzyme retained more than 80 per cent activity.

Varghese (2002) evaluated the cleaning efficiency of an enzyme based cleaning formulation after artificially soiling the milk plates. Soiling was done with pasteurized whole milk as this was the most commonly encountered type of

soil. The author suggested the use of borax methylene blue and sudan III to spot the protein residues and fat residues respectively. Enzyme detergent was three times better than its alkaline counterpart in dislodging the soil from substrate.

Adinarayana *et al.* (2003) reported that the addition of 10 mM $CaCl_2$ and 1M glycine individually and in combination improved the stability and compatibility of a thermostable alkaline protease from *Bacillus subtilis* PE-11 with commercially available local detergents They conducted a washing test using an enzyme based preparation, to assess their efficiency to remove blood stain in the presence of commercial detergents. The preparation contained 2 ml of alkaline protease from *Bacillus subtilis* and 7 mg of wheel detergent made up to 100 ml with distilled water. Blood stained cloths were washed with the cleaning solution at 60°C for 15 minutes. Visual examination of washed clothes revealed that enzyme based preparation gave a better performance. Untreated blood stained cloth served as the control.

Joo *et al.* (2003) reported that *Bacillus clausii* 1-52 produced an alkaline protease that exhibited significant compatibility and stability towards both surfactants and oxidising agents. It retained 73 per cent and 116 per cent activity after incubation for 72 h with five per cent SDS and five per cent H_2O_2. As the enzyme was found to be stable over a wide range of pH and temperature, they opined that the enzyme can be successfully used as an additive in detergent industry.

According to Aftab *et al.* (2006) enzymes recovered from alkalophilic thermophiles conferred stability in a wide pH range but was usually thermolabile. They found that the alkaline protease enzyme of *Bacillus brevis* SSA1 isolated from soil was not only stable in the presence of commercial detergents but gave an improved performance in the presence of detergents.

The protease enzyme of *Bacillus cereus* MCMB-326 isolated from buffalo hide was stable and active in the presence of five per cent and 10 per cent H_2O_2, such that it could be used in bleach based detergent formulations (Nilegaonkar *et al.*, 2007).

Materials and Methods

3. MATERIALS AND METHODS

3.1 ASSESSMENT OF SPOILAGE POTENTIAL OF THE ISOLATES

3.1.1 Collection of Samples

Two representative samples each of raw milk, pasteurized milk and skim milk were aseptically collected from Kerala Agricultural University Dairy Plant at weekly intervals for five weeks spread over a period of two months. The samples were brought to the lab in an insulated container. After being brought to the lab, skim milk was subjected to laboratory sterilization in the same container.

To get a representative flora of water from the dairy plant, tap water as well as middle stream alkaline water washings during cleaning in place (CIP) were collected aseptically. Two samples each of tap water and hot alkaline water washings (pH 12 and temperature 85°C) were collected weekly for five consecutive weeks. The samples were brought to the lab in insulated container.

3.1.2 Isolation and Characterization of the Isolates

The appropriately diluted samples of raw milk, pasteurized milk, sterilized skim milk, tap water and alkaline water washings were pour plated in Nutrient agar (Himedia) and incubated at 37°C for 48 h so as to get discreet colonies. After considering the colony characteristics, single colonies were selected and streaked to purity in nutrient agar plates. The interesting isolates were transferred to nutrient agar slants, incubated at 37°C for 48 h and then maintained at 4°C. Sub culturing was done at monthly intervals. At regular intervals the purity of culture was confirmed by staining. The isolates were subjected to biochemical characterization after activating the culture in nutrient broth at 37°C for 24 h (Barrow and Feltham, 1993).

3.1.3 Qualitative Assessment of Ability of the Isolates to Produce Spoilage Enzymes

The spoilage potential of isolates was assessed in terms of its ability to produce the extra cellular enzymes: proteases, lipases and lecithinases (Harrigan, 1998; Dogan and Boor, 2003).

Protease enzyme

The potential of the isolates to produce extra cellular proteolytic enzymes was determined by streaking on Plate count agar (Himedia) containing 10 per cent skim milk. After incubation at 37°C for 48 h, the plates were flooded with 1N Hydrochloric acid for observation of clearance zone formed by protease positive strains. For each isolate, clearance zone around two to five colonies were measured and the ratio of clearance zone diameter to the colony diameter was calculated. Accordingly, protease activity was categorized on a scale of one to four where one represented mild protease activity and four represented marked protease activity.

Lipase enzyme

Production of extra cellular lipase was assessed by streaking on Spirit blue agar (Himedia) containing tributyrin at a level of three per cent. The plates were incubated at 37°C for 48 h, and observed for the presence of dark blue colonies. Lipase activity was assessed by noting the color intensity. To rule out lipase activity, the negative plates were transferred to 7°C and examined again after five days. After a visual inspection, the color intensity was categorized on a scale of one to four, where one represented light blue and four represented dark blue color.

Lecithinase enzyme

Production of extra cellular lecithinase enzyme was assessed qualitatively on Egg yolk agar (Himedia) to which ten per cent egg yolk emulsion (Himedia) was added. After incubation at 37°C for 48 h, the plates were examined for opaque zones around the colony. The plates that were negative for lecithinase activity were transferred to 7°C and examined after five days. Diameter of the opaque zone

surrounding lecithinase positive colonies was measured and the ratio of the size of the opaque zone to that of colony was calculated. Lecithinase activity was categorized on a scale of 1 to 4, where 1 represented mild lecithinase activity and 4 represented marked lecithinase activity.

Based on their ability to produce spoilage enzymes, the isolate P12 and S4 were selected for further work. The two selected isolates that showed comparable potential to produce the spoilage enzymes proteases, lipases and lecithinases were also qualitatively assessed for their ability to produce the industrially significant enzymes, amylase and gelatinase. The potential of the isolate to produce amylase enzyme was assessed by streaking on the Starch agar (Himedia). After getting sufficient growth, starch agar plate was flooded with lugol's Iodine solution. Colorless zone around the growth was indicative of hydrolysis of starch by amylase enzyme. The medium turned blue where starch was not hydrolysed. To detect the presence of gelatinase, the isolate was streaked on the gelatin agar medium (Himedia) and incubated at 37°C. After getting sufficient growth, the agar plate was flooded with acidic Mercuric chloride. Mercuric chloride precipitates the gelatin such that media turn opaque wherever unhydrolysed gelatin is present. Absence of opaqueness/clearing of media around the colony was taken as an indication of gelatin hydrolysis.

3.1.4 Identification of the Selected Isolates P12 and S4

The isolates P12 and S4 were streaked on nutrient agar and colony characteristics like color, form, elevation and margin were observed and recorded. The isolates were examined for shape, arrangement, Grams reaction, spore position and motility by microscopic examination. Biochemical characterization of the isolates was carried out (Barrow and Feltham, 1993). After the preliminary identification, P12 was streaked on *Pseudomonas* agar and S4 on *Bacillus* cereus differential agar to appreciate the colony characteristics in selective media. Genetic identification (16SrRNA sequencing) was done by sending the isolate to Central Institute of Fisheries and Technology, Wellington Island, Cochin.

3.2 IMPACT OF PSYCHROTROPHIC SPOILAGE ENZYMES IN CURD AND STERILISED MILK

3.2.1 Preparation of Curd (dahi)

Fresh good quality raw cow milk was procured from Kerala Agricultural University Dairy Plant. Milk was subjected to boiling temperature for three minutes. After cooling to room temperature, milk was divided into three portions for different treatments. The first portion that was not inoculated served for the preparation of control curd (CC). Second portion was inoculated with the isolate P12 (CT1) and third portion with the isolate S4 (CT2).

3.2.1.1 Preparation of Inoculum

One hundred and fifty aliquots of nutrient broth in 250 ml conical flasks were inoculated with active culture of P12 and S4 seperately at a level of 2.5 per cent. After overnight incubation at 37°C, the optical density of broth was adjusted to 0.6 at 600 nm. The quantity of broth required to be added to one portion of milk so as to get P12 and S4 at a level of 10^3cfu/ml was calculated from the corresponding standard curves (Optical density at 600 nm plotted against viable count in cfu/ml). Accordingly, volume of nutrient broth required for inoculation of P12 and S4 was taken separately and subjected to refrigerated centrifugation at 8000 rpm for 30 minutes. The cell pellets so obtained were washed twice in normal saline and then suspended in minimum quantity of sterilized skim milk.

The entire suspension of P12 in skim milk was added to the second portion (TI) and the suspension of S4 in skim milk was added to the third portion (T2) so as to get P12 and S4 at a level of 10^3cfu/ml respectively. The uninoculated first portion was used for the preparation of the control. The three sets of milk were then kept under refrigeration for 72 h. This milk was used for the preparation of curd. Curd prepared from milk that was not inoculated acted as control (CC). Milk inoculated with PI was used for the preparation of curd CT1 and milk inoculated with S4 was used to prepare the curd CT2.

3.2.1.2 Starter Culture

Lyophilized cultures of *Streptococcus salivarius* ssp *thermophilus* (NCDC No.074) and *Lactobacillus delbrueckii* ssp *bulgaricus* (NCDC No.09) were procured from National Dairy Research Institute, Karnal. Cultures were activated and maintained in ten per cent skim milk. Only pure and active cultures were used in the preparation of curd. Samples of milk that were kept under refrigeration were (CC, CT1 and CT2) boiled and cooled to room temperature. To this, starters were added in the ratio 1:1 at a level of two per cent. Samples were then dispensed in 30 ml volumes and kept for incubation at 37°C for 16 h. After incubation, curd samples were transferred to refrigerator. Analysis of physico-chemical parameters was done on zero, fifth and tenth day. A total of six replications were carried out to assess the statistical significance of the results.

3.2.2 Evaluation of Physico-chemical Quality of Curd

Preparing cultured dairy products that are free from defects continues to be a challenging problem in dairy industry. The appearance of whey detracts consumer appeal and is indicative of improper formulation, culturing or processing. Objective of this trial was to assess the impact of spoilage enzymes of P12 and S4 on the physico-chemical characteristics, texture and sensory parameters of curd.

3.2.2.1 Titratable Acidity

Titratable acidity of treatment curd samples was determined as per the procedures of Scott *et al.* (2001).

3.2.2.2 Measurement of pH

The samples were tempered to room temperature. The pH of the sample was measured using a digital pH meter (Elico LI 610).

3.2.2.3 Syneresis

Spontaneous syneresis of undisturbed set curd was determined using a siphon method, designed by Lucey (2001), with slight modifications.

The cup of curd was taken out from the refrigerator and weighed (W1). It was then kept at an angle of 45° for ten minutes to allow whey on the surface to collect on the side of cup. Liquid whey from the surface of sample was siphoned out carefully using a syringe. Siphoning was carried out within 10s to prevent further leakage of whey from the curd. The sample was weighed again after removal of whey (W2). The syneresis was expressed as the percentage weight of whey over the initial weight of curd sample.

$$\frac{W1 - W2}{W1} \times 100$$

3.2.2.4 Texture Analysis

The texture analyzer (TA-X T2 texture analyzer – Stable Microsystem) was used to evaluate the firmness. and viscosity index of curd. The equipment was set in the back extrusion mode with the following settings; Pretest-speed - 1.00 mm/sec, Test-speed - 1.00 mm/sec, Post-test speed - 10.00 mm/sec, Distance – 30mm and Trigger force – 2g. Probe with a diameter of 35mm was used for the test. The mode was set to measure the force in compression.

3.2.2.5 Sensory Evaluation

The samples were subjected to sensory evaluation by a four member panel under standard conditions using the score card prescribed by Ranganadham and Gupta (1987), with modification in weightage given to flavour, body and texture, color and appearance and overall acceptability. Average score obtained from four members of the panel for each sample was used for statistical analysis (Appendix- I).

3.2.3 Sterilized Milk

Fresh good quality skim milk procured from Kerala Agricultural University Dairy Plant was subjected to boiling temperature for three minutes. After cooling, skim milk was divided into three portions. The first portion that was not inoculated served as the control. After preparing the inoculum as described in 3.2.1.1, P12 and S4 were inoculated into second and third portions so as to have 10^3cfu/ml.The samples (SC, ST1 and ST2) were then kept under refrigeration for 72h. The milk samples were dispensed in 50 ml volume in 100 ml conical flasks. A temperature of 121°C at 15 lb pressure for 15 minutes was employed to achieve the laboratory sterilization of milk samples. Samples were stored at room temperature and analysis was done on 0, seventh and 15th day of storage. Results were compared with that of control to determine the influence of spoilage enzymes. A total of six replications were carried out to assess the statistical significance of the results.

3.2.3.1 pH of Sterilised Milk

The pH of the sample was measured using a digital pH meter (Elico LI 610). The electrodes were cleaned after every observation.

3.2.3.2 Index of Proteolysis

Tyrosine content of the sterilized milk was determined on 0 day, seventh day and 15th day by Hull's method (1945). To five milliliter of sample, equal quantity of 12 per cent Trichloro acetic acid (TCA) was added. After vortexing, mixture was allowed to stand for 15 minutes. Then it was filtered using Whatman No.42 filter paper. To one milliliter of TCA filtrate, added five millilitersof five per cent Sodium carbonate and one milliliter of Folin – Ciocalteau reagent. After thorough mixing, reaction mixture was incubated at room temperature for 20 minutes. Optical density was measured at 660 nm using UV VIS Spectrophotometer (SL 159, Elico).Tyrosine content was calculated by comparing with the standard curve.

The Non Protein Nitrogen value of sterilised milk on 0, seventh and 15th day was determined by Kjeldahl method according to ISI Handbook of food analysis, 1981. To two millilitersof TCA filtrate in the Kjeldahl flask, a pinch of copper sulphate and potassium sulphate were added along with five ml of concentrated sulphuric acid. The contents were digested in a digestion flask until a clear and colorless solution was obtained. After cooling to room temperature, solution was made alkaline by adding 40 per cent NaOH. The contents were steam distilled and the ammonia liberated was collected in 20 ml of saturated boric acid solution containing 2-3 drops of mixed indicator (methyl red and methylene blue). The distillation was continued until about 65-75 ml of distillate was collected. This was titrated against N/10 H_2SO_4 until the grass-green colour changed to steel-gray, a further drop then giving purple colour. The NPN value in per cent nitrogen was calculated using the formula

$$\text{Nitrogen per cent by weight} = \frac{14 \times X \times 100}{10000 \times W}$$

Where, X is the volume of standard sulphuric acid required in millilitre
W is the weight of sample in grams.

3.2.3.3 Evaluation of Organoleptic Quality

Extending the shelf life of fluid milk products will contribute to the competitiveness of dairy industry in the beverage market. Sterilization is a processing strategy to extend shelf life at room temperature. As product quality drives consumer acceptance, it is important to evaluate the impact of thermostable enzymes on the sensorial characteristics of the product. A quantitative descriptive analysis approach was followed for the sensory evaluation of sterilized milk. (Chapman *et al.*, 2001; Meilgaard *et al.*, 2007).

The panel comprising of four members were initially familiarized with the grading of samples on the intensity scale of 1 to 15 for the attributes viscosity, colour intensity, cooked flavour, unclean flavour, bitter taste, sour taste and after taste by providing physical reference standards (Appendix-II).

Fifty milliliter samples in screw capped conical flask along with 100 ml paper cups were given to the judges. The panelists evaluated the sample individually. Overall quality rating in the intensity scale of 1 to 15 was also done simultaneously. Mean panelist ratings of attribute intensities and overall acceptability of sterilized skim milk on zero day, 7th day and 15th day at room temperature were recorded. A total of six replications were done and results were analyzed for statistical significance. To create a visual profile or fingerprints of product attributes, spider plots were created by plotting average intensity values on each scale and then joining the points.

3.3 ALKALINE PROTEASES

3.3.1 Production of Protease in Alkaline pH

The isolate S4 that exhibited comparable protease activity on skim milk agar was assessed for its ability to grow and produce protease enzyme in an alkaline medium. For this an appropriately diluted sample of the active culture was streaked on Glucose Peptone Yeast extract Carbonate medium (GPYC medium, pH-10.0) fortified with 10 per cent skim milk (Kumar, 1997). The plates were incubated at 37°C for 48 h and were examined for zone of clearance.

GPYC medium

Glucose	-	10.0 g
Peptone	-	5.0 g
Yeast extract	-	1.0 g
K_2HPO_4	-	1.0 g
$MgSO_4$	-	0.2 g
Agar	-	18 g

Distilled water to make up 1000 ml (pH-10.0)

Sodium carbonate (two per cent) was sterilized separately and added aseptically to get a pH – 10.0. Skim milk was sterilized separately and added aseptically at a level of 10 per cent (v/v) at the time of plating.

The selected isolate S4 was streaked on Nutrient agar slant, incubated at 37°C for 24 h and then stored under refrigeration. Subculturing was done at monthly intervals. Purity of culture was checked periodically by microscopic examination.

3.3.2 Determination of Protease Activity

The protease activity of the sample was determined at pH 11.0 using casein as substrate according to the method of Hagihara *et al.* (1958), with slight modifications.

Five milliliter of casein substrate (Casein 0.5 per cent (Sisco) dissolved in 20 mM sodium borate – NaOH buffer at pH – 11.0) was equilibrated to 55°C and one milliliter of suitably diluted enzyme solution (cell free supernatant) previously equilibrated to the same temperature was added. After vortexing, the mixture was incubated at 55°C for 10 minutes. The reaction was then terminated by the addition of 5.0 ml of 12 per cent TCA. The TCA precipitated protein was separated by filtration through Whatman No.42 filter paper (ash less). Tyrosine content was estimated as explained in 3.2.3.2. The TCA filtrate without the addition of enzyme was used for the preparation of blank.

To prepare the standard, one millilitre aliquots of tyrosine solutions with varying known concentrations of tyrosine were treated as above for developing colour. By plotting the optical density values at 660 nm against the tyrosine concentrations (μg/ml), the standard curve was constructed. The regression equation obtained from the standard curve was $Y = 141.75X - 0.0003$ where Y is the optical density at 660 nm and x is the concentration of tyrosine (μg/ml). One unit of enzyme activity is defined as that amount of enzyme required to produce TCA reagent soluble fragments equivalent to one micromole of tyrosine from casein under the standard conditions of assay.

Protein was quantified by Lowry's method (Genei, Bangalore) using Bovine serum albumin as the standard. Specific activity of the enzyme is defined as the number of units of tyrosine per milligram of protein.

3.3.3 Optimization of Conditions for Production of Alkaline Protease

The influence of various nutritional and environmental factors on the production of extra cellular alkaline protease by the selected alkalophilic isolate (S4) was assessed with a view to optimize the parameters for maximizing the enzyme production. In this part of study, the isolate S4 was grown under different culture conditions and alkaline protease production was monitored as described in 3.3.2. The strategy of varying one parameter at a time keeping others unaltered was employed. Once a particular parameter was found to show maximum enzyme activity, that parameter was incorporated while designing the next trial for optimization. Using this approach, different environmental and nutritional factors affecting the enzyme production were studied for the sequential optimization of conditions for maximum production of alkaline protease. All the trials on enzyme production were carried out in triplicate.

One hundred milliliters of nutrient broth was inoculated with a loopful of 24 h old slant culture and incubated at 37°C for 24 h. The absorbance of broth culture at 600 nm was adjusted to 0.6. The culture so standardized was used at a level of two per cent throughout the production studies aimed at optimizing the environmental parameters.

3.3.3.1 Environmental Factors

The impact of different environmental factors like temperature of incubation, period of incubation, initial pH of the medium, agitation and rate of inoculum on the production of alkaline protease enzyme were assessed using nutrient broth containing skim milk (10 g skim milk powder in 100 ml distilled water) at a level of two per cent (Shafee *et al.*, 2005; Olajuyigbe and Ajele, 2005).

3.3.3.1.1 Temperature and Period of Incubation

Replicates of culture flasks with nutrient broth having two per cent skim milk were inoculated at a level of two per cent. Incubation was done at 37°C, 42°C and 55°C. Protease activity of the samples at the selected temperature of incubation was determined after 24 h, 48 h and 72 h.

3.3.3.1.2 pH of the Medium

To study the influence of pH on production of protease enzyme pH of the media (nutrient broth with two per cent skim milk) was adjusted to 8.0, 9.0, 10.0 and 11.0 by adding calculated quantities of two per cent sodium carbonate, which was sterilized separately. Protease production was determined after incubation at 37°C for 24 h.

3.3.3.1.3 Agitation

To test the effect of aeration, two sets of flasks containing nutrient broth fortified with two per cent skim milk were inoculated with the test organism (optical density adjusted to 0.6 at 600 nm) at a level of two per cent. One set was incubated at 37°C in an ordinary incubator and the other at 37°C in a shaking incubator (125 rpm). Production of protease enzyme after 24 h of incubation was determined.

3.3.3.1.4 Rate of Inoculum

The nutrient broth fortified with two per cent skim milk was inoculated with the standardized inoculum at a level of one, two, three and four per cent. The protease activity was determined after incubation at 37°C for 24 h.

3.3.3.2 Effect of Some Nutrients on Production of Protease Enzyme

The basal fermentation medium comprised of skim milk powder and whey powder both at a level of 0.5 per cent in distilled water. The pH of the medium was adjusted using two per cent sodium carbonate. Both the fermentation media and sodium carbonate solution were sterilized separately by autoclaving. Before

inoculation, pH of the medium was adjusted to 10.0 by adding calculated quantity of sodium carbonate solution (Kumar, 1997; Shafee *et al.*, 2005).

3.3.3.2.1 Nitrogen Sources

To the basal fermentation medium, various organic nitrogen sources like tryptone, peptone, beef extract and yeast extract were added at a level of 0.5 per cent. Incubation was done at 37°C for 24 h and amount of protease enzyme liberated was determined.

To study the influence of inorganic nitrogen sources, ammonium carbonate, ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate were individually added to the basal fermentation medium at a level of 0.5 per cent. Protease production was assessed after incubation at 37°C for 24 h. Protease production in the basal fermentation medium was taken as control.

3.3.3.2.2 Carbon Sources

Considering the superior influence of tryptone on production of protease enzyme, the basal fermentation medium was modified so as to have the following composition (0.5 per cent whey powder, 0.5 per cent skim milk powder, 0.5 per cent tryptone (pH – 10.0). To this modified medium, different carbon sources like glucose, sucrose, maltose, corn starch, potato starch and trisodium citrate were added at a level of 0.5 per cent. The influence of various carbon sources on protease production was assessed by determining the protease activity after incubation at 37°C for 24 h. Protease enzyme in the modified basal fermentation medium without the addition of any carbohydrates acted as the control.

3.3.4 Characterization of Crude Enzyme

Characterization of crude protease enzyme was carried out with appropriate modifications of procedures of Nascimento and Martins (2004) and Nilegaonkar *et al.* (2007).

3.3.4.1 Production of Crude Protease Enzyme

Standardized inoculum (OD at 600 nm – 0.6) of active culture of S4 was inoculated at a level of three per cent to the optimized media with the following composition.

Whey powder	-	5 g
Skim milk powder	-	5 g
Tryptone	-	5 g
Trisodium citrate	-	5 g
Sucrose	-	5 g
Magnesium sulphate	-	5 g
Distilled water	-	1000 ml
pH	-	11.00

Whey powder and skim milk powder dissolved in required volume of water were sterilized together. Rest of the ingredients – tryptone, trisodium citrate, sucrose and magnesium sulphate in required quantities were sterilized together. At the time of inoculation these two parts were mixed and calculated quantity of two per cent Na_2CO_3 was added so as to get a pH 11.0.

After incubation at 37°C for 24 h, the media were subjected to refrigerated centrifugation (Eltek, RC 4100 D) at 8000 rpm for 40 min at 10°C. The clear cell free supernatant obtained was then filtered through 0.45 μ Millipore filter. This filtrate containing the extracellular protease served as the crude protease enzyme.

3.3.4.2 pH Stability

The crude protease solutions were exposed to different pH of 7.0, 8.0, 9.0, 10.0 11.0 and 12.0 (Tris-Hcl and glycine-NaOH buffer) by mixing the enzyme with appropriate buffer in the ratio 1:1. The enzyme solutions at different pH values were incubated at 30°C for 24 h. The residual protease activity was subsequently determined in terms of tyrosine value, using 0.5 per cent casein as

substrate. The original enzymatic activity before incubation at various pH values was taken as 100 per cent.

3.3.4.3 Temperature Stability

The crude enzyme solution was exposed to different temperatures of 20°C, 40°C, 60°C and 80°C for a total duration of 90 min. Aliquots of enzyme solution were drawn after 30, 60 and 90 min of exposure to different temperatures. The heat treated enzyme samples were then immediately cooled and the residual enzyme activity was determined using 0.5 per cent casein as substrate under standard assay conditions. The enzyme activity at '0' time was taken as 100 per cent.

3.3.4.4 Effect of Metal Ions

The influence of different metal ions on the activity of crude protease was determined at a concentration of 0.5 mM. The metal ions tested were Na^{+}, Ca^{++}, Mg^{++}, Hg^{++}, Mn^{++} and Co^{++}. The enzyme solutions were incubated with selected metal ions in the ratio 1:1 at 60°C for ten min. Subsequently the enzyme was assayed for residual protease activity using 0.5 per cent casein as substrate, under standard assay conditions.

3.3.4.5 Effect of Inhibitors on the Activity of Protease Enzyme

The crude protease enzyme solution was preincubated with chemical inhibitors at 10 mM concentration in the ratio 1:1 at 60°C for 30 min. The residual protease activity was determined on 0.5 per cent casein substrate under standard assay conditions. Activity of enzyme without the addition of inhibitor was taken as 100 per cent.

3.3.5 Assessment of Cleaning Efficiency of Inbuilt Detergent

3.3.5.1 Cleaning of Milk Soiled Plates

The cleaning efficiency of the inbuilt formulation was compared with that of a standard cleaning solution generally used in the laboratory, after artificially soiling the plates (Varghese, 2002). To soil the plates, one millilitre of pasteurised

whole milk was spread evenly on Petri dishes (diameter 9 cm) and air dried. The plates were kept in the incubator overnight to ensure complete and uniform drying of the milk films on to the plates.Uncleaned Petri dish was taken as control. The cleaning efficiency of the following combinations was evaluated. The cell free supernatant (CFS) used in the trial possessed an activity of 25 U/ml.

I.	Water	- 50 ml
II	CFS	– 25ml
	Water	- 25 ml
III	Water	-50 ml
	Labolene	- 0.25 ml
IV	Water	- 50 ml
	Tween 80	- 0.01 g
	NaOH	- 0.001 g
V	CFS	- 25 ml
	Tween 80	- 0.001 g
	NaOH	- 0.001 g
	Water	- 25 ml

The cleaning protocol comprised of the following steps

1) A pre rinse with 10 ml water by gently rotating the plates five times clockwise and five times anticlockwise so as to have a total contact time of five minutes.

2) A wash by 10 ml test solution by gently rotating the plates five times clockwise and five times anticlockwise so as to have a total contact time of ten minutes.

3) A post rinse with 10 ml water so as to have a total contact time of five minutes.

4) Drainage of excess water by keeping the plates in slanting position. The entire procedure was carried out at room temperature. Utmost care was taken to carry out the cleaning protocol in a uniform way for all test solutions.

To facilitate better visualization of proteinaceous milk residues in the cleaned assay plates, methylene blue staining was resorted to. For this 2 ml of methylene blue stain was added and the plate was gently rotated so as to spread the stain in a uniform manner. After a contact period of three minutes, drained out the stain. After air drying, the plates were carefully examined for milk residues that took blue stain. Dried plates were again rinsed with 10 ml of distilled water. Protease activity of the rinse was determined to rule out residual enzyme activity.

3.3.5.2 Wash Test

The efficiency of the crude protease enzyme (cell free supernatant with an activity of 25 U/ml) in combination with a locally available detergent in removing blood stain was also assessed by a wash test. A preliminary trial conducted in this regard revealed that, of all the detergents tested, the protease enzyme used in this trial was maximally compatible with the detergent Wheel (Procter and Gamble).

The possibility of using this enzyme as a detergent additive was looked into by washing blood stained cloth pieces with different combination of test solution (Adinarayana *et al.*, 2003). Blood (0.5 ml) was spread over a fixed area of a white cotton cloth piece and allowed to dry so as to get cloth pieces stained uniformly with blood. Unwashed blood stained cloth piece served as control. The following sets of test solutions were prepared and compared.

1) Water - 50 ml

2) Wheel - 0.1 g
   Water - 50 ml

3) Wheel - 0.1 g
   CFS - 10 ml
   Tween 80 - 0.01 g
   H_2O_2 - 0.005 ml
   Water - 50 ml

The wash cycle comprised of the following steps:

1) The stained cloth pieces were kept in 50 ml water at 55°C for 10 minutes.
2) It was then kept in a shaking incubator (125 rpm) at 55°C for 5 minutes to get a rinsing effect.
3) Drained out the test solution and washed again with 50 ml water at 55°C in a shaking incubator for 5 minutes.
4) The cloth pieces were taken out and dried. Untreated cloth piece stained with blood was taken as control.
5) Efficiency to remove blood stain was assessed by visual examinationof dried cloth pieces.

3.3.6 Purification of Protease Enzyme

3.3.6.1 Production of Enzyme

The crude protease enzyme of *Bacillus cereus* (isolate S4) was collected as detailed in 3.3.4.1. Purification of the crude enzyme was attempted (Son and Kim, 2002; Vidyasagar *et al.*, 2006).

3.3.6.2 Precipitation with Ammonium Sulphate

Fractional precipitation of extracellular enzyme was done using Ammonium sulphate. Fifty milliliter of crude enzyme (CFS) was precipitated with solid ammonium sulphate at 40 per cent saturation. For this calculated quantities of ammonium sulphate was added slowly with constant stirring. The precipitate obtained after refrigerated centrifugation at 8000 rpm for 30 min was discarded. The supernatant was again brought to 80 per cent saturation with solid ammonium sulphate and the precipitate was collected by refrigerated centrifugation at 8000 rpm for 30 min. The protein pellet so obtained was dissolved in minimum quantity of 20 mM Tris HCl buffer (pH 11.0). This fraction was used for further work, as preliminary trial indicated that 40-80 per cent fraction has considerably more enzyme activity. The resuspended 40-80 per cent fraction was dialysed using dialysis tubing 25mm x 16 mm (Sigma) extensively against three changes of three litres of distilled water and then against the same buffer for 24 h.Precipitate formed was removed by centrifugation and the resultant solution was used for further purification.

3.3.6.3 Gel Filtration Chromatography

For further purification, gel filtration with Seralose 4B and Seralose 6B was resorted to. After thorough washing of Seralose 4B (SRL) with distilled water, it was packed into a column (500 mm x10 mm) and equilibrated with Tris HCl buffer (pH – 11.0) for one hour. One millilitre of dialysed enzyme solution was then allowed to percolate through the packed column. Same buffer was used to elute it. Seventy five fractions of two millitre each were collected at the flow rate

of 30 ml/h. The fractions, containing protein was detected by measuring the absorbance at 280 nm using UV VIS Spectrophotometer (SL 159, Elico). Specific activity of all the fractions containing protein was determined.

Total protein was determined by the method of Lowry *et al* (1951) using bovine serum albumen as standard. Protease activity of the sample was determined using casein as substrate. The amount of enzyme required to release one micromole of tyrosine from casein per millilitre in one minute under standard assay conditions was taken as one unit of protease activity. Specific activity is the number of units of activity per milligram of protein.

Fraction with maximum specific activity was then passed through Seralose 6B (SRL) packed column as detailed before. Specific activity of active fractions was determined and that fraction with specific activity was rechromatographed with seralose 6B, again. The most active fraction so obtained was than assessed for its homogenity by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) (Laemmli, 1970).

3.3.6.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.6.4.1 Reagents Used

Acrylamide-bisacrylamide stock (30: 0.8)

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water to make up	100 ml

Filtered through Whatman No.1 filter paper and stored at 4°C.

1.5 M Tris pH 8.8

Tris base	181.7 g

Distilled water to make up	1000 ml

Adjusted pH to 8.8 with 4N HCl. and stored at 4°C.

0.5 M Tris pH 6.8

Tris base	60.6 g.
Distilled water to make up	1000 ml

Adjusted pH to 6.8 with 4 N HCl. and stored at 4°C.

Resolving gel (12.5 per cent)

Acrylamide: bisacrylamide (30: 0.8)	12.5 ml
Tris hydrochloride (1.5 M) pH 8.8	7.5 ml
Sodium dodecyl sulphate (ten per cent)	0.3 ml
Ammonium persulphate (ten per cent)	0.15 ml
N, N, N, N - tetra methyl ethylenediamine (TEMED)	0.01 ml
Distilled water	9.6 ml

Stacking gel (four per cent)

Acrylamide stock (30.8 per cent)	0.67 ml
Tris hydrochloride (1.5 M) pH 6.8	1.25ml
Sodium dodecyl sulphate (ten per cent)	0.05ml
Ammonium persulphate (ten per cent)	25 µl
N, N, N, N - tetra methyl ethylenediamine (TEMED)	2.5 µl
Distilled water	3.00 ml

Electrophoresis buffer

Tris base	3.0 g
Glycine	14.4 g
Sodium dodecyl sulphate	1.0 g
Distilled water to make up	1000 ml

Sample preparation buffer (2x)

0.5 M Tris hydrochloride, pH 6.8	2.5 ml
Glycerol	2.0 ml
Sodium dodecyl sulphate (ten per cent)	4.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.5 mg
Distilled water to make up	10.0 ml

Distributed in small aliquots and stored at 4°C.

Coomassie brilliant blue (SRL) staining solution

Coomassie brilliant blue (R250)	0.25 g
Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water to make up	1000 ml

Destaining solution I

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water to make up	1000 ml

Destaining solution II

Glacial acetic acid	70 ml
Methanol	50 ml
Distilled water to make up	1000 ml

3.3.6.4.2 Method

The thirty nineth fraction obtained after passing through Seralose 6B was subjected to 12.5 per cent SDS PAGE. Resolving gel solution of 12.5 per cent was poured between two glass plates so as to form a gel of one millimeter thickness. Distilled water was layered on the top to ensure uniformity of the gel surface. After ensuring polymerization, the distilled water was pipetted out. Stacking gel (four per cent) was then poured between the glass plates. The comb was inserted and the apparatus was left as such for two hours for complete polymerization. After polymerization, the comb was removed and the wells were washed thoroughly with running buffer. A small quantity of running buffer was added into each of these wells. Ten µl of the sample was mixed with equal volume of sample preparation buffer and kept in boiling water bath for five minutes. These samples were loaded into individual wells under a column of buffer. Standard medium range molecular weight marker (GENEI) was loaded in one of the wells. The glass plates were fixed onto the vertical slab gel electrophoresis apparatus. Electrophoresis buffer was carefully poured into the top and bottom reservoirs. Electrophoresis was carried out at 10 mA constant current till the bromophenol blue marker reached near the bottom of the resolving gel. The gel was removed from the glass plate; after snipping off the stacking gel, gel was transferred to a Petri dish containing Coomassie brilliant blue staining solution for two to three hours. The gels were then destained with destaining solution I and then with destaining solution II till the background became clear. It was viewed in white light and photographed.

3.3.6.4.3 Estimation of Molecular Weight

The molecular weight of purified protein fraction was obtained by comparing the distance migrated with that of the standard marker proteins of known molecular weights. A standard graph was plotted with the $\log_{10}$ Da values of the marker protein on the Y-axis and the mobility of the known bands of the marker on the X-axis. The values of the distance migrated by the sample proteins were interpolated with the standard curve to determine the molecular weight of the polypeptide band.

Results

4. RESULTS

4.1 ASSESSMENT OF SPOILAGE POTENTIAL OF THE ISOLATES

The isolates obtained from raw milk, processed milk, tap water and alkaline water washings collected from Kerala Agricultural University Dairy Plant were qualitatively assessed for its ability to produce the spoilage enzymes: proteases, lipases and lecithinases. The biochemical characterization was done to identify the isolate at genus level. The identity of the isolate along with its ability to produce the spoilage enzymes is given in Appendix III.

Gram negative rods constituted 69.2 per cent of the raw milk isolates. In this category, coliforms were the highest in proportion (50 per cent), closely followed by *Pseudomonas* (35.3 per cent). Gram positive isolates coming to 30.8 per cent of raw milk isolates comprised mainly of *Bacillus* and members of *Micrococcaceae.* Of the twenty six isolates obtained from raw milk, nine belonged to coliforms (34.6 per cent), seven to *Pseudomonas* (26.9 per cent) three to *Staphylococcus* (11.5 per cent), and two each to the genera *Bacillus*, *Chromobacterium* and *Micrococcus* (7.7 per cent) and one to the genus *Lactobacillus* (3.8 per cent).

In pasteurized milk 50 per cent of the isolates were *Pseudomonas* and coliforms contributed to 16.7 per cent. The proportion of *Bacillus* in pasteurized milk increased to 22.2 per cent when compared to raw milk (7.7 per cent). Out of the eighteen isolates obtained from pasteurized milk, one each belonged to the genus *Micrococcus* and *Staphylococcus*. Seventy five per cent of Gram negative rods were found to belong to *Pseudomonas*. Ninety per cent of the sterilized milk isolates belonged to the genus *Bacillus*. One sterilized milk isolate was found to be *Micrococcus.*

Identification of the isolates obtained from tap water samples showed that Gram negative rods contributed to 66.7 per cent of the total flora. Of the Gram negative flora *Pseudomonas* constituted 50 per cent. Rest comprised of *Chromobacterium* (25 per cent), *Flavobacterium* (12.5 per cent) and *E .coli* (12.5

per cent). The isolates from hot alkaline water washings contained only genus *Bacillus*, all of which possessed significant lecithinase activity. Of the total isolates from dairy environment, 31.4 per cent belonged to *Bacillus* and 27.5 per cent to *Pseudomonas*.

Ninety per cent of *Bacillus* isolates and cent per cent of *Pseudomonas* isolates obtained in this trial showed protease activity (plate1). Lipase activity was evident in 72 per cent of *Bacillus* isolates and 100 per cent of *Pseudomonas* isolates (plate 2). In general, lipase activity was more for *Pseudomonas* spp than for *Bacillus* spp. Other genera isolated in this work exhibited comparatively weak enzyme activity. All *Staphylococcus* isolates got in this trial exhibited only lipase activity. Moreover lipase production was found to be more at 7°C than at 37°C. In spite of considerable growth at 37°C, only two isolates showed lipase activity at this temperature. All the *Bacillus* isolates obtained from alkaline water washings showed strong lecithinase activity at 37°C (plate 3). None of the *E. coli* isolates exhibited any enzyme activity under the experimental conditions of this work.

4.1.1 Screening of Proteolytic Isolates for Growth in Alkaline pH

As stability in alkaline pH is a desirable character, all the isolates that showed remarkable protease activity were assessed for their ability to produce protease in alkaline medium. Of the tested isolates, isolate S4 exhibited the maximum activity in alkaline pH. Though P12 showed a weak proteolytic activity in alkaline pH, it was selected for further work, taking into consideration its predominant occurrence in milk sample. Isolate P12 exhibited strong proteolytic activity in ordinary skim milk agar.

4.1.2 Identification of Selected Isolates- P12 and S4

Details of morphological and biochemical characteristics of P12, isolated from pasteurised milk and S4, isolated from sterilised milk are given in Appendix IV and plates 4a, 4b, 5a and 5b. In nutrient agar, P12 gave rise to small, circular, entire, smooth, convex, shining, transluscent colonies with a slight bluish tinge. Gram staining showed P12 as Gram negative, non-sporulating, small rods. In

➤ Zone of proteolysis (S4)

Plate 1. Protease production in skim milk agar

➤ Lipase negative (R26)

➤ Lipase positive (P12)

Plate 2. Lipase production in spirit blue agar with tributyrin

➤ Opaqueness due to lecithinase action (A1)

Plate 3. Lecithinase production in eggyolk agar

Plate 4a. Colony morphology of P12

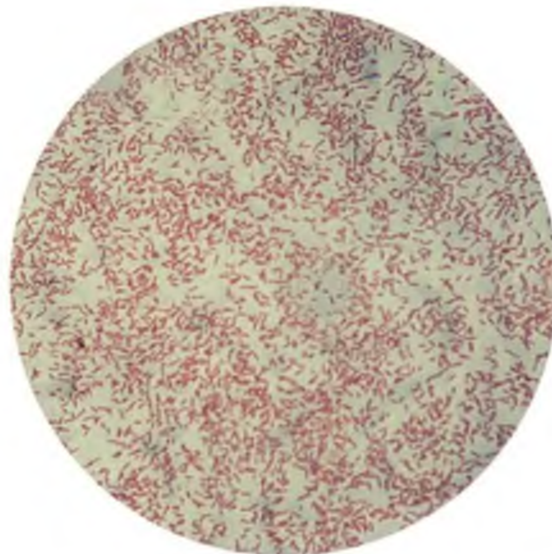


Plate 4b. Microscopic view of Gram negative P12

Plate 5a. Colony morphology of S4

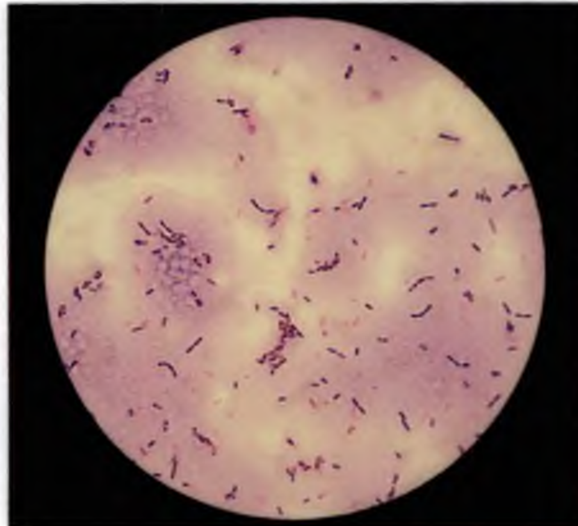
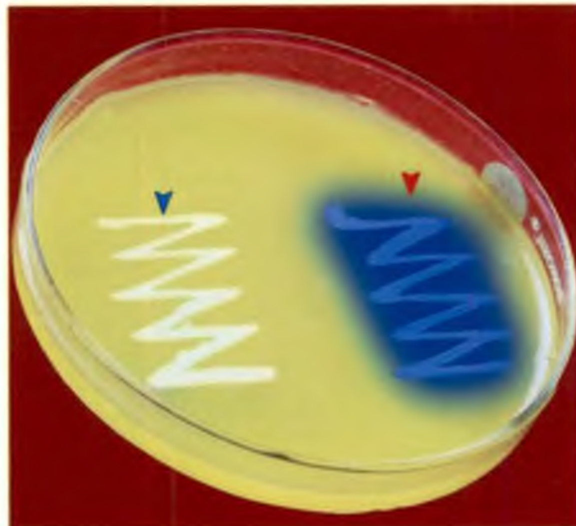


Plate 5b. Microscopic view of Gram positive S4

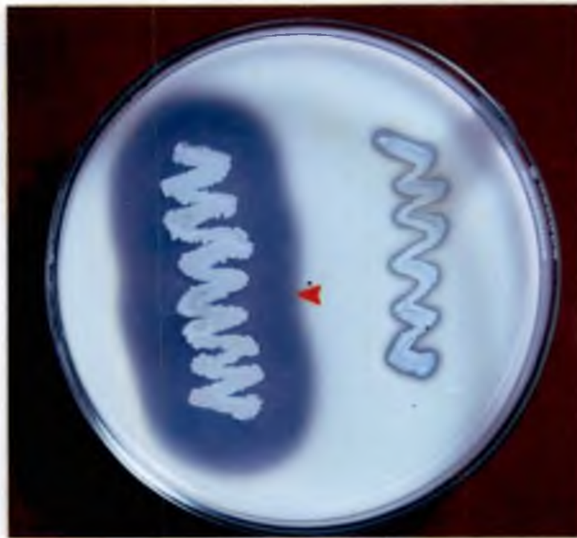
➤ Diffusible fluorescent pigment production (P12)

Plate 6. Pseudomonas agar for fluorescein production



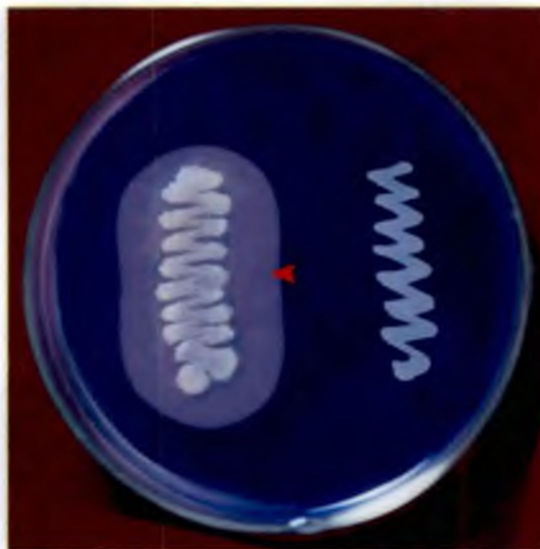
➤ Mannitol positive Lecithinase Negative (P12)
➤ Mannitol negative & Lecithinase positive (S4)

Plate 7. Bacillus cereus differential agar



➤ Zone of clearing in case of gelatin liquefaction (P12)

Plate 8. Production of gelatinase in gelatin agar



➤ Amylase positive (S4)

Plate 9. Production of amylase in starch agar

Nutrient agar, isolate S4 gave rise to large, circular, rough, raised, dry and opaque colonies. Gram staining revealed that S4 was a Gram positive rod in chains, with central or sub terminal ellipsoidal spores with no swelling of the body.

The typical colony characteristics of P12 in *Pseudomonas* agar and S4 in *Bacillus cereus* differential agar are depicted in plates 6 and 7. In *Pseudomonas* agar, P12 produced diffusible pigment that showed fluorescence under UV light. In *Bacillus cereus* differential agar, isolate S4 gave turquoise blue colonies (mannitol negative) with an opaque halo (egg yolk reaction) that indicated the production of lecithinase enzyme. Isolate P12 was found to produce gelatinase (plate 8) and S4 was found to produce amylase enzyme (Plate 9).

Identity of the isolate P12 and S4 was confirmed as *Pseudomonas aeruginosa* and *Baciilus cereus,* respectively based on 16S rRNA sequencing by sending the cultures to Central Institute of Fisheries and Technology, Wellington Island, Cochin.

4.2 IMPACT OF PSYCHROTROPHIC SPOILAGE ENZYMES ON CURD (DAHI) AND STERILISED MILK

4.2.1 Curd

4.2.1.1 Titratable Acidity and pH

The mean titratable acidity (in percentage of lactic acid) and pH of control and treatment groups are given in Tables 1a and 1b. On zero day, fifth day and tenth day sample CT2 (inoculated with the isolate S4) showed maximum titratable acidity, followed by CT1 (inoculated with the isolate P12) and control. The titratable acidity of all samples was significantly different between each other on all the tested days of storage. For all the samples titratable acidity increased significantly with storage (Fig.1a, 1b, 1c).

Significant difference in pH between samples was evident on 0 day itself. On all the tested days, maximum pH was observed for control followed by CT1

and CT2. The pH decreased significantly with storage. Highest titratable acidity and lowest pH values were recorded after 10 days of storage for all the samples.

Table 1a. Mean titratable acidity of curd on storage

Days of storage	Titratable acidity (Percentage of lactic acid)		
	Control	CTI	CT2
0 day	0.67 ± 0.01^{Aa}	1.09 ± 0.02^{Ba}	1.18 ± 0.02^{Ca}
5 days	0.70 ± 0.01^{Aa}	1.15 ± 0.02^{Ba}	1.73 ± 0.02^{Cb}
10 days	0.87 ± 0.02^{Ab}	1.37 ± 0.03^{Bb}	1.81 ± 0.07^{Cc}

Table 1b. Mean pH of curd on storage

Days of storage	pH		
	Control	CTI	CT2
0 day	4.80 ± 0.02^{Aa}	4.65 ± 0.01^{Aa}	3.76 ± 0.05^{Ca}
5 days	4.60 ± 0.02^{Ab}	3.91 ± 0.02^{Bb}	3.54 ± 0.10^{Cb}
10 days	4.34 ± 0.05^{Ac}	3.71 ± 0.05^{Bc}	3.54 ± 0.13^{Cb}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.

Capital letters denote comparison between rows; small letters denote comparison between columns

4.2.1.2 Syneresis

To assess the impact of psychrotrophic spoilage enzymes on the physical characteristics of dahi, spontaneous syneresis that occurred for CT1 and CT2 were compared with that of control on 0 day, fifth day and tenth day.The mean syneresis value obtained for three treatments are shown in Table 2a. On all tested days of storage syneresis values for CT1 and CT2 were significantly different from control (Fig. 2a, 2b, 2c). However the difference in syneresis value between CT1 and CT2

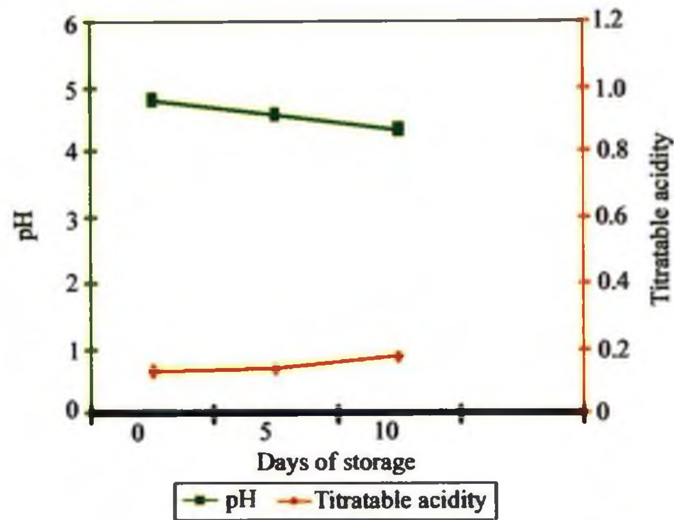


Fig.1a. pH and titratable acidity of control curd during storage

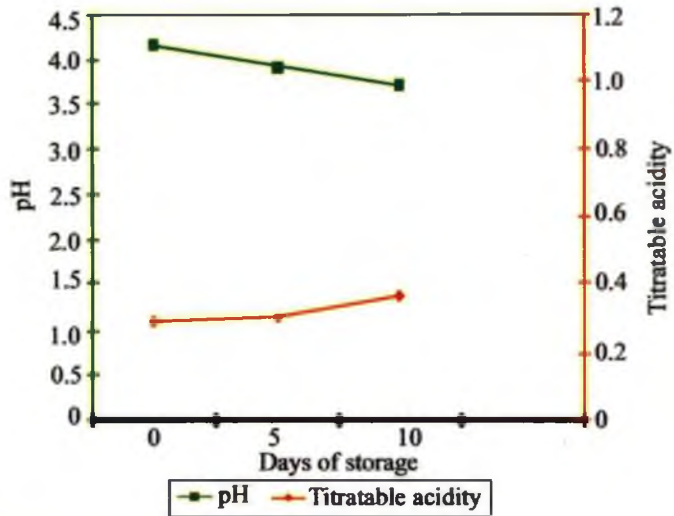


Fig.1b. pH and titratable acidity of curd CT1 during storage

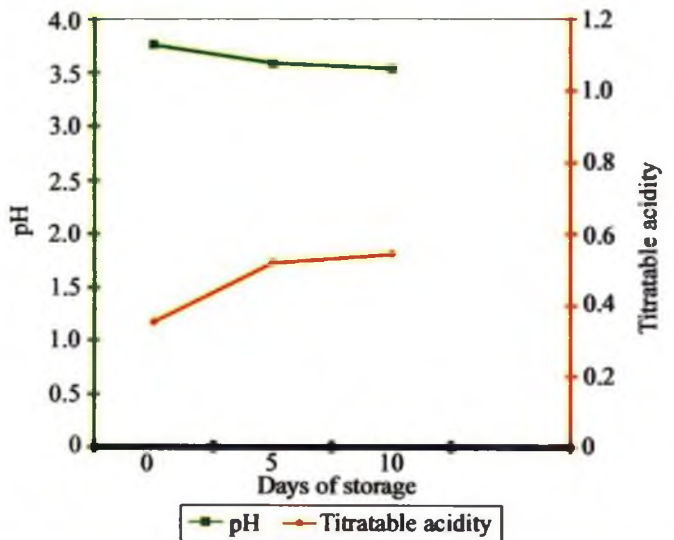


Fig.1c. pH and titratable acidity of curd CT2 during storage

was not significant on 0 day and tenth day; but the syneresis value of CT2 was significantly higher than for CT1 on fifth day of storage. In general for all the samples, syneresis increased with storage. For the control group, increase became significant only after 10 days of storage. Significant increase in value was observed from fifth day of storage itself for CT1 and CT2. Syneresis value differed significantly between all days of storage for both CT1 and CT2.

4.2.1.3 Texture Analysis

As a part of storage study, firmness of the three treatment groups was assessed using TA.XT2 texture analyzer. The firmness values are given in Table 2b. Statistical analyses of the results showed that firmness of the control group was significantly lower than the treatment groups CT1 and CT2 on all tested days of storage. Curd CT2 showed significantly higher firmness values than CT1 throughout storage.

Firmness value generally showed a tendency to increase with storage. Firmness of control group differed significantly between 0 and fifth day and also between 0 and 10 days but not between fifth and tenth day. For CT1 and CT2, firmness differed significantly between all days of storage (Fig. 2a, 2b, 2c).

Viscosity is important for the organoleptic quality of the product as it contributes to appealing appearance and pleasant mouth feel. Viscosity of experimental curd samples was measured using TA.XT2 texture analyzer by way of back extrusion. The values obtained for three treatment groups on 0, five and ten days of storage are presented in Table 2c.

Viscosity of the control group was significantly higher than CT1 and CT2 on all the three tested days of storage. Curd CT2 exhibited lowest viscosity on all days. Viscosity tended to decrease with storage. Reduction in viscosity at different days of storage for control and CT2 were significant. But for curd CT1 viscosity decreased significantly only after 10 days of storage.

Table 2a Mean syneresis values of curd on storage.

Days of storage	Syneresis (Percentage)		
	Control	CTI	CT2
0 day	2.49 ± 0.20^{Aa}	4.24 ± 0.34^{Ba}	4.20 ± 0.37^{Ba}
5 days	3.12 ± 0.20^{Aa}	7.01 ± 0.32^{Bb}	8.21 ± 0.47^{Cb}
10 days	4.05 ± 0.28^{Ab}	11.08 ± 0.39^{Bc}	11.92 ± 0.34^{Bc}

Table 2b. Mean firmness values of curd on storage

Days of storage	Firmness (Kilogram)		
	Control	CTI	CT2
0 day	0.0971 ± 0.0181^{Aa}	0.2269 ± 0.0129^{Ba}	0.2497 ± 0.0116^{Ca}
5 days	0.1581 ± 0.0067^{Ab}	0.4196 ± 0.0157^{Bb}	0.4258 ± 0.0175^{Cb}
10 days	0.1647 ± 0.0057^{Ab}	0.5267 ± 0.0161^{Bc}	0.5986 ± 0.0180^{Cc}

Table 2c Mean viscosity index of curd on storage.

Days of storage	Viscosity index		
	Control	CTI	CT2
0 day	0.0301 ± 0.0035^{Aa}	0.0133 ± 0.0007^{Ba}	0.0127 ± 0.0006^{Ba}
5 days	0.0177 ± 0.0005^{Ab}	0.0120 ± 0.0004^{Ba}	0.0092 ± 0.0005^{Cb}
10 days	0.0130 ± 0.0009^{Ac}	0.0076 ± 0.0008^{Bb}	0.0028 ± 0.0014^{Cc}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.

Capital letters denote comparison between rows; small letters denote comparison between columns

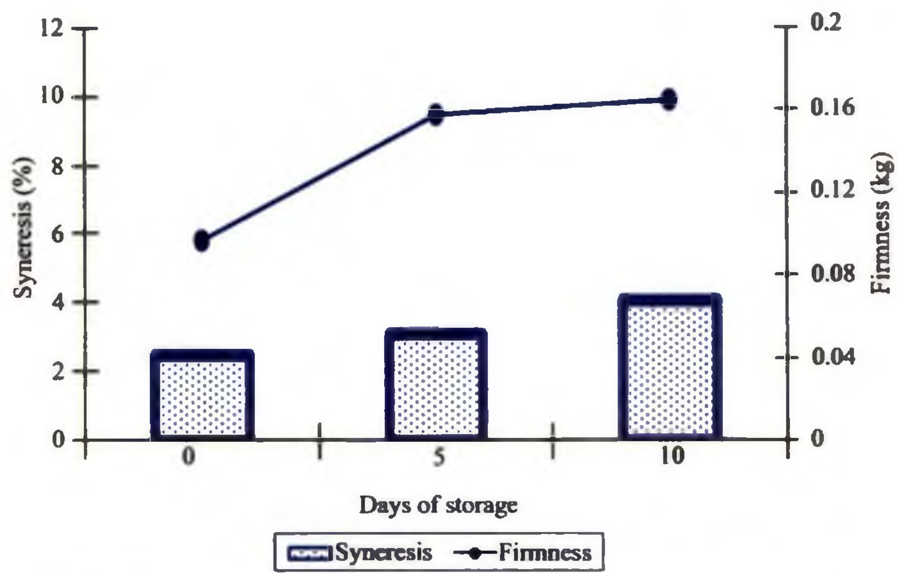


Fig. 2a. Firmness Vs syneresis values of control curd during storage

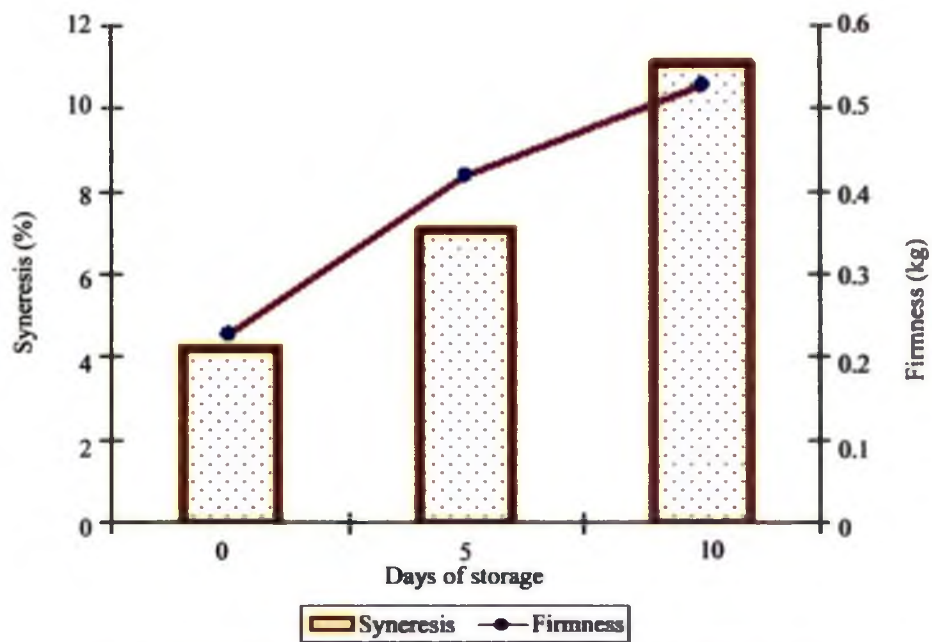


Fig. 2b. Firmness Vs syneresis values of curd CT1 during storage

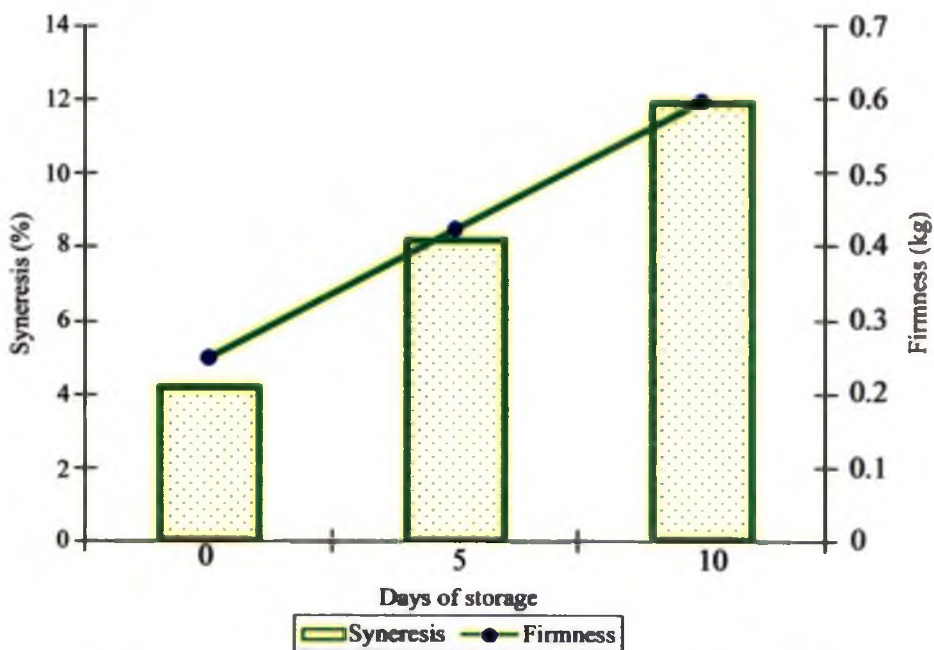


Fig. 2c. Firmness Vs syneresis values of curd CT2 during storage

4.2.1.4 Sensory Evaluation

A semi-trained four member panel evaluated flavour, body and texture, colour and appearance and overall acceptability of the experimental curd samples on 0 day, fifth day and tenth day of storage.

The mean flavour scores obtained for curd samples as given in Table 3a were analysed using the non-parametric test; Kruskalwal test to know the difference between treatments and Wilcoxon test to know the difference between days of storage within treatment. Results showed that CT1 and CT2 were significantly different from control on 0 day itself. The same trend existed on fifth day and tenth day of storage. Wilcoxon signed rank tests revealed that flavour scores were significantly different between 0 and fifth day, 0 and tenth day, and fifth day and tenth day for both CT1 and CT2. Flavour score of control lowered significantly by five days. However the score on tenth day was not significantly different from that on fifth day (Fig. 3a).

The mean scores for body and texture obtained for curd samples are given in Table 3b. Body and texture scores for CT1 were not significantly different from control on 0 day. When compared to control, CT2 had a significantly lower score on 0 day itself. Difference between CT1 and CT2 was also significant on 0 day of storage. After five days of storage, body and texture score showed a sharp decline when compared to control. The difference in score between CT1 and CT2 were not significant on fifth day. The same trend was evident on tenth day of storage also. For all the three groups, body and texture score decreased significantly with storage (Fig. 3b).

Table 3a Mean flavour score of curd

Flavour	Control	CTI	CT2
0 day	34.88 ± 0.43^{Aa}	32.79 ± 0.52^{Ba}	32.25 ± 0.95^{Ba}
5 days	32.17 ± 0.61^{Ab}	18.66 ± 1.82^{Bb}	26.50 ± 0.60^{Cb}
10 days	31.38 ± 0.64^{Ab}	18.68 ± 0.64^{Bc}	14.58 ± 0.60^{Cc}

Table 3b Mean body and texture score of curd

Body and texture	Control	CT1	CT2
0 day	26.17 ± 0.59^{Aa}	24.66 ± 0.55^{Aa}	23.17 ± 0.74^{Ba}
5 days	24.0 ± 0.33^{Ab}	18.29 ± 1.15^{Bb}	15.70 ± 1.12^{Bb}
10 days	19.13 ± 0.84^{Ac}	11.38 ± 0.65^{Bc}	11.58 ± 0.60^{Bb}

Table 3c Mean colour and appearance score of curd

Color and appearance	Control	CT1	CT2
0 day	13.25 ± 0.36^{Aa}	11.33 ± 0.47^{Ba}	11. 54 ± 0.41^{Ba}
5 days	12.54 ± 0.53^{Aa}	9.33 ± 0.60^{Bb}	9.25 ± 0.55^{Bb}
10 days	11.54 ± 0.51^{Aa}	6.25 ± 0.68^{Bc}	4.88 ± 0.63^{Bc}

Table 3d Mean overall acceptability score of curd

Overall acceptability	Control	CT1	CT2
0 day	13.29 ± 0.26^{Aa}	11.71 ± 0.50^{Ba}	11.83 ± 0.51^{Ba}
5 days	13.58 ± 0.23^{Aa}	7.67 ± 0.36^{Bb}	7.75 ± 0.36^{Bb}
10 days	11.70 ± 0.33^{Ab}	3.96 ± 0.39^{Bc}	4.71 ± 0.39^{Bc}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
Capital letters denote comparison between rows; small letters denote comparison between columns

With respect to colour and appearance, CT1 and CT2 got significantly lower scores when compared to control on 0 day itself (Table 3c, Fig. 3c). The same trend persisted on fifth and tenth day of storage also. For the control curd, colour and appearance were not affected by storage. The score reduced significantly for CT1 and CT2 with storage.

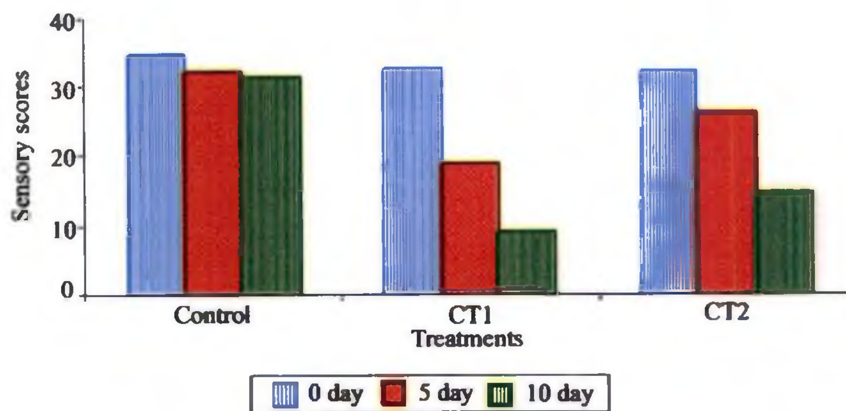


Fig. 3a. Flavour score of control and treatment groups on storage

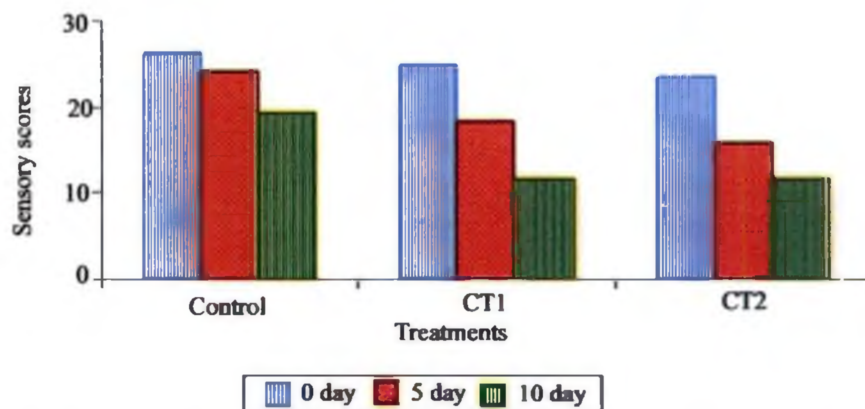


Fig. 3b. Body and texture score of control and treatment curd on storage

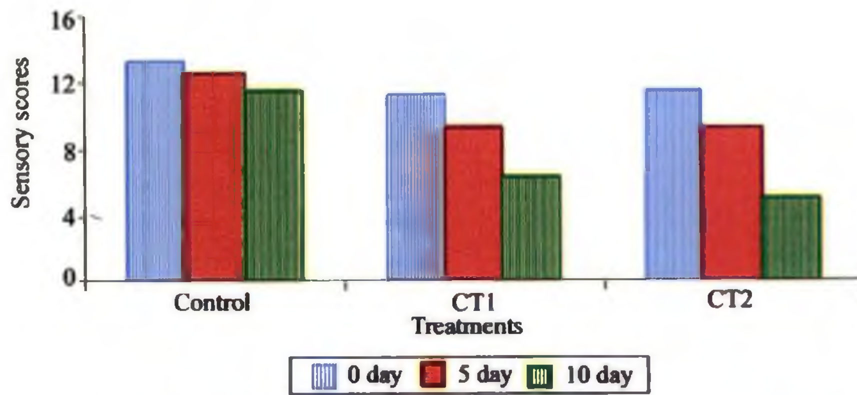


Fig. 3c. Colour and appearance score of control and treatment groups on storage

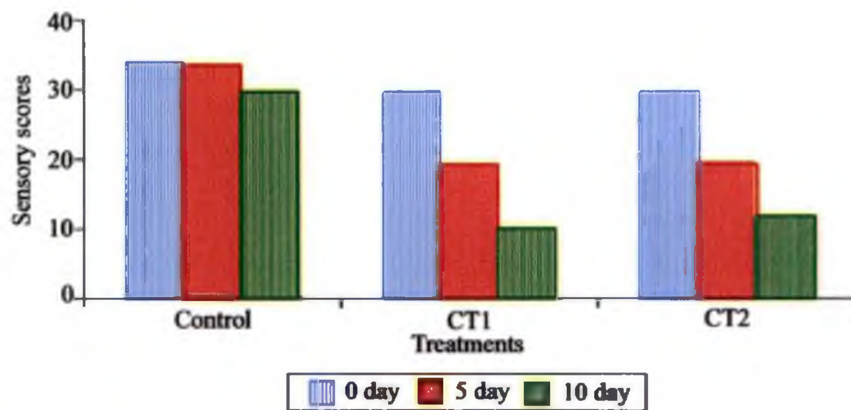


Fig. 3d. Overall acceptability score of control and treatment groups on storage

On all tested days of storage, control curd possessed significantly higher overall acceptability scores than CT1 and CT2 (Table 3d). The scores were not significantly different between CT1 and CT2 on any of the days tested. Scores for overall acceptability lowered drastically for both CT1 and CT2 (Fig.3d) on the fifth day of storage. For the control, decrease in score became significant only on tenth day of storage.

4.2.2 Sterilized Milk

4.2.2.1 pH

Mean pH of sterilised milk for control ST1 and ST2 on 0day, seventh day and fifteenth day are given in Table 4. In general, pH values for ST1 and ST2 were lower than that of control. Between treatments, pH value differed significantly only at 15 days. Within treatment, pH value showed a tendency to decrease. But the decrease was found to be significant for ST2 between 0 day and fifteenth day. Lowest value of pH was recorded for ST2 at 15 days of storage (Fig. 4).

4.2.2.2 Index of Proteolysis

Mean tyrosine and NPN value of sterilised milk on 0, seventh and fifteenth day are given in Table 5a and 5b. Though tyrosine and NPN values did not differ significantly between treatments on 0 day, the differences became significant by seventh and fifteenth day. In general both values increased with storage (Fig.5a,5b,5c). For the control group, values became significantly higher from that of 0 day only after 15days of storage. In the case of ST1 and ST2, the increase of tyrosine and NPN values became highly significant by seven days itself. By 15 days, the values increased still further. The maximum tyrosine and NPN values were observed for the treatment group ST1 at 15 days of storage.

Table 4. Mean pH of sterilised skim milk on storage

Days of storage	pH		
	Control	STI	ST2
0 day	6.67 ± 0.02^{Aa}	6.60 ± 0.02^{Aa}	6.64 ± 0.02^{Aa}
7 days	6.57 ± 0.02^{Aa}	6.51 ± 0.02^{Aab}	6.55 ± 0.02^{Aa}
15 days	6.57 ± 0.01^{Aa}	6.41 ± 0.01^{Bbc}	6.50 ± 0.03^{Ca}

* The mean difference is significant ($p \leq 0.05$).

Table 5a. Mean tyrosine values for sterilised skim milk on storage

Days of storage	Tyrosine value (μ moles of tyrosine/ ml)		
	Control	STI	ST2
0 day	11.51 ± 0.72^{Aa}	13.48 ± 0.73^{Aa}	12.55 ± 0.72^{Aa}
7 days	11.75 ± 0.46^{Aa}	19.35 ± 0.81^{Bb}	16.35 ± 0.55^{Cb}
15 days	13.53 ± 0.83^{Aab}	102.97 ± 4.74^{Bc}	62.36 ± 2.33^{Cc}

Table 5b. Mean NPN values for sterilised skim milk on storage

Days of storage	Tyrosine value (Percentage of Nitrogen)		
	Control	STI	ST2
0 day	0.036 ± 0.002^{Aa}	0.039 ± 0.004^{Aa}	0.004 ± 0.003^{Aa}
7 days	0.043 ± 0.001^{Ab}	0.114 ± 0.004^{Bb}	0.06 ± 0.002^{Cb}
15 days	0.044 ± 0.001^{Ab}	0.235 ± 0.003^{Bc}	0.1 ± 0.004^{Cc}

* The mean difference is significant ($p \leq 0.01$).
* Figures bearing same superscript do not differ significantly.

Capital letters denote comparison between rows; small letters denote comparison between columns

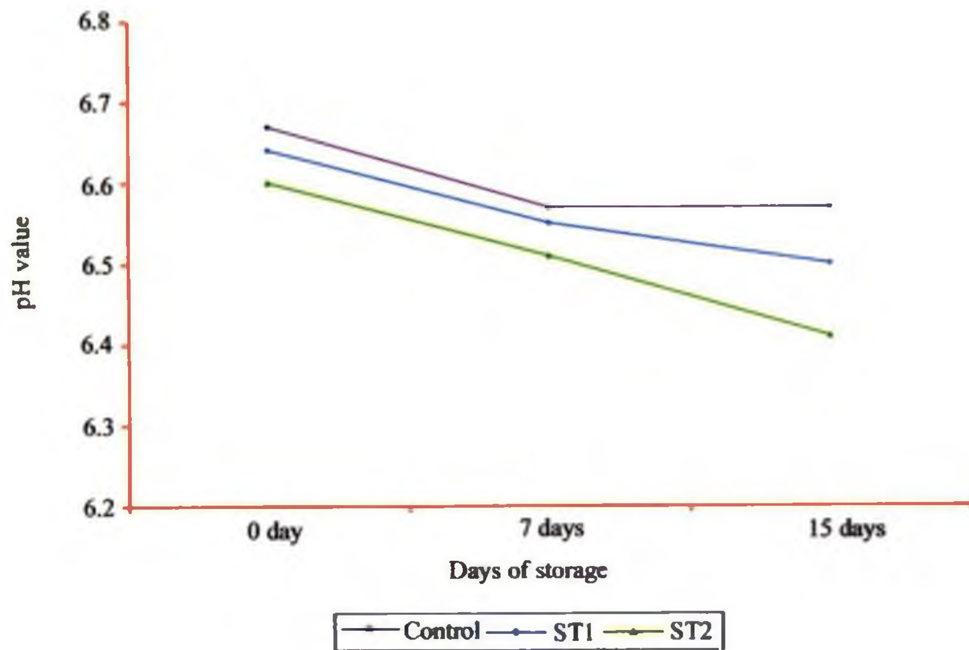


Fig. 4 pH of sterilized skim milk on storage

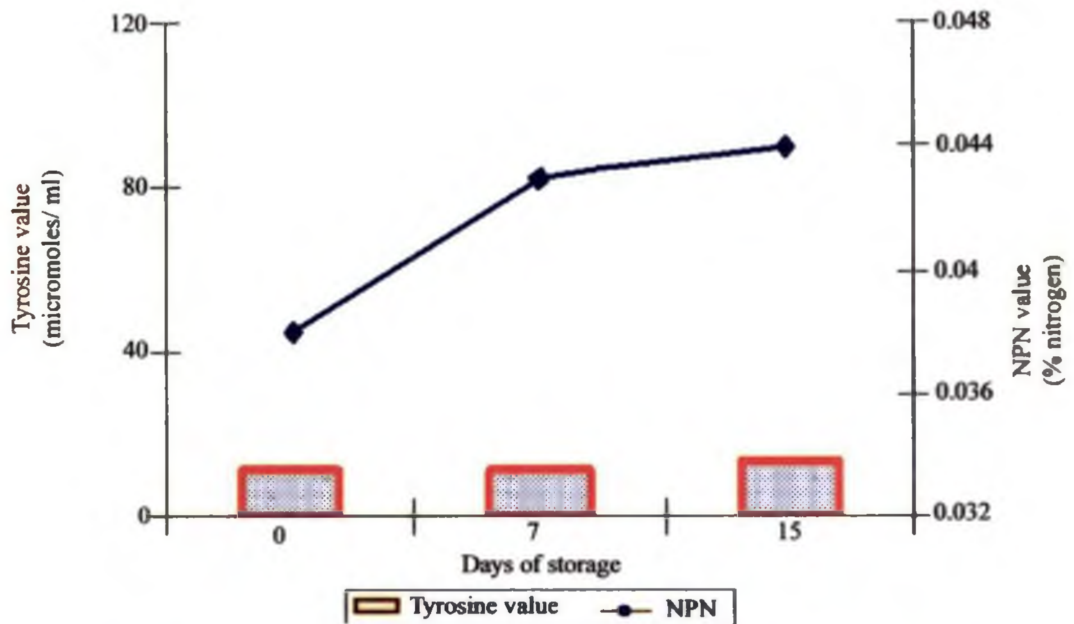


Fig. 5a. Tyrosine Vs NPN values for control sterilized milk during storage

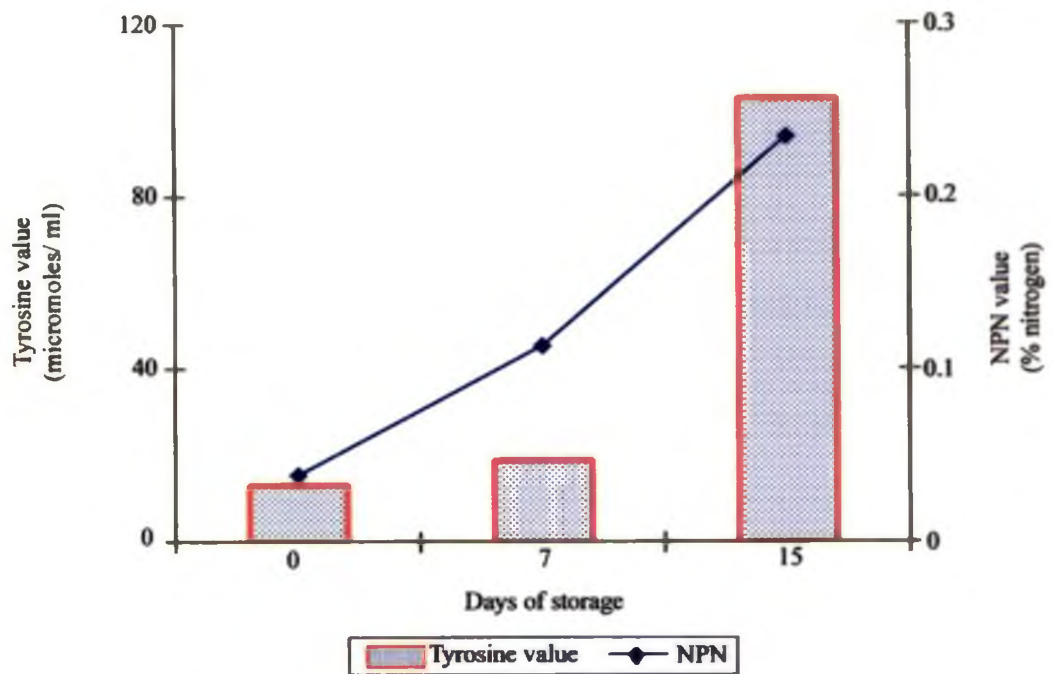


Fig. 5b. Tyrosine Vs NPN values for ST1 during storage

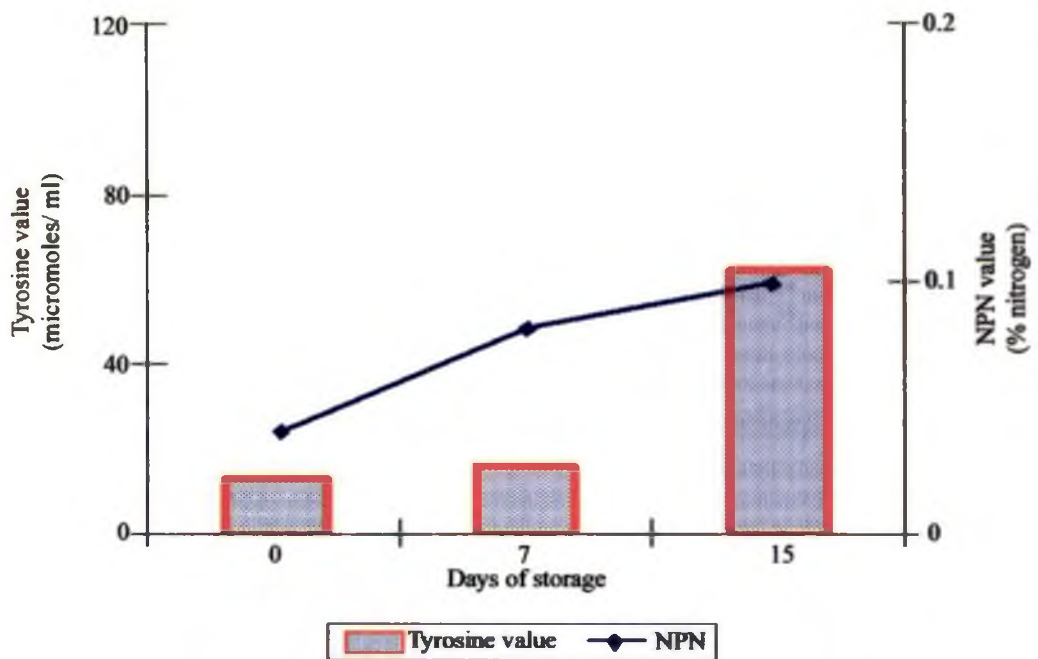


Fig. 5c. Tyrosine Vs NPN values for ST2 during storage

4.2.2.3 Sensory Evaluation

Consumer acceptance of any product is strongly determined by its sensory characteristics. The objective of present trial was to find out if the treatment groups were detectably different for controls. Cooked flavour was significantly higher for control than ST1 and ST2 on 0, 7 and 15 days of storage. At 7 days of storage, intensity of cooked flavour was significantly high for ST2 than ST1. In general, intensity of cooked flavour decreased with storage. But the difference was insignificant for ST2 between all days whereas for control and ST1 difference was not significant between 7 and 15 days of storage (Table 6a).

On 0 day none of the sample possessed detectable unclean flavour. On 7 days and 15 days intensity was significantly highest for ST1, followed by ST2 and control. With storage, intensity of unclean flavour increased significantly for both ST1 and ST2 on 7 days and still further by 15 days of storage. Unclean flavour was negligible in control throughout storage (Table 6b).

Table 6a. Panelist ratings of sterilised milk – cooked flavour

Cooked flavour	Mean ± SE Control	STI	ST2
0 day	5.17 ± 0.35^{Aa}	3.83 ± 0.31^{Ba}	4.0 ± 0.37^{Ba}
7 days	3.00 ± 0.26^{Ab}	1.83 ± 0.48^{Bb}	3.83 ± 0.31^{Ca}
15 days	2.83 ± 0.31^{Ab}	1.33 ± 0.42^{Bb}	2.67 ± 0.43^{Aa}

Table 6b. Panelist ratings of sterilised milk – unclean flavour

Unclean flavour	Control	STI	ST2
0 day	(ND)	(ND)	(ND)
7 days	0.50 ± 0.18^{Aa}	6.67 ± 0.49^{Bb}	3.17 ± 0.40^{Cb}
15 days	0.25 ± 0.17^{Aa}	$10.5\ 0\pm 0.76^{Bc}$	8.83 ± 0.47^{Cc}

Table 6c. Panelist ratings of sterilised milk – Bitter taste

Bitter taste	Control	STI	ST2
0 day	(ND)	0.83 ± 0.17^{Ba}	(ND)
7 days	0.20 ± 0.10^{Aa}	6.83 ± 0.31^{Bb}	7.50 ± 0.43^{Bb}
15 days	0.50 ± 0.18^{Aa}	8.33 ± 0.42^{Bc}	11.50 ± 0.43^{Cc}

Table 6d. Panelist ratings of sterilised milk – Viscosity

Viscosity	Control	STI	ST2
0 day	1.5 ± 0.22^{Aa}	1.5 ± 0.22^{Aa}	1.33 ± 0.21^{Aa}
7 days	1.5 ± 0.22^{Aa}	1.67 ± 0.33^{Aa}	5.17 ± 0.31^{Bb}
15 days	3.67 ± 0.33^{Ab}	2.0 ± 0.36^{Ba}	6.0 ± 0.26^{Cb}

Table 6e. Panelist ratings of sterilised milk – Colour intensity

Color intensity	Control	STI	ST2
0 day	4.17 ± 0.31^{Aa}	3.83 ± 0.31^{Aa}	3.83 ± 0.31^{Aa}
7 days	4.5 ± 0.22^{Aa}	4.00 ± 0.26^{Aa}	6.33 ± 0.33^{Bb}
15 days	4.16 ± 0.31^{Aa}	8.50 ± 0.43^{Bb}	9.67 ± 1.33^{Cc}

Table 6f. Panelist ratings of sterilised milk – Sour taste

Sour taste	Control	STI	ST2
0 day	(ND)	(ND)	(ND)
7 days	(ND)	(ND)	2.33 ± 0.33^{Bb}
15 days	(ND)	(ND)	4.17 ± 0.40^{Bc}

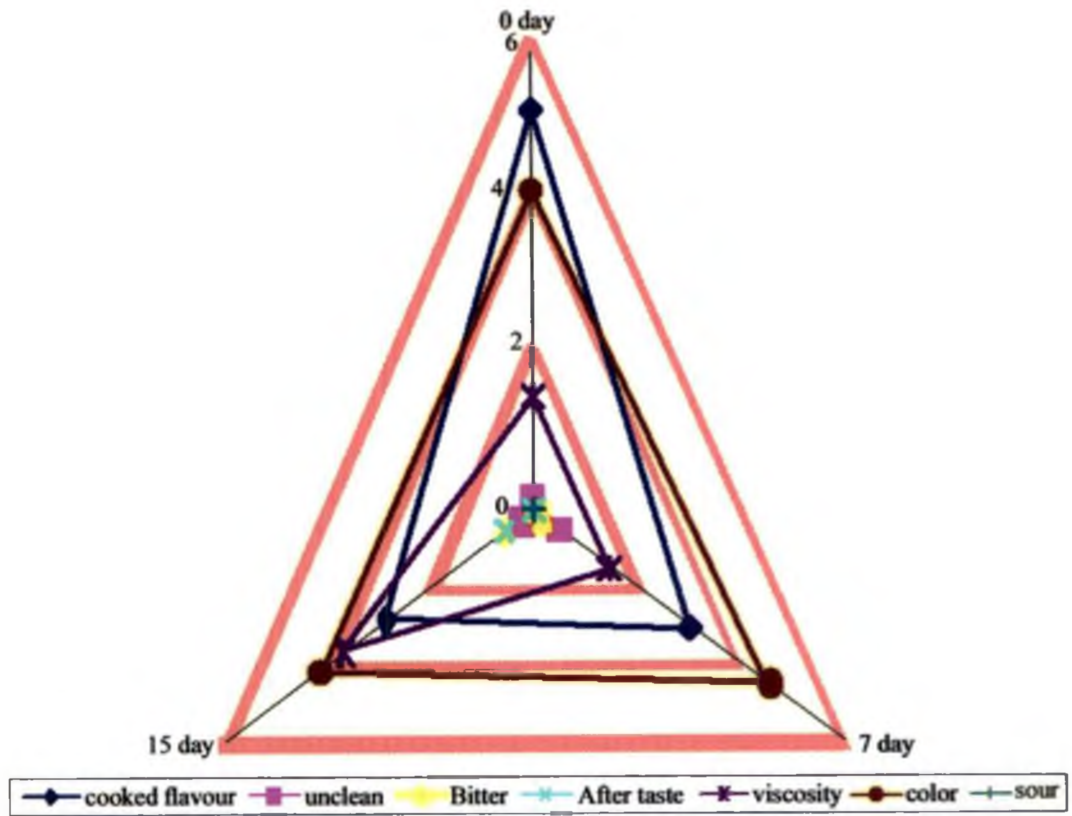


Fig. 6a. Quantitative descriptive analysis of control sterilized milk

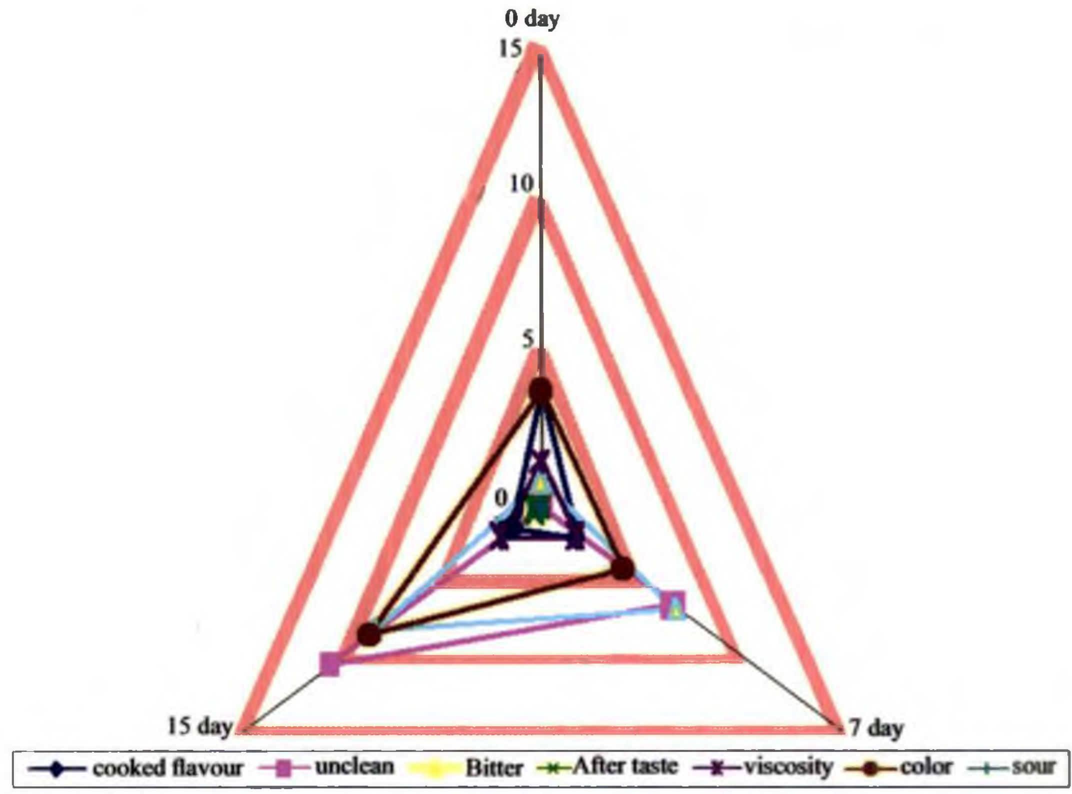


Fig. 6b. Quantitative descriptive analysis of treatment sterilized milk ST1

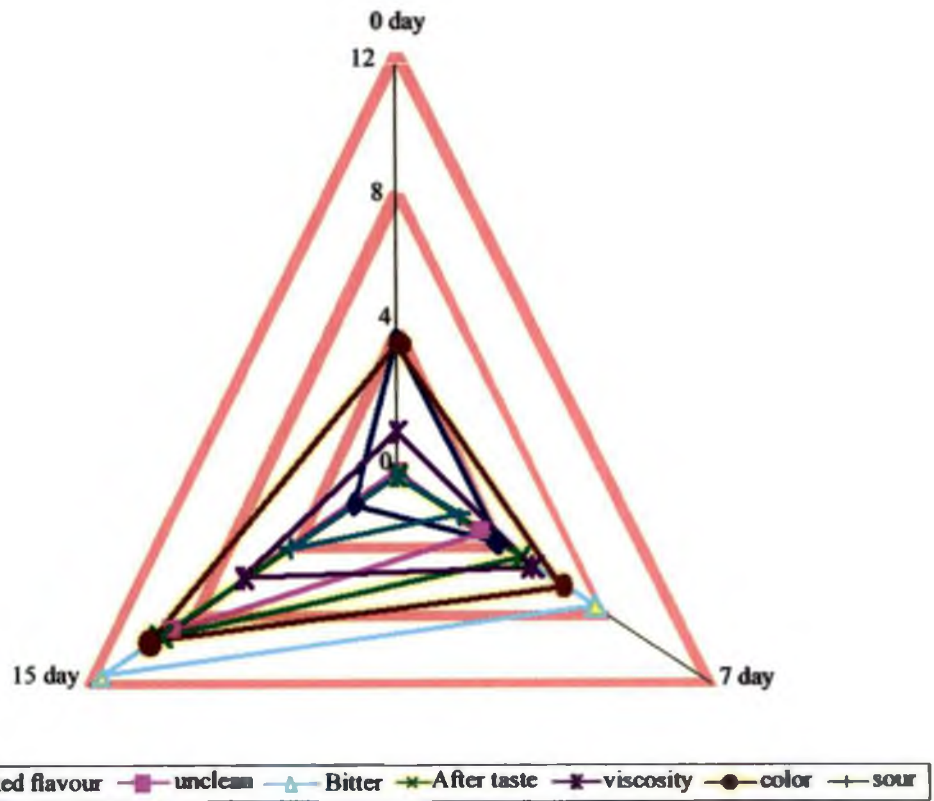


Fig. 6c. Quantitative descriptive analysis of treatment sterilized milk ST2

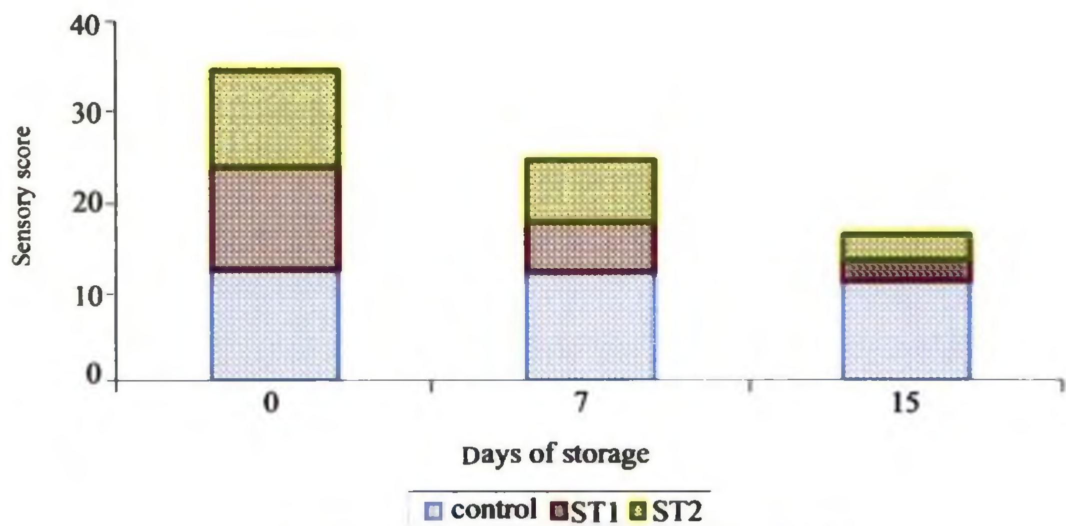


Fig. 6d. Overall quality rating of sterilized milk

Table 6g. Mean panelist ratings of sterilised milk – Overall quality

Overall quality rating	Control	STI	ST2
0 day	12.67 ± 0.33^{Aa}	11.17 ± 1.31^{Ba}	11.00 ± 0.37^{Ba}
7 days	12.17 ± 0.31^{Ab}	5.5 ± 0.42^{Bb}	6.83 ± 0.31^{Bb}
15 days	14.17 ± 0.31^{Ab}	2.33 ± 0.33^{Bc}	3.00 ± 0.37^{Bc}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
* ND- Not detectable

Capital letters denote comparison between rows; small letters denote comparison between columns

On all tested days of storage, bitter taste was negligible in the control group. Intensity of bitter taste increased sharply in ST1 and ST2 at 7 days of storage. This trend continued on 15th day of storage also. Significant bitterness was not detected in control throughout storage period (Table 6c).

Viscosity of the three treatments did not differ significantly on 0 day (Table 6d). However after seven days, ST2 showed significantly higher viscosity than control. On the contrary, when compared to control viscosity values were lower for ST1, though not significant.

Colour intensity was more or less same for all treatments on 0 day. However, browning increased significantly for ST2 from day seven onwards. Color intensity was highest for ST2 after 15 days of storage. With storage, colour intensity increased significantly for ST1 and ST2 especially during later stages of storage. Color intensity remained unchanged for control throughout storage (Table 6e). Panelists could detect sour taste only in ST2, after keeping the sterilized milk at room temperature for 7 days. The intensity increased further by 15 days (Table 6f).

Overall quality rating, a reflection of totality of above said parameters was significantly lower for ST1 and ST2 when compared to control on 0 day itself.

Overall quality rates declined sharply with storage (Table 6g). The results of quantitative descriptive analysis are depicted in Fig. 6a, 6b, 6c and 6d.

4.3 ALKALINE PROTEASES

4.3.1 Environmental Factors Affecting Production of Alkaline Protease

The growth and biosynthetic activities of microorganisms vary considerably depending on the availability of the required nutrients, and environmental conditions. It is essential to evaluate the performance of selected strain under different nutritional and environmental conditions to obtain high and commercially viable yields of protease.

4.3.1.1 Temperature and Period of Incubation

The mean protease activity (expressed in terms of μmoles of tyrosine) after 24 h of incubation at 37°C, 42°C and 55°C were 114.21 ± 0.80, 98.36 ± 1.27 and 97.48 ± 3.48 respectively (Table 7a). After 48 h of incubation protease activity did not show remarkable change for samples kept at 37°C (112.90 ± 1.07) and 55°C (79.94 ± 2.96).

However for cultures kept at 42°C, protease activity increased to 105.41 ± 0.97. The protease activity of samples at 37°C remained at 114.62 ± 1.51 even after 72 h of incubation. The enzyme activity of the culture kept at 42°C increased to 137.87 ± 1.26 and that at 55°C to 126.24 ± 2.04 after 72 h of incubation (Fig. 7a).

Statistical analysis of the results indicate that the differences in the protease activity between the cultures maintained at 37°C, 42°C and 55°C were highly significant at 24, 48 and 72 h of incubation.

4.3.1.2 pH of the Medium

The pH of the fermentation medium has a marked effect on the growth of cells and thereby on the enzyme production also. Hence, the protease production by the selected alkalophilic organism was monitored in the medium (nutrient broth

with 0.5 per cent skim milk) after adjusting the pH to 8, 9, 10 and 11 using calculated quantity of two per cent sodium carbonate. Protease assay was done using the cell free supernatant obtained after 24 h incubation at 37°C. Maximum activity of 118.60 ± 0.84 (Table 7b and Fig.7b) obtained at pH 11 was significantly higher than that at pH 8 and 9; but the difference was not significant when compared to the activity at pH 10. The enzyme activity obtained at pH 8, 9 and 10 were significantly different between each other.

Table 7a. Effect of temperature and period of incubation on production of protease enzyme

Temperature	Activity		
	Activity after 24 hr.	Activity after 48 hr.	Activity after 72 hr.
37°C	114.21 ± 0.80[a]	112.90 ± 1.07[a]	114.62 ± 1.51[a]
42°C	98.36 ± 1.27[b]	105.41 ± 0.97[b]	137.87 ± 1.26[b]
55°C	97.48 ± 3.48[c]	79.94 ± 2.96[c]	126.24 ± 2.04[c]

Table 7b. Effect of pH on production of protease enzyme

pH	Activity after 24 hrs of incubation
8	80.46 ± 1.06[a]
9	95.01 ± 0.64[b]
10	116.78 ± 1.54[c]
11	118.60 ± 0.84[c]

Table 7c. Effect of aeration on the production of protease enzyme

Activity after 24h (with aeration)	Activity after 24h (without aeration)
108.54 ± 2.36^{a}	119.39 ± 3.10^{b}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
* Activity is expressed as μ moles of tyrosine released under standard assay conditions.

4.3.1.3 Aeration

The results pertaining to the effect of aeration on the production of alkaline protease is presented in Table 7c, Fig.7c. Under the test conditions in this trial, the mean enzyme activity obtained for culture kept without aeration was 119.39 ± 3.10 μmoles of tyrosine/ml under standard assay conditions. This was significantly higher than the activity achieved with aeration (108.54 ± 2.36).

4.3.1.4 Rate of Inoculum

The amount of inoculum used to initiate the fermentation also affects the production of protease enzyme. In this trial, media was inoculated at a level of one, two, three and four per cent. Protease assay was done after 24 h of incubation at 37°C. The results are given in Table 7d and Fig.7d. Maximum protease yield was observed when three per cent rate of inoculum was used. This value was significantly higher than the values obtained with one, two and four per cent rate of inoculation. When multiple comparisons were done, enzyme activity at two and four per cent level was not significantly different. Between all other treatments, difference was significant. Least activity was recorded when inoculation was done at one per cent level.

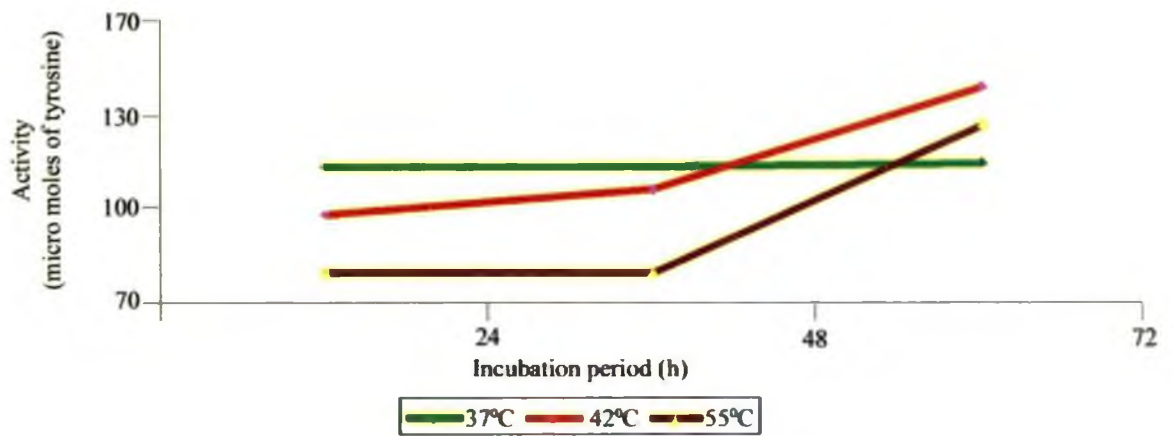


Fig. 7a. Effect of temperature and period of incubation on production of protease enzyme

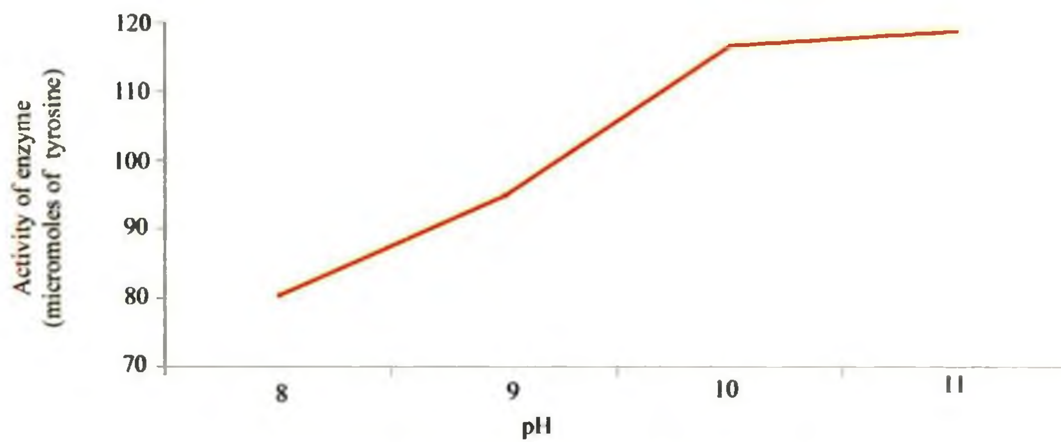


Fig. 7b. Effect of pH on production of protease enzyme

Table 7d. Effect of rate of inoculum on production of protease enzyme

Rate of inoculum (per cent)	Activity after 24h
One	120.56 ± 1.32^{a}
Two	149.11 ± 1.70^{b}
Three	176.57 ± 3.84^{c}
Four	143.93 ± 1.15^{b}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
* Activity is expressed as μ moles of tyrosine released under standard assay conditions.

4.3.2 Nutritional Factors Affecting Production of Alkaline Protease

4.3.2.1 Nitrogen Sources

To the basal fermentation medium, various organic nitrogen sources were added individually at a level of 0.5 per cent. The influence of each source on production of protease was assessed by comparing the enzyme activity with that obtained in basal fermentation media that served as control. Mean protease activity obtained after the addition of various organic nitrogen sources are given in Table 8a. Incorporation of all the organic nitrogen sources tested in this trial showed a significant increase in enzyme activity when compared to control (Fig. 8a). Maximum enzyme activity of 247.60± 3.86 was obtained when tryptone was added. Supplementation of basal fermentation medium with beef extract improved the enzyme yield when compared to control. However the activity got with beef extract was significantly lower than that obtained with peptone and yeast extract. Yeast extract and peptone supported protease production similarly.

Different inorganic nitrogen sources were also assessed for their ability to enhance the yield of protease enzyme. This was done by incorporating various inorganic nitrogen sources at a level of 0.5 per cent into the basal medium and by assaying the enzyme activity of cell free supernatant after 24 h of incubation at

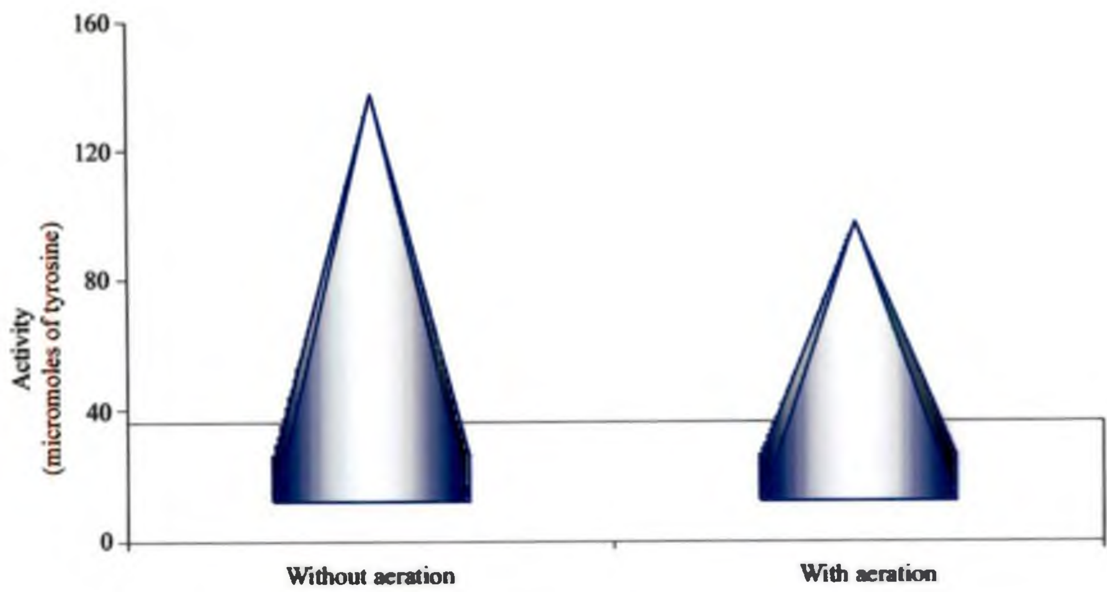


Fig. 7c. Effect of aeration on the production of protease enzyme

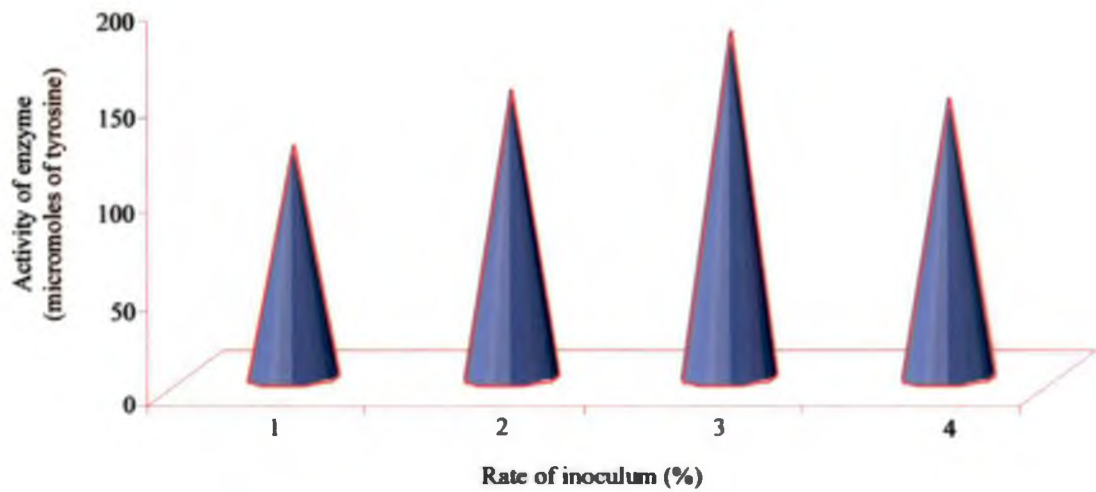


Fig. 7d. Effect of rate of inoculum on enzyme production

37°C. The mean protease activity of different treatments is recorded in Table 8b. The mean protease activity obtained after incorporation of ammonium carbonate, ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate were 109.75 ± 3.07, 150.34 ± 2.21, 244.09 ± 2.51, 175.66 ± 3.58 and 110.35 ± 3.12 micromoles/ml respectively (Fig. 8b). Highest activity was obtained with ammonium sulphate, followed by ammonium chloride and ammonium nitrate. Both of these showed significantly higher yield when compared to the control. Ammonium carbonate and potassium nitrate supported the enzyme activity similarly.

Table 8a. Effect of organic nitrogen sources on the production of protease enzyme

Organic Nitrogen Sources	Activity
Control	88.83 ± 3.05^{a}
Tryptone	247.60 ± 3.86^{b}
Peptone	213.06 ± 3.64^{c}
Beef extract	101.39 ± 1.90^{d}
Yeast extract	206.44 ± 2.53^{c}

Table 8b. Effect of inorganic nitrogen sources on the production of protease enzyme

Inorganic Nitrogen Sources	Activity
Control	88.83 ± 3.05^{a}
Ammonium Carbonate	109.75 ± 3.07^{b}
Ammonium Nitrate	150.34 ± 2.21^{c}
Ammonium Sulphate	244.09 ± 2.51^{d}
Ammonium Chloride	175.66 ± 3.58^{e}
Potassium nitrate	110.35 ± 3.12^{b}

Table 8c. Effect of different carbon sources on the production of protease enzyme

Carbon Sources	Activity
Control	252.15 ± 2.56[a]
Glucose	249.81 ± 2.01[a]
Sucrose	248.90 ± 1.84[a]
Maltose	236.31 ± 3.10[b]
Corn starch	236.69 ± 3.22[c]
Potato starch	252.54 ± 2.95[a]
Trisodium citrate	272.14 ± 2.61[d]

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
* Activity is expressed as μ moles of tyrosine released under standard assay conditions.

4.3.2.2 Carbon Sources

The mean protease activity after the addition of different carbon sources at a level of 0.5 per cent to the modified whey based fermentation medium (0.5 per cent tryptone, 0.5 per cent whey powder and 0.5 per cent skim milk powder) is presented in the Table 8c. Activity obtained in modified whey based fermentation medium without addition of any carbon sources acted as control. Of the various carbon sources tested, only trisodium citrate was found to be capable of enhancing the protease yield significantly. Glucose, sucrose and potato starch were found to have no significant impact on the enzyme yield. Addition of maltose and corn starch to the fermentation medium had a significant adverse effect on the enzyme yield (Fig. 8c).

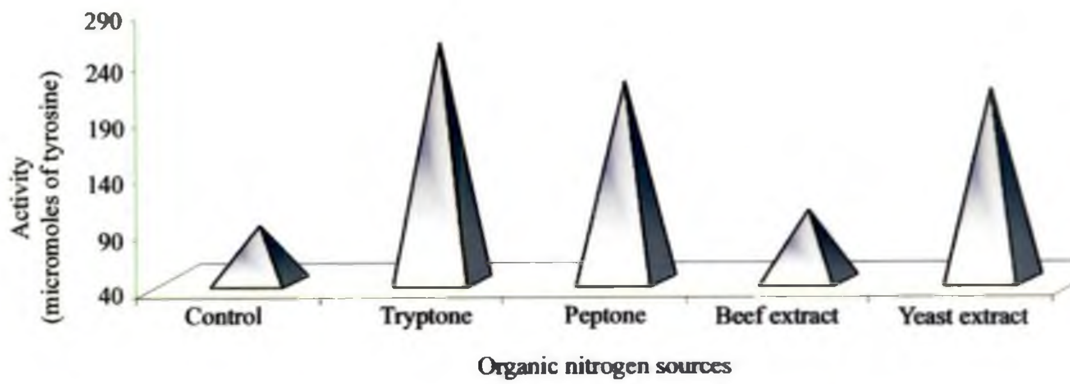


Fig. 8a. Effect of organic nitrogen sources on production of protease enzyme

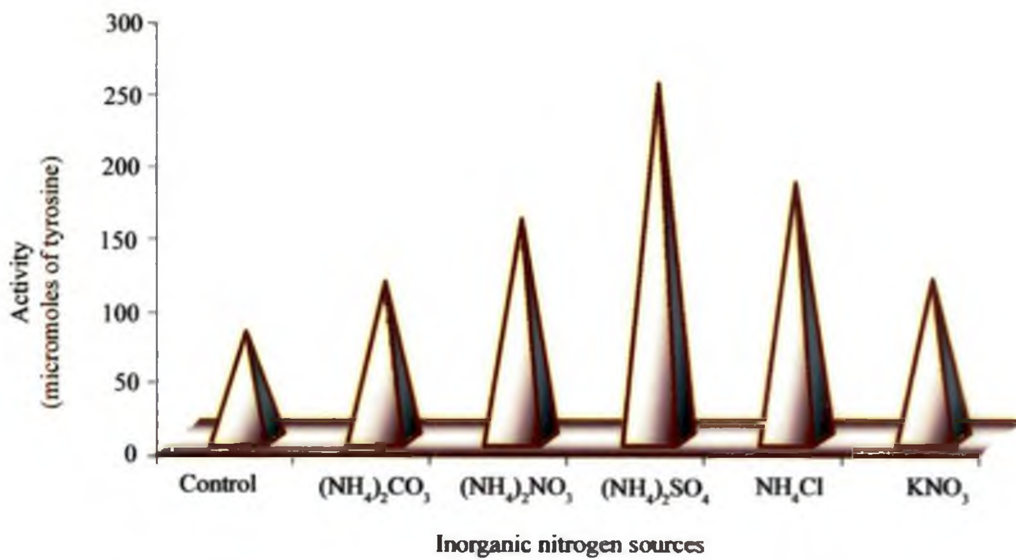


Fig. 8b. Effect of inorganic nitrogen sources on production of protease enzyme

4.3.3 Partial Characterization of Enzyme

4.3.3.1 pH Stability

Bacillus derived alkaline proteases are the major industrial work horses in different process applications. This essentiates the assessment of enzyme potential to remain stable in a wide range of pH. The stability of enzyme with an activity over a wide pH range was assessed by pre incubation of the sample at pH 7, 8, 9, 10, 11 and 12 for 24 h. The mean activity obtained after exposure to different pH for 24 h is as shown in Table 9a. The initial activity of enzyme (283.8 ± 3.69) was taken as 100 per cent. The protease activity increased with increase in alkalinity. In this trial, maximum activity was recorded at pH 12.0. The activity observed after 24 h at pH 12 was significantly higher than that observed at pH 11.0 (Fig. 9a).

Table 9a. Stability of enzyme at different pH

pH of the medium	Protease activity (μmoles of tyrosine/ ml)	Residual activity (per cent)
7	314.34 ± 3.53	92.6^{a}
8	325.41 ± 3.69	110.8^{b}
9	336.97 ± 6.54	114.3^{b}
10	355.15 ± 2.06	118.7^{bc}
11	385.53 ± 3.13	125.1^{d}
12	262.74 ± 2.59	135.8^{e}

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.
* Activity is expressed as μ moles of tyrosine released under standard assay conditions.

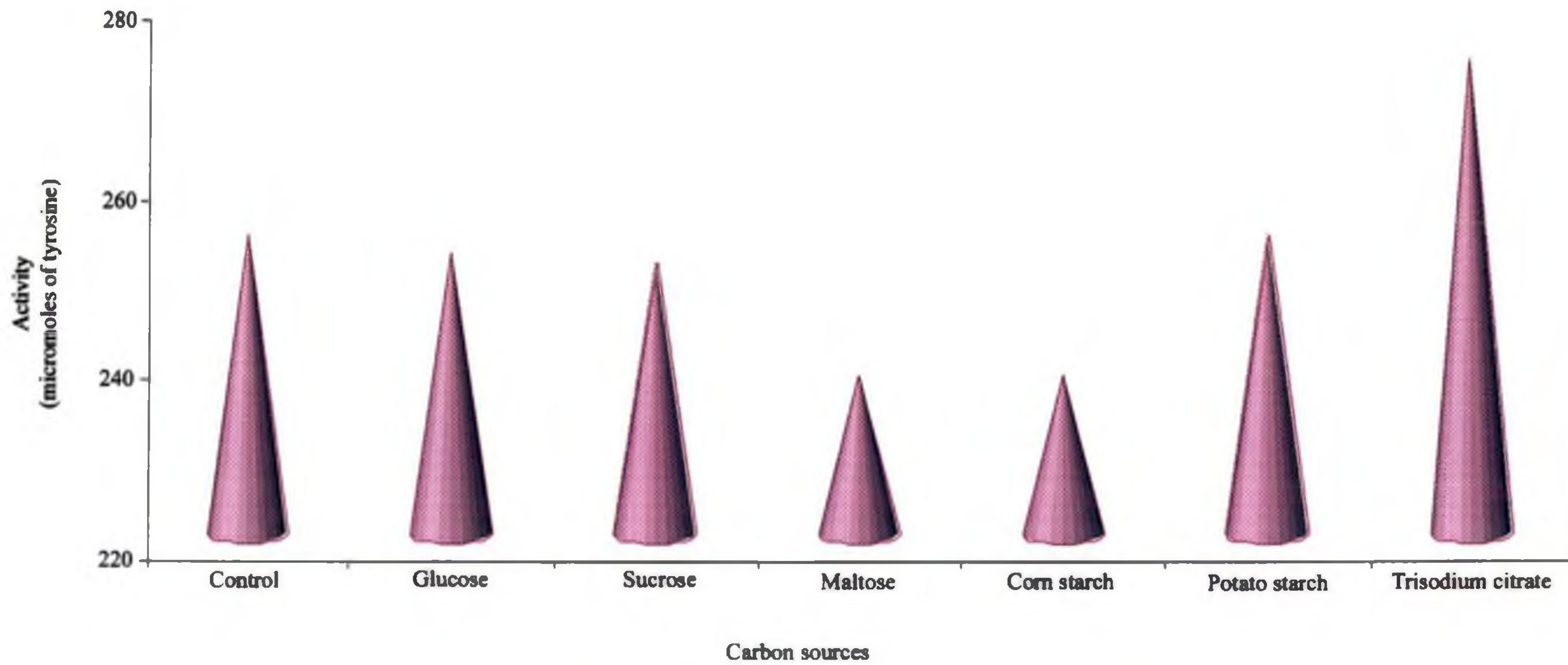


Fig. 8c. Effect of carbon sources on production of protease enzyme

4.3.3.2 Temperature Stability

Enzymes that are capable of maintaining activity and stability in a wide range of temperature is of great significance as this can be made use of in both cold and hot washing cycles. To evaluate the performance of protease enzyme at different temperatures, the crude enzyme was subjected to 20°C, 40°C, 60°C and 80°C. Residual activity at these temperatures was assessed after 30, 60 and 90 minutes of exposure. Activity prior to the exposure (300.98 ± 3.00) to the selected temperatures was taken as 100 per cent. The detailed results of the trial are depicted in Table 9b, Fig. 9b.

Table 9b Stability of enzyme at high temperature

Temper-ature (°C)	Duration of exposure (minutes)							
	0 hr.		30 min		60 min		90 min	
	Activity (µ moles of tyrosine/ml)	Residual activity (per cent)	Activity (µ moles of tyrosine/ml)	Residual activity (per cent)	Activity (µ moles of tyrosine/ml	Residual activity (per cent)	Activity (µ moles of tyrosine/ml	Residual activity (per cent)
20	300.98 ± 3.00	100	240.78 ± 0.47	80	212.49 ± 1.50	70.6	210.69 ± 1.86	70.0
40	300.98 ± 3.00	100	235.97 ± 3.60	78.4	206.17 ± 1.11	68.5	184.80 ± 0.74	61.4
60	300.98 ± 3.00	100	238.98 ± 0.96	79.4	183.00 ± 0.85	60.8	180.08 ± 1.11	60.1
80	300.98 ± 3.00	100	139.65 ± 1.18	46.4	224.80 ± 2.18	74.7	224.83 ± 1.36	67.2

Storage stability of the crude enzyme was also assessed at 10 days interval for 40 days after keeping the enzyme at -10° and 10°C. The initial activity of 300.98 ± 3.00 was taken as 100 per cent. Mean values of residual activity are given in Table 9c. Under both storage conditions initially there was a gradual increase in activity. Activity reached a peak at 20 days after which activity started to decline. After 40 days of storage residual activity was 77.4 per cent and 69.5 per cent respectively (Fig. 9c).

Table 9c. Stability of enzyme at low temperature

Days of storage / Temperature	10°C		-10°C	
	Activityμ moles of tyrosine/ml	Residual activity	Activityμ mole of tyrosine/ml	Residual activity
0	300.98 ± 3.00	100	300.98 ± 3.00	100
10	378.62 ± 2.43	125.8	370.83 ± 2.18	123.2
20	437.75 ± 1.90	145.4	389.01 ± 2.78	129.3
30	384.3 ± 1.86	127.7	360.0 ± 1.77	119.6
40	233.04 ± 2.37	77.4	209.16 ± 1.73	69.5

4.3.3.3 Effect of Metal Ions

The stability of crude protease enzyme in the presence of metal ions like Zn^{++}, Ca^{++}, Mg^{++}, Na^{+}, Hg^{++}, Mn^{++} and Co^{++} was investigated. The activity obtained (271.70 ± 3.45) prior to incubation with metal ions was taken as control. In general all metal ions except Mn^{++} exhibited a significant stimulatory effect (Table 9d, Fig. 9d). Inhibitory effect imparted by Co^{++} was not significant.

Table 9d. Stability of enzyme in the presence of different metal ions

Metal ions	Activity (μmoles of tyrosine /ml)	Residual activity (per cent)
Control	271.70 ± 3.45[a]	100
Zn^{++}	302.16 ± 2.93[b]	111.4
Ca^{++}	364.11 ± 4.20[c]	134.3
Mg^{++}	363.46 ± 3.15[d]	134.0
Na^{+}	346.70 ± 4.58[e]	127.7
Hg^{++}	328.40 ± 4.92[f]	121.0
Mn^{++}	279.83 ± 3.34[a]	103.0
Co^{++}	270.45 ± 4.39[a]	99.6

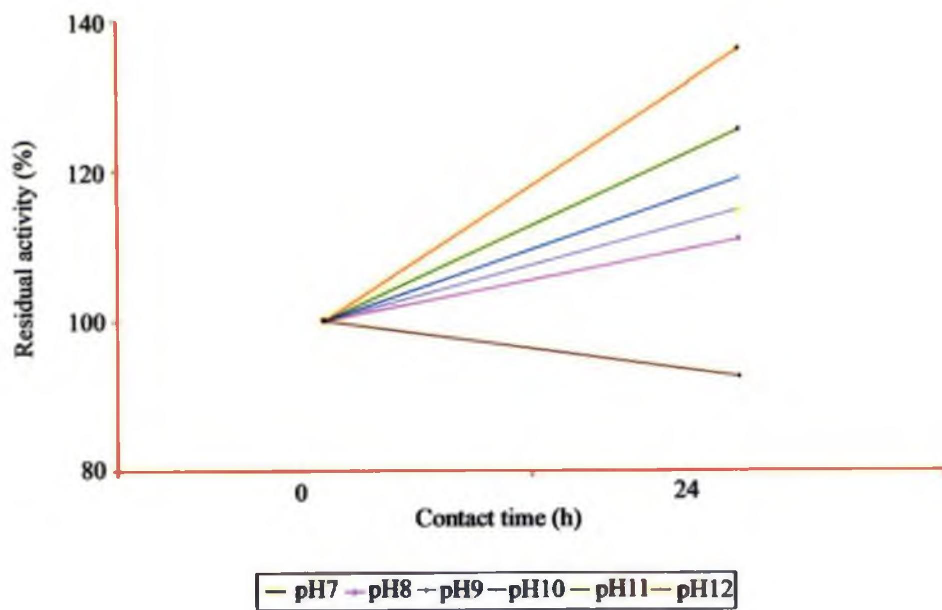


Fig. 9a. Stability of enzyme at different pH

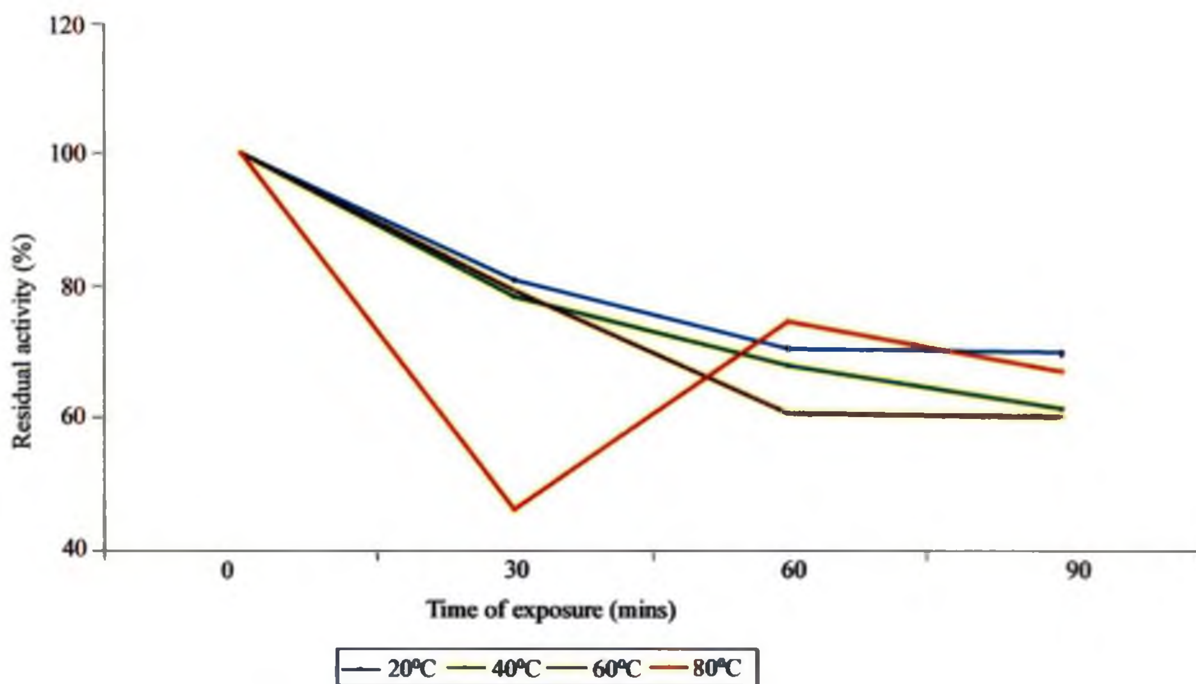


Fig. 9b. Stability of enzyme at high temperature

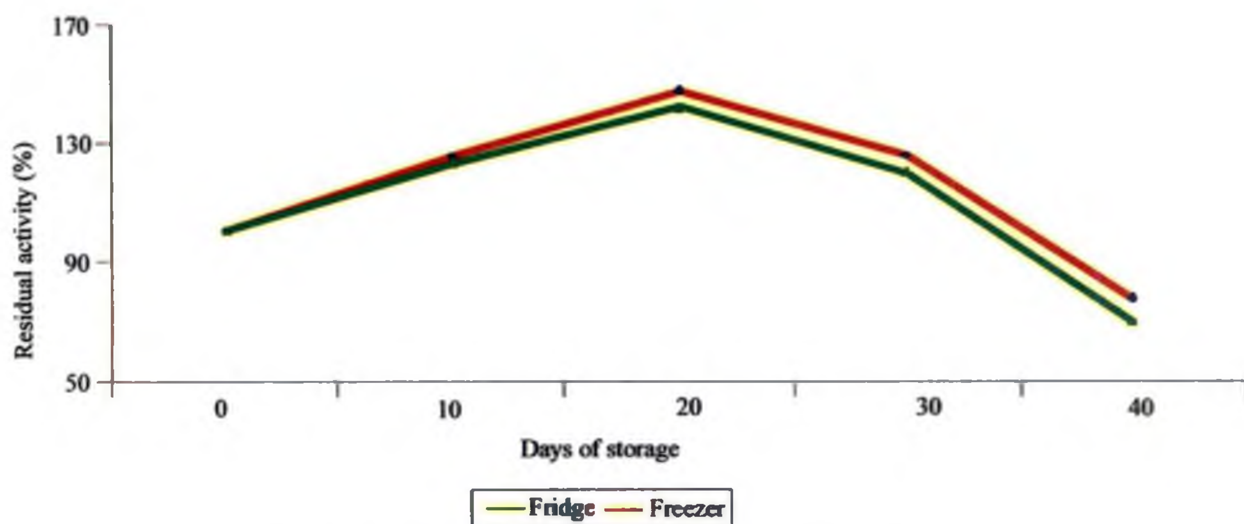


Fig. 9c. Storage stability of enzyme at low temperatures

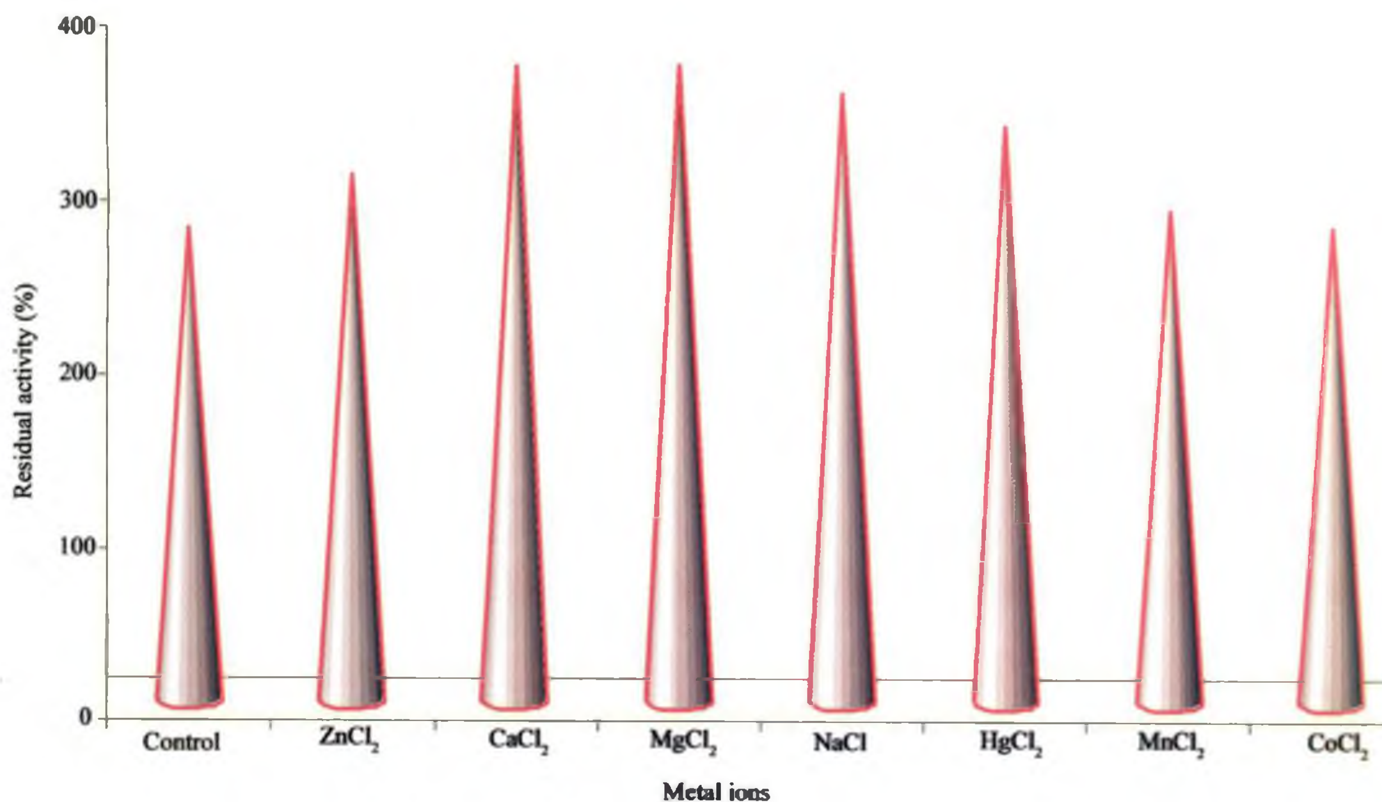


Fig. 9d. Stability of protease enzyme in the presence of metal ions

Table 9e. Effect of inhibitors on the activity of protease enzyme

Compounds	Activity (μmoles of tyrosine/ml)	Residual activity (per cent)
Control	273.20 ± 5.18^{a}	100
Phenylmethyl sulfonyl fluride	105.82 ± 4.21^{b}	38.5
Ethylene diamine tetra acetate	175.95 ± 2.68^{c}	64.5
β -mercaptoethanol	254.39 ± 3.21^{d}	93.1
Hydrogen peroxide	272.17 ± 2.99^{a}	99.6
Tween - 80	303.67 ± 3.80^{e}	111.2
Sodium dodecyl sulphate	597.97 ± 8.86^{f}	218.9

* The mean difference is significant ($p \leq 0.05$).
* Figures bearing same superscript do not differ significantly.

4.3.3.4 Effect of Inhibitors

The effects of chemical inhibitors, surfactants reducing agents and oxidizing agents on enzyme activity give an insight for partial characterization of enzyme. Activity of the enzyme after preincubation with phenylmethyl sulfonyl fluride, Ethylene diamine tetra acetate, Hydrogen peroxide, β-mercaptoethanol, Sodium dodecyl sulphate and Tween 80 were determined using casein as the substrate. Activity of enzyme prior to preincubation (273.20 ± 5.18) was taken as 100 per cent for calculation of percentage residual activity. The results of inhibitor study are given in Table 9e and Fig.9e.

Phenylmethyl sulfonyl fluride at 10 mM concentration and EDTA at a level of 1mM caused significant inhibition in enzyme activity. Beta mercaptoethanol decreased the activity significantly. Hydrogen peroxide did not interfere with the enzyme activity. The nonionic surfactant Tween 80 enhanced the enzyme activity

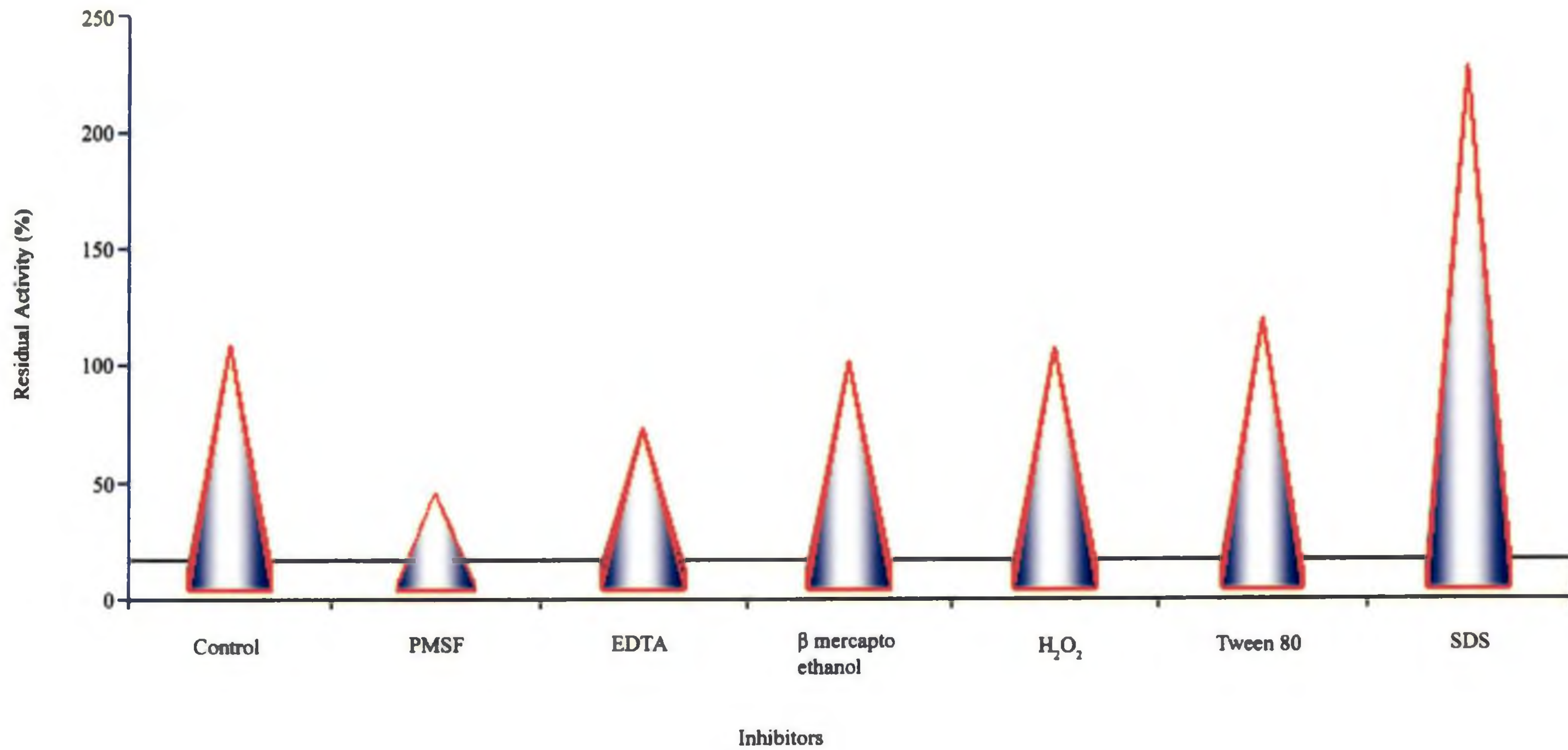


Fig. 9e. Effect of inhibitors on the activity of protease enzyme

by 11.2 per cent. In the presence of SDS, protease activity showed a remarkable increase, which was more than double the activity of control.

4.3.4 Assessment of Cleaning Efficiency of Inbuilt Detergent

4.3.4.1 Cleaning of Milk Soiled Plates

The results of cleaning of artificially soiled Petri plates using different formulations along with control and water wash are depicted in plates 10a, 10b, 10c, 10d, 10e and 10f. Uniform procedure for soiling and cleaning were strictly adhered to for all plates and test solutions. Results portray that water alone and crude enzyme as such (cell free supernatant) was not capable of giving satisfactory results. Though labolene was effective to a certain extent, blue stained protein residues were very prominent and significant, especially in the periphery of the plate. Incorporation of enzyme in the cleaning formulation very much improved the efficiency in cleaning the dried up residue, especially in the corners and periphery. Cleaning efficiency of the preparation containing all the ingredients but the enzyme was poor. Superiority of the enzyme based cleaning formulation is confirmed from the result.

4.3.4.2 Wash Test

Proteases represent one of the most important groups of industrial enzymes. Use of alkaline proteases as laundry detergent additive is one of its industrial application. The crude protease enzyme obtained from S4 was also assessed for its efficiency to remove proteinaceous stain by conducting a wash test to remove blood stains. The results of wash trial are given in plates 11a, 11b, and 11c and11d. The visual assessment of stained cloth pieces clearly demonstrates the superior cleaning efficiency of enzyme based formulation over detergents.

4.3.5 Purification of Enzyme

The results of purification are summarized in Table 10. The cell free supernatant (CFS) obtained after growing the culture under optimized growth conditions had a specific activity of 765. Most of the enzyme activity was

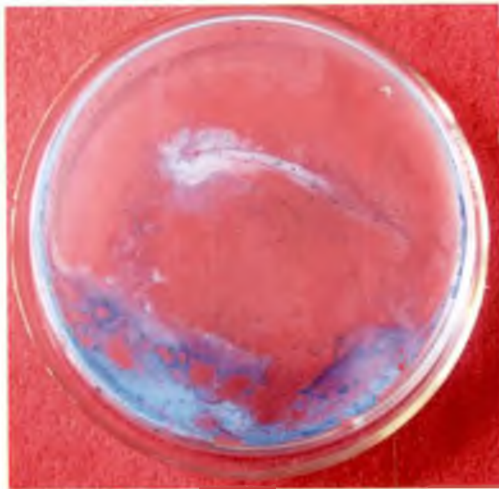


Plate 10a. Control

Plate 10b. Water wash

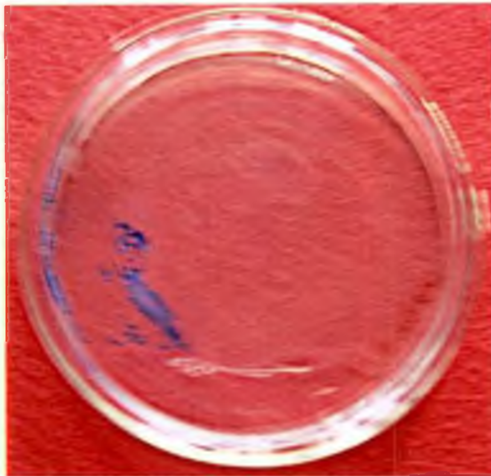


Plate 10c. Crude enzyme wash

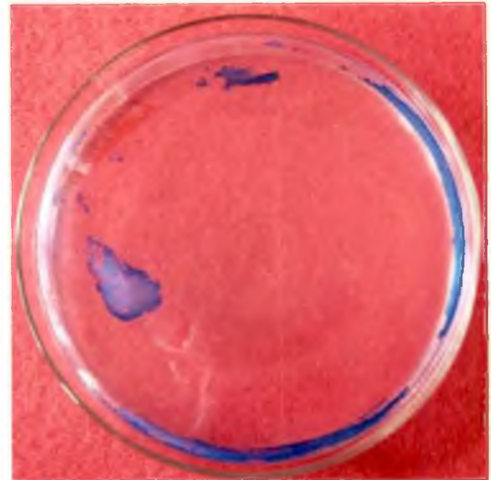


Plate 10d. 0.5 per cent Labolene wash

Assessment of cleaning efficiency - milk soil

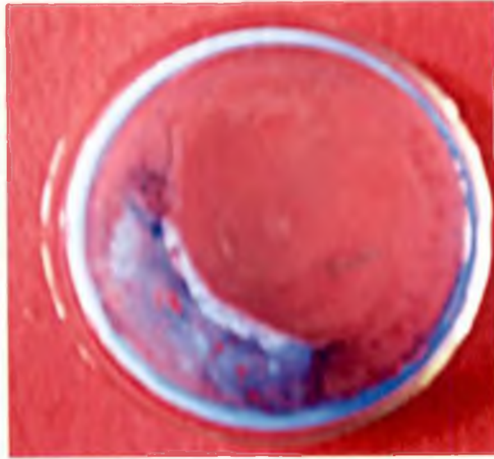


Plate 10e. Inbuilt detergent wash without enzyme

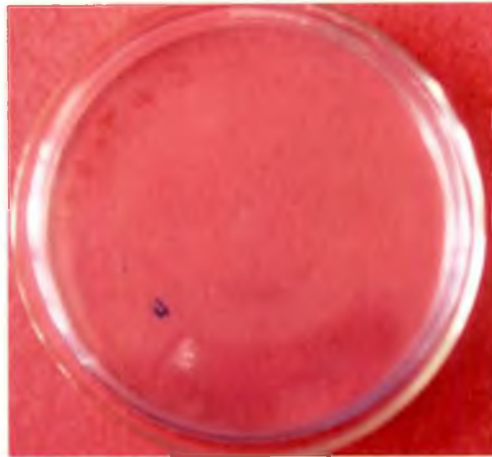


Plate 10f. Inbuilt enzyme detergent wash

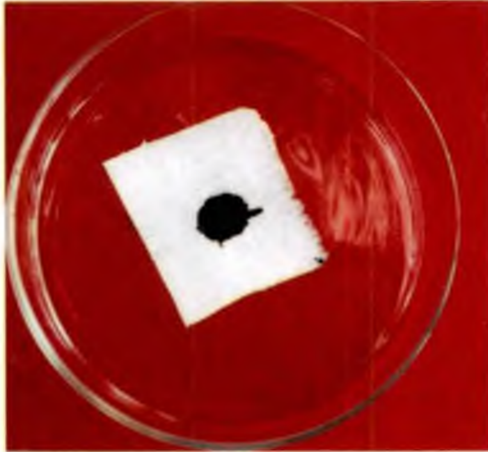


Plate 11a. Control

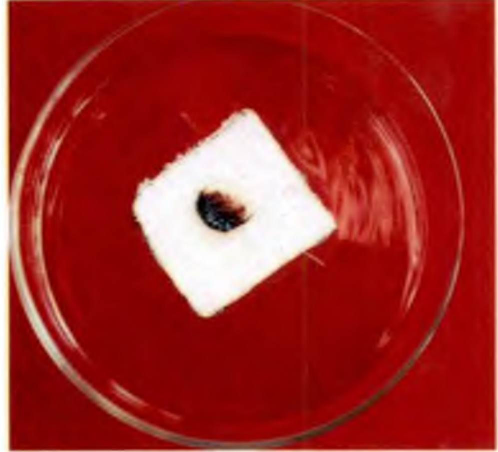


Plate 11b. Water wash

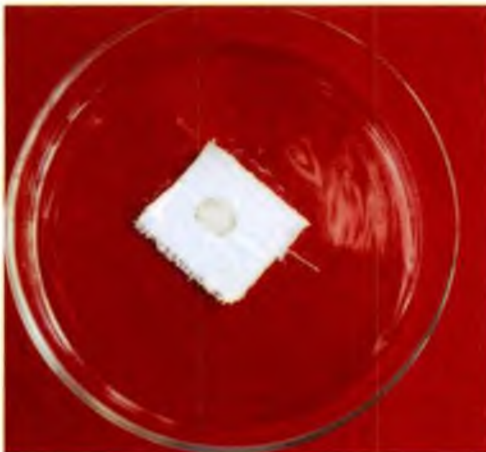


Plate 11c. Detergent wash

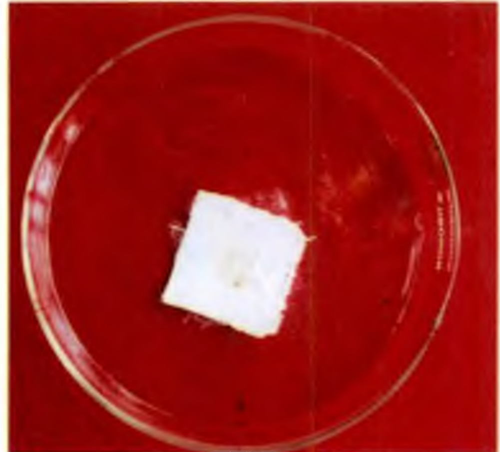


Plate 11d. Inbuilt enzyme detergent wash

Assessment of cleaning efficiency - blood stain

recovered in 40 to 80 per cent ammonium sulphate fractionation. Specific activity of the dialysate suspension was 18730.91 with a purificatiuon fold of 24.48. Gel filtration on seralose was quite effective to remove contaminating protease. The elusion profile of protein obtained on application of clear dialysate on sertalose 4B is shown in fig.10a. The 19th ,26th,27th and 38th fractions showed the presence of proteins.The 27th fraction that showed most of the enzyme activity had a specific activity of 95672 indicating a purification fold of 125.06. This fraction gave many peaks on further gel filtration using seralose 6B (fig.10b). Of all the fractions collected 39th fraction exhibited the maximum specific activity of 102428.2 indicating a purification fold of 133.89. When this fraction was again rechromatographed, a single symmetrical peak was obtained. With a specific activity 108100, a purification fold of 141.31 could be achieved.

Table 10. Enzyme activity at different steps of purification

Media	Tyrosine (µmoles/ml/min)	Protein (mg/ml)	Specific activity	Purification fold
CFS	92.6	0.121	765.00	1
40-80 per cent precipitate	412.08	0.022	18730.91	24.48
Seralose 4B	765.38	0.008	95672.50	125.06
Seralose 6B (27th fraction)	819.42	0.008	102428.20	133.89
Seralose 6B (39th fraction)	864.8	0.008	108100.00	141.31

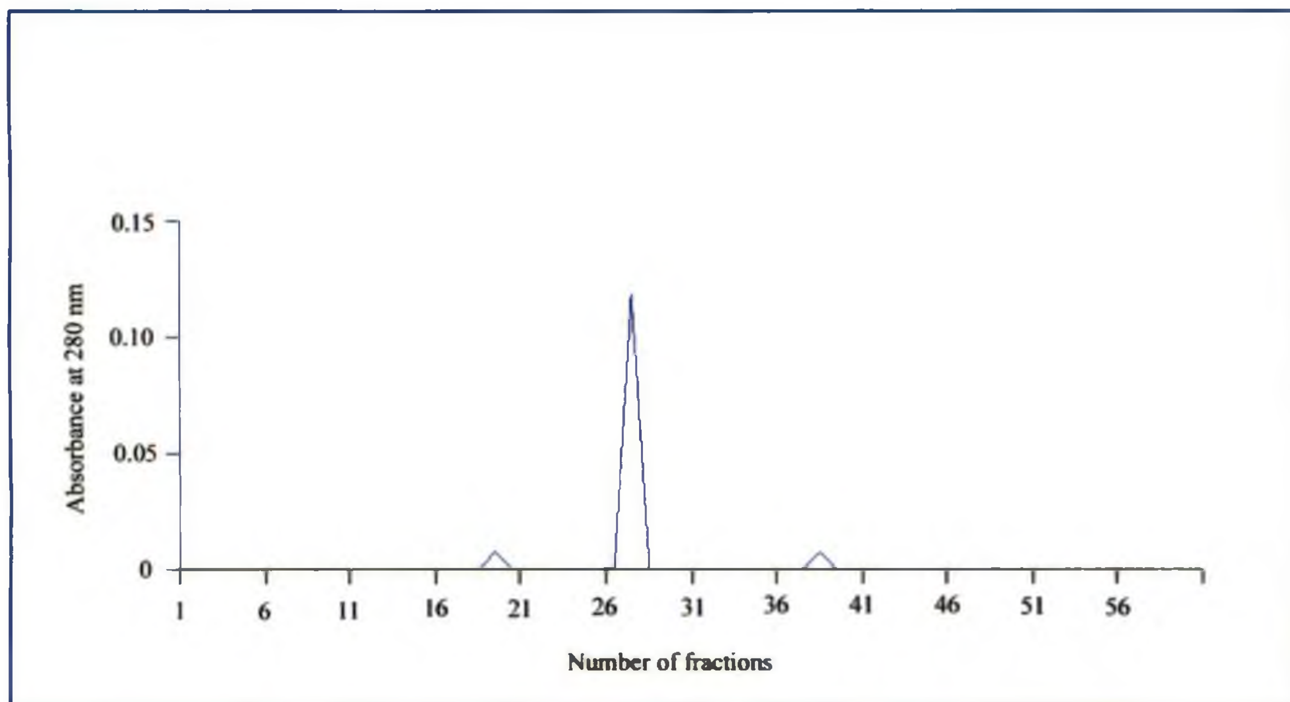


Fig. 10a. Column chromatography using seralose 4B

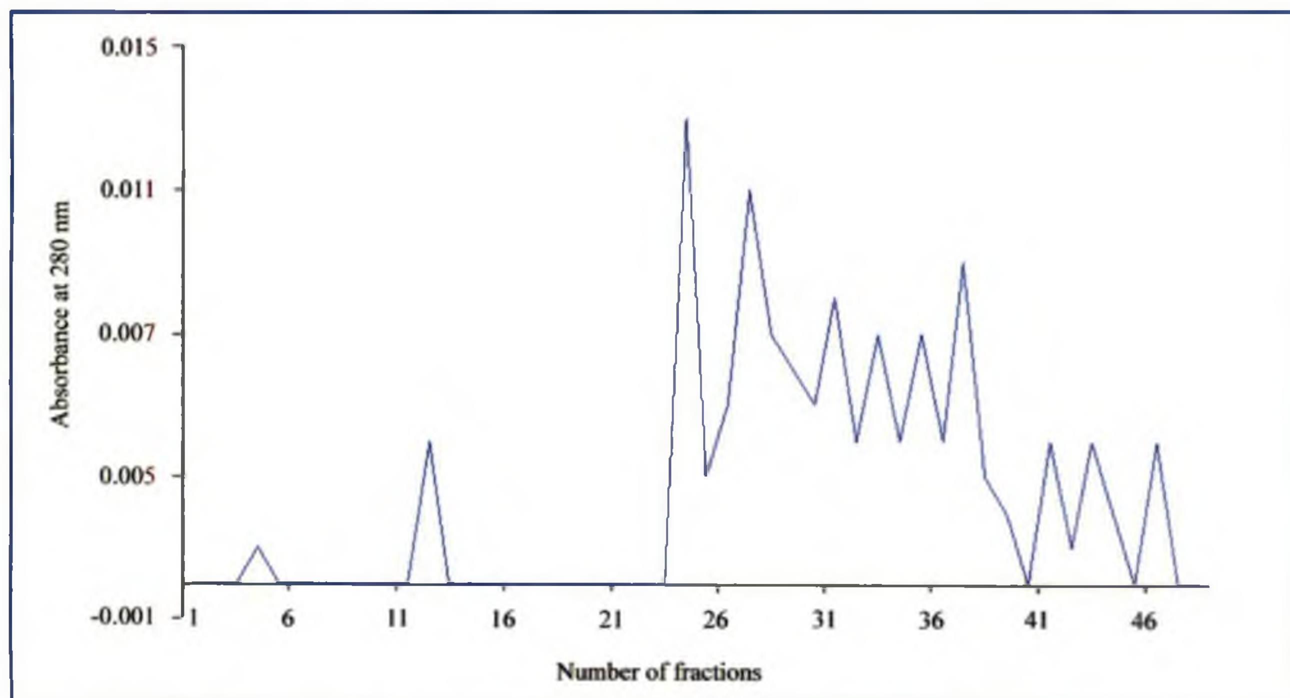


Fig. 10b. Column chromatography of 27th fraction using seralose 6B

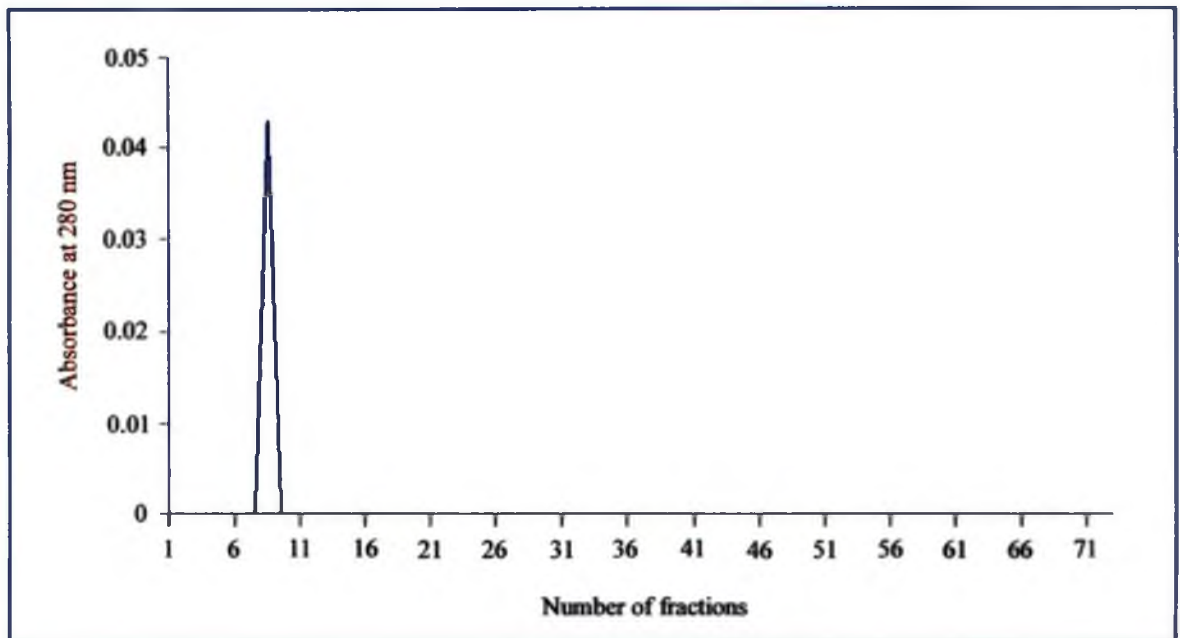


Fig. 10c. Column chromatography of 39th fraction using seralose 6B

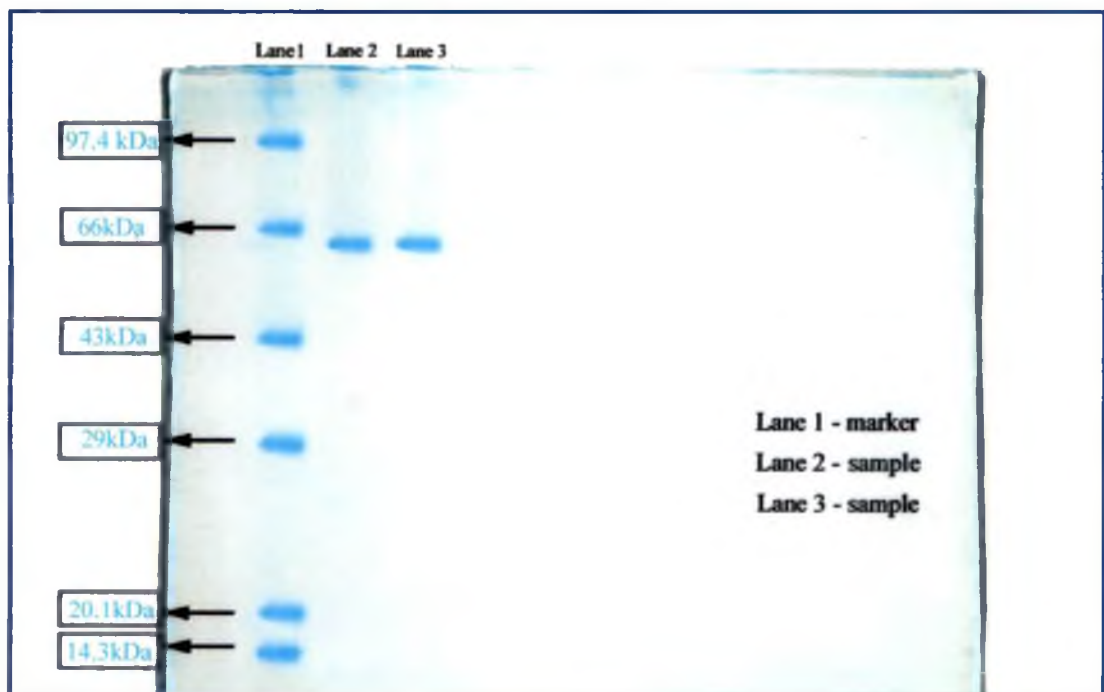


Fig. 10d. SDS PAGE of 39th fraction

The homogenity of the active fraction obtained was assessed by SDS PAGE (Fig.10d). The presence of a single band in Lane 2 and Lane 3 confirms the homogenity of purified enzyme. The molecular weight of enzyme was found to be 50.5 kDa. by SDS PAGE.

Discussion

5. DISCUSSION

5.1 ASSESSMENT OF SPOILAGE POTENTIAL OF THE ISOLATES

According to the results, Gram negative flora contributed to 69.2 per cent of isolates obtained from raw milk. Jayarao and Wang (1999) divided the Gram negative organisms associated with lowering of milk quality into coliforms and non coliforms. They observed that Gram negative non coliforms contributed to 67.1 per cent of bulk tank milk isolates with *Pseudomonas* as the most prominent genus. However in this trial, coliforms and *Pseudomonas* contributed to 50 per cent and 35.3 per cent respectively of the Gram negative flora. Predominance of *Pseudomonas* as the dominant flora in refrigerated raw milk has been reported previously (Ternstrom *et al.,* 1993; Desmasures *et al.,* 1997; Dunstall *et al.*, 2005). Coliform is considered as index of hygiene. Higher proportion of coliforms in raw milk reflects the poor hygienic conditions adopted at the farm level. The Gram negative isolates from raw milk in this trial included members of *Pseudomonas, Chromobacterium, Enterobacter* and *Escherichia.* Sorhaug and Stepaniak (1997) also reported the incidence of these genera in raw milk.

A clear shift in the dominant microflora was evident in pasteurised milk kept under refrigeration. In pasteurized milk *Pseudomonas* and coliforms contributed to 50 per cent and 16.7 per cent of the total isolates respectively. Psychrotrophic nature of *Pseudomonas* has been reported by Mc Phee and Griffiths (2002). The bacterial profile of retail milk samples as reported by Asha (2007) also supports the prevalence of *Pseudomonas* species. Eneroth *et al.* (1998) reported the occurrence of *Pseudomonas, Bacillus* and *Enterobacteriaceae* in refrigerated pasteurized milk. In this work also members of these genera were isolated. Eneroth *et al.* (1998) also observed that Gram negative psychrotrophs that spoiled refrigerated processed milk comprised mainly of *Pseudomonas,* followed by members of *Enterobacteriaceae.* A similar picture was obtained in this trial also. Stepaniak (1991) identified *Pseudomonas* spp as the single most important detrimental factor determining keeping quality of pasteurised milk. The prevalence

of higher proportion of *Pseudomonas* followed by *Bacillus* in pasteurized milk is in accordance with the findings of Mahare and Gashe (1990). The reports of Sorhaug and Stepaniak (1997) and Garcia *et al.* (1989) indicating *Pseudomonas* species as the most predominant organism that limit the shelf life of processed milk at 4°C, endorses the observations got in this trial.

Nanu *et al.* (2007) succeeded in isolating *P. aeruginosa* from 10.8 per cent of raw milk sample collected at farmers' level. However, in this trial a distinct shift to predominance of *Pseudomonas* happened after processing of milk. An interesting observation noticed in this trial was that a typical type of colony outnumbered others in all pasteurized milk samples. This predominant type (P12) was identified as *P. aeuruginosa*. Incidence of *P.aeuruginosa* in pasteurized milk has been reported by Kumaresan and Annal Villi (2008). According to them, post processing contamination led to the predominance of heat sensitive *Pseudomonas* in pasteurized milk. Anderson *et al.* (1990) found that *P.aeruginosa* could survive within exopolysaccharide masses in the interior walls of piping. Eneroth *et al.* (1998) reported that filling step was the most critical contamination site for Gram negative rods, especially *Pseudomonas*. These reasons could be attributed to the high occurrence of *Pseudomonas* in pasteurised milk.

Proportion of *Bacillus* isolates was 7.7 per cent in raw milk, 22.2 per cent in pasteurized milk and 90 per cent in sterilized milk. With processing treatment (heat treatments), the proportion of thermo resistant spore formers increased steadily. This clearly shows that processing treatment, to which a product is subjected to, is very important in deciding the spoilage flora of significance in that product. *Bacillus* species are so widely distributed in the environment that, it can be introduced into milk and milk products during production handling and processing (Murphy *et al.*, 1999). With heat treatment, heat sensitive organisms are killed such that thermoduric flora need to face less competition. Hence there is an increased proportion of *Bacillus* species in sterilized milk and pasteurized milk, when compared to raw milk. The high number of spore formers could arise from the poor environment hygiene at the farm level. Spores in raw milk have been identified as the major source of *B. cereus* in processed milk. In contrast to the case

of *Pseudomonas*, post pasteurization contamination is rarely a reason for the incidence of this organismin processed milk (Lin *et al.*, 1998).

Isolate S4 (identified as *B. cereus)* selected for further work, based on the production of spoilage enzymes was obtained from sterilized skim milk that showed signs of spoilage after one week of refrigeration. Psychrotolerant strains of *B. cereus* have been reported by Meer *et al.* (1991). Creilly *et al.* (1994) identified *B. cereus* as one of the most commonly isolated *Bacillus* species from milk. They opined that in milk, *Bacillus* was mainly associated with cattle feed. Incidence of psychrotolerant strains of *Bacillus cereus* had been reported earlier by Shehata *et al.*, (1983); Sharma *et al.*, (1984) and Pacova *et al.* (2003). The isolate S4 with remarkable potential to produce the spoilage enzymes, later identified as *B.cereus* was also capable of multiplication at 7°C.

Water flora also comprised mainly of *Pseudomonas*. *Pseudomonas* was isolated from raw milk, pasteurized milk and water samples. The ubiquitous nature of *Pseudomonas* resulted from its capacity to adapt to various situations and degrade a wide range of substrates (Palleroni, 1992). Dogan and Boor (2003) suggested that, existence of high level of diversity among *Pseudomonas* isolates from different processing environment was due to the lack of long term colonization of these species in processing environment. The alkaline water washings used in this trial had a temperature of 85°C and pH 12. In such a harsh environment, majority of the flora may be incapable of survival. Only the highly resistant spore formers are expected to survive in such extreme environment. The findings in this trial are also in unison with this fact. The fact that only *Bacillus* spp was isolated from alkaline water washings is indicative of loss of heterogenity of flora in water, which had extreme conditions of pH and temperature. Ibrahim *et al.* (2007) also succeeded in isolating *Bacillus* species from the extreme conditions of Egyptian Soda Lake.

Wiedmann (2000) and Prakash *et al.* (2007) reported protease, lipase and lecithinase activity for putative *Pseudomonas*. All the isolates of *Pseudomonas* assessed in this trial exhibited protease and lipase activity and 52.6 per cent

showed lecithinase activity. Dogan and Boor (2003) reported more than 80 per cent of *Pseudomonas* isolates to have proteolytic potential. In general, lipase activity was more relevant for *Pseudomonas* than for any other genera. Fifty eight per cent of *Pseudomonas* in this trial showed both lipase and lecithinase activity; 42 per cent showed only lipase activity and none showed lecithinase activity alone. A similar trend in the production of lipase and lecithinases by *Pseudomonas* had been reported by Dogan and Boor (2003). Zacharov and Halpern (2007) also observed lipase activity mainly for *Pseudomonas*. Of all the tested isolates only 10.5 per cent showed lipase activity at 37°C. All other isolates which showed considerable growth at 37°C with minimal lipase activity exhibited higher lipase activity when kept at 7°C. The optimum temperature for lipase production is often lower than optimum growth temperature (Andersson, 1980). The spoilage enzyme profile of *Pseudomonas* isolates obtained in this trial was of diverse nature. Zacharov and Halpern (2007) observed that even species with same identity possessed different enzyme profile. Heterogenity in the enzymatic ability of strains belonging to the same species of *Pseudomonas* has been confirmed by them. Ma *et al.* (2003) proposed that proteolytic activities of the psychrotrophs in general and *Pseudomonas* species in particular were valuable tools for detection of spoilage and shelf life prediction.

Phillips and Griffiths (1990) reported that 86 per cent of the thermoduric psychrotrophic bacteria belonged to genus *Bacillus*. The predominance of *B.cereus* in proteolytic spore formers of raw milk had been observed by Sharma *et al.*(1984). Isolate S4 (*B.cereus*) obtained from sterilized milk, exhibited remarkable potential to produce all the spoilage enzymes assessed in this trial. The high lipase activity of *B. cereus* (S4) is in line with the reports of Matta and Punj (1999).

Absence of remarkable enzyme activity for *E. coli* is in agreement with the reports of Prakash *et al.* (2007). The weak proteolytic activity of *Micrococcus* observed in this trial is also supported by the observations made by Prakash *et al.* (2007). The weak enzymatic ability of *Lactobacilli* obtained in this trial is in consonance with the reports of Zacharov and Halpern (2007).

Of the total isolates from the dairy environment, 31.4 per cent belonged to *Bacillus* spp and 27.5 per cent to *Pseudomonas* spp. Adams *et al.* (1975) reported that microbiological activities of enzymatic origin were better indices of microbiological quality than the number of microorganisms. Both the isolates (P12 and S4), selected based on the potential to produce spoilage enzymes were psychrotrophs. Zacharov and Halpern (2007) proposed to monitor the dominant psychrotrophic species with the potential to produce thermostable enzymes as a sensitive and efficient tool to monitor the milk quality. Even isolates identified to same genus was found to possess different enzyme profile. The thermostable nature of enzymes makes it a critical factor in determining the shelf life of products. Existence of such diverse enzymatic potential necessitates the development of sensitive and efficient tools to monitor the enzyme producers – the key players in determining the quality of processed products.

5.1.1 Screening of Proteolytic Isolates for Growth in Alkaline pH

Screening of isolates for their potential to produce extra cellular protease in alkaline medium revealed that isolate S4 showed maximum activity under alkaline conditions. Biochemical characterization suggested that S4 belonged to the genus *Bacillus*. Isolate S4 was isolated from sterilized skim milk that showed signs of spoilage after refrigerated storage of one week. Incidence of such psychrotolerant strains of *Bacillus* in milk had been reported earlier (Meer *et al.*, 1991; Pacova *et al.*, 2003). Murphy *et al.* (1999) opined that *Bacillus* species with adverse proteolytic activities are so widely distributed in the environment that they could be introduced into milk and milk products at any stage of production and processing. Isolation of *Bacillus* species from sterilized milk is in agreement with Bellow *et al.* (2007) who have reported occasional occurrence of *Bacillus* originating from raw milk in high heat treated milks like UHT milk.

In the present study, members of genus *Bacillus* were found to exhibit more alkaline protease activity than *Pseudomonas, Enterobacter* and *Klebsiella*. The reports of Yang *et al.* (2000) that majority of commercially available alkaline proteases were derived from *Bacillus* species affirms these observation. *Bacillus*

species with potential to produce alkaline proteases have been isolated from a wide variety of sources like soil (Nascimento and Martins, 2004), buffalo hide (Nilegaonkar, *et al.*, 2007); Egyptian soda lake (Ibrahim *et al.*, 2007) and waste water near milk processing plant (Chu, 2007). The *Bacillus* isolate from sterilized skim milk which was used in this trial also showed the ability to produce extracellular alkaline protease with possible industrial applications.

5.1.2 Identification of selected isolates

Biochemical characterization results of the short Gram negative rods suggested the isolate to be *Pseudomonas aeruginosa.* Soluble blue pigment is produced by certain strains of *P.aeruginosa.* Formation of fluorescent pigments of *P. aeruginosa* is related to the ability of sulfite to act as a specific sulfur source (Palumbo, 1972). Isolate P12 showed distinct fluorescence when streaked on *Pseudomonas* agar for fluorescin production. Prevalence of *P.aeruginosa* in milk had been reported by Nanu *et al.*, 2007; Prakash *et al.*, 2007 and Yagoub *et al.*, 2008.

Morphological and biochemical characteristics suggested that isolate S4 was *B.cereus* . Peacock blue colonies indicated the isolate to be mannitol negative. Egg yolk precipitation was evident as an opaque halo around the colony. Growth in *Bacillus cereus* differential agar exhibited the typical characters of *B.cereus.* High incidence of *B.cereus* in milk had been reported by Sharma *et al.* (1984); Pacova *et al* (2003) and Bartoszewickz *et al.* (2008).

5.2 IMPACT OF PSYCHROTROPHIC SPOILAGE ENZYMES IN CURD AND STERILIZED MILK

Conservation and preservation of food is a prerequisite to food security as it assures stability and self reliance. Food processing techniques that extend shelf life make an important contribution to world food security as it provide consumers with food whose safety and nutritional quality is assured. Though food industry has undergone progressive refinement and processing sophistication during recent

years, spoilage mediated by bacterial enzymes, especially thermostable enzymes, still cause significant economic losses for food industry. While designing processing techniques to assure quality food, extra care is to be given to the prevalent flora especially those which possess high potential to produce spoilage enzymes.

5.2.1 Curd

5.2.1.1 Titratable Acidity and pH

In general, changes observed in pH correlated well with titratable acidity. Titratable acidity increased with decrease in pH. Such decrease in pH with concomitant increase in acidity had been reported by Kamruzzaman *et al.* (2002), Soomro *et al.* (2003) and Praseeda (2005). The mean titratable acidity of fresh curd samples was low and pH was high when compared to the values reported by Kamruzzaman *et al.* (2002) and Praseeda (2005). This difference could be attributed to the difference in type of culture and the processing conditions followed during the preparation of product (Rao *et al.*, 2002). A significant decrease in pH along with increase in titratable acidity after 10 days of storage is indicative of continued acid production by starters under storage conditions. Similar findings have been reported by Salji and Ismail (1983), Al-Kadamany *et al.*(2002) and Aly *et al.* (2004). In contrast to these observations, Salvador and Fiszman (2004) observed that after storage pH values barely changed under refrigerated storage. Kroger (1975) opined that a pH above 4.5 produced a weak coagulum. In this trial also, control with a mean pH of 4.80 ± 0.01 gave a weaker coagulum than CT1 and CT2, as reflected in the firmness values.

The treatment groups CT1 and CT2 made from milk precultured with P12 and S4 showed significantly lower pH and higher titratable acidity on 0 day itself. The same trend continued till 10 days of storage. The proteolysis initiated by P12 and S4 would have stimulated lactic acid production by starters during storage (Cousin and Marth, 1977). Both CT1 and CT2 showed higher titratable acidity than prescribed by BIS (1980) from fifth day of storage itself. Between CT1 and

CT2, acidity was more for CT2. This might be due to the production of lactic acid by S4 along with starters but not by P12. Lactic acid production of S4 has further been confirmed by litmus milk reactions during characterization of the isolate. Vassiliadou, (1992) had reported several species of *Bacillus* to produce lactic acid. Free fatty acids released by *Pseudomonas* could inhibit the growth of lactic acid bacteria (Shah, 1994; Jaspe *et al.* 1995). This inhibition of lactic acid bacteria by isolate P12 can also be a reason for lower titratable acidity of CT1 when compared to CT2.

For CT2 the difference in the pH between 5 and 10 days was not significant. But the simultaneous change in titratable acidity was significant. Changes in buffering capacity of milk can happen with no effect on pH, but with an effect on titratable acidity values (Gould and Frantz, 1945). The titratable acidity of CT1 at 5 days was not significantly different from that at 0 day. According to Gassem and Frank (1991) enzymes which were actively involved in proteolysis of milk (pH 6.3) might not be that active in yogurt (pH 4.0). Same reason can be attributed to the absence of increase in acidity after five days of storage. However titratable acidity gradually increased to significantly higher level by 10 days. This shows that proteases got adapted to acidic pH such that it became more active during later stages of storage.

5.2.1.2 Syneresis

Appearance and physical characteristics are important quality parameters of any fermented milk product. Syneresis is the process by which whey component of milk is expelled following curd formation. Though a natural phenomenon, if present in higher levels, it will interfere with the appeal of the product. The values of spontaneous syneresis obtained for CT1 and CT2 clearly suggest that psychrotrophic spoilage enzymes can influence syneresis value significantly. This could be due to the variations in the protein quality of milk due to the action of proteolytic enzymes of P12 and S5 when kept under refrigeration. Mc Phee and Griffiths (2002) reported that majority of proteases of *Pseudomonas* retained their activity at 4°C. As the micellar content of β-casein was affected, cold storage of

milk affected syneresis (Ali *et al.,* 1980). Reduction in protein and fat content of milk samples kept under refrigeration after inoculation with *P.aeruginosa* and *Bacillus cereus* had been reported by Yagoub *et al.* (2008) and Bellow *et al.* (2007) respectively. Cousin (1982) and Schroder *et al.* (1982) also reported proteolysis during cold storage by the action of psychrotrophs. The observation of increased syneresis in CT1 and CT2 is in line with the findings of Gassem and Frank (1991) who reported that variations in syneresis could be attributed to the variations in protein quality of milk. Any alterations in the composition of casein micelle which form the gel network affect coagulation and subsequent syneresis (Pearse and Mackinlay, 1989). The difference in pH resulting from the action of P12 and S4 under refrigeration, influence the pattern of casein – whey protein aggregation (Anema and Li, 2003) and thereby coagulum formation. Yogurt made from milk treated with psychrotrophic protease had lower water holding capacity and protein hydration than untreated control. This also would have contributed to the increased syneresis observed in this trial (Gassem and Frank, 1991). According to Castillo *et al.* (2006) syneresis parameters are highly correlated to rate of acidification.

Syneresis rate increased with lowering of pH as a result of reduction in net micelle charge and subsequent electrostatic repulsion between micelle (Marshall, 1982). The results of this trial are in line with these observations. Salvador and Fiszman (2004) and Sodini *et al.,* (2004) opined that increase in syneresis was more pronounced during initial stages of storage than in later stages. In the present trial, study was made only for a shorter period of 10 days. Syneresis values were significantly higher for both CT1 and CT2 when compared to control after 10 days.

According to the results of this trial pH decreased and syneresis increased with storage. Grundelins *et al.* (2000) opined that syneresis was more pronounced at a lower pH. Observations in this trial are endorsed by the findings of Jaros and Rohm (2003), who reported that enhanced acidification to pH below 4.0 lead to increased syneresis. The excessive rearrangement of particles in gel net work before and during gelation could also be implicated as being responsible for

increased whey separation in treatment groups when compared to control (Lucey, 2001).

Psychrotrophic proteases preferentially attack casein (Kappa casein and Beta casein) over whey proteins (Triantafyllidou and Roussis, 1999). Syneresis is influenced by changes in concentration of β-casein (Pearse and Mackinlay, 1989). Moreover psychrotrophic proteases can act as plasminogen activators and activate plasminogen which attack β-casein (Mitchell *et al.*,1986; Lira and Neilsen,1998). Changes in β-casein concentration occurring from the action of proteolytic enzymes in CT1 and CT2 would have contributed to the observations of this trial.

With more fat content there will be an increased number of interstices within the reticulum occupied by fat globules such that there will be increased impedance to whey drainage (Storrey *et al.*, 1983). Lipolytic activity of P12 and S4 might have resulted in decreased number of fat interstices (Bellow *et al.*, 2007 and Yagoub *et al.*, 2008) and thereby lesser impedance to whey drainage. This could also be a reason for significant increase in syneresis especially in CT1 and CT2 when compared to control on storage.

5.2.1.3 Texture Analysis

Texture analysis is primarily concerned with the evaluation of mechanical characteristics of a food. These mechanical characteristics are proven to be correlated with sensory perception and can be assessed from the deformation curve generated, by subjecting the food material to a controlled force. The texture criterion like firmness is an important parameter that decides the quality criteria and consumer acceptability of fermented milk products.

Firmness, viscosity and extent of syneresis are very important criteria in deciding consumer acceptability of fermented milk products (Muir and Hunter, 1992). The firmness of control curd was significantly lower than CT1 and CT2 on all tested days of storage. This observation is supported by the findings of Cousin and Marth (1977), who reported an increase in firmness for yogurt made from milk precultured with psychrotrophic bacteria. Preculturing of milk with proteolytic

isolates P12 and S4 would have caused differences in protein structure thereby influencing firmness. Tye *et al.* (1988) observed a supportive role for *Pseudomonas* proteases in accelerating cheddar cheese ripening. In this trial also, coagulum formation was faster in CT1 and CT2. The possibilities of differences in protein structure affecting firmness have been put forward by Labropoulos *et al.* (1981). According to Totosaus *et al.* (2002) protein concentration and degree of denaturation very much influenced the characteristic of gel. Moreover, the peptides and amino acids released by way of proteolytic action of P12 and S4 would have stimulated curd starters (Cousin and Marth, 1977; Gassem and Frank, 1991), improved lactic acid production, increased curd contraction and syneresis, thereby enhancing firmness. This finding is also in compliance with the earlier observations made with titratable acidity and syneresis. In contrast to this result, Serra *et al.* (2009) reported that firmness value hardly increased during cold storage. Higher level of protease liberated by elevated number of microbial cells in CT1 and CT2 would have contributed to the denatured protein matrix (Tamime and Marshall, 1997), thereby positively influencing the firmness values (Parnell-clunies *et al.*, 1988 and Lucey *et al.*, 1998).

The significant differences in firmness of CT1 and CT2 after 10 days of storage reflect the differences in the specificity of casein hydrolysis by the proteases released by P12 and S4 (Gassem and Frank, 1991). Acidification to pH values below 4 could lead to body and texture defects such as gel shrinkage and syneresis (Jaros and Rohm, 2003) which in turn increased firmness. In all the three treatments pH lowered with storage. Curd CT1 showed a pH below 4.0 on 0 day itself. Curd CT2 showed a pH below 4.0 from 5th day of storage. In this trial also, lowering of pH correlated well with increase in firmness.

The result obtained indicates that in general the viscosity values of CT1 and CT2 were significantly lower than control on 0 day and 5 days and 10 days of storage. However values differed significantly between CT1 and CT2 only from 5th day. The protease action of P12 and S4 would have contributed to the denaturation of protein such that denatured protein matrix was more for CT1 and CT2 when compared to control. Krasaekoopt *et al.* (2004) observed that yogurt.

prepared from milk with higher protein denaturation had lower viscosity. They also observed that viscosity decreased along with an increase in firmness during storage. Firmness viscosity profile obtained in this trial is in compliance with this report.

5.2.1.4 Sensory Evaluation

Sensory evaluation offers the opportunity to obtain a complete analysis of the textural properties of food as perceived by human senses. Sensory methods of measuring food quality may appear to lack precision that is desirable in scientific research because of the variability from person to person and variability from hour to hour in likes and dislikes of each person. In spite of these drawbacks, sensory evaluation is still the ultimate method for calibrating the instrumental methods of texture measurement.

Analysis of sensory scores revealed that score values reduced significantly with storage. Flavour scores reduced significantly after 5 days of storage, even for control. Onset of off-flavour was appreciated much earlier than other defects throughout the storage (Al-kadamany *et al.*, 2002). Vargas *et al.* (1989) also proposed flavour as an important parameter to determine the end point of storage of soya - whey yogurt. Flavour scores of CT1 and CT2 were significantly lower than that of control. The proteolytic and lipolytic activities of P12 and S4 in CT1 and CT2 can be considered to be responsible for the adverse effect on the flavour profile of CT1 and CT2. Lipases from *Pseudomonas* spp have been reported to retain 55 to 100 percent activity after heat treatment at 63°C for 30 min and 75 to 100 percent activity after heat treatment at 100°C for 30 sec in skim milk (Law *et al.*, 1977). Moreover flavour changes can arise from differences in acidity (Ott *et al.*, 2000). In this trial also, significant differences in acidity might have influenced flavour profile of the samples.

Body and texture changes showed significant differences between control and CT2 on 0 day itself.Curd CT1 differed significantly from control after five days of storage. Within treatment also scores reduced significantly with storage.

According to Modler *et al.* (1983) body firmness increase with protein content (Modler *et al.*, 1983), *Pseudomonas aeruginosa* (P_{12}) and *Bacillus cereus* (S_4) inoculated into base milk might have reduced protein content (Younus *et al.*, 2002) of milk base. This reason can be attributed to body and texture changes of CT1 and CT2. The pH changes that would have occurred during refrigerated storage for 72 h might have affected casein whey protein aggregation (Anema and Li, 2003) and thereby body and texture. Even small variation in pH affected integrity of casein micelle and their susceptibility to proteolysis has been reported by Mc kellar (1989). Lower body and texture scores for CT1 and CT2 can be due to the poor quality of base milk used in the preparation of curd. Rangappa and Achaya (1973) proposed that initial acidity and physical quality of milk had significant effect on the texture of dahi prepared out of it. They reported that milk stored too long before inoculation gave rise to broken curd with poor taste. In contrast to this observation, Praseeda (2005) reported that body and texture score remained same throughout the storage period. Differences in the cultures used and the processing conditions adopted in the laboratory would have contributed to this effect (Gupta *et al.*, 2000).

The poor performance of CT1 and CT2 with respect to color and appearance on all tested days of storage can be under the influence of spoilage enzymes released by P12 and S4 in CT1 and CT2 respectively. Syneresis value of CT1 and CT2 were significantly higher than control. Greater whey separation on the top would have hindered the 'appearance' of product, thereby reducing the score for color and appearance.

The poorer performance of CT1 and CT2 when compared to control, for all the parameters tested is responsible for the significantly lower scores for overall acceptability. The results of sensory evaluation indicated that metabolic activities of P12 and S4 had a significant negative impact on the overall quality and shelf life of a fermented milk product, curd.

5.2.2 Sterilized Milk

5.2.2.1 pH of Sterilised Milk

Reduction in pH of ultra heat treated milk on storage is due to precipitation of calcium phosphate, dephosphorylation of casein and interaction between lactose and protein (Samel *et al.*, 1970; Gaucher *et al.*, 2008). Same reason would have contributed to the decrease in pH in sterilised milk. The pH value differed significantly between different groups at 15 days of storage. This can be because of the difference in the concentration of unprotonated amines in different groups (Whitelaw and Weaver, 1988; Celestino *et al.*, 1997). The degradation of lactose with formation of formic acid also explains the decrease in pH during storage. Andrews *et al.* (1977) ascribed the drop in pH to the maillard that led to the loss of positive charge on the protein molecules. Observations in this treatment are also in accordance to this. Sterilized milk ST2 which showed maximum decrease in pH was adjudged to have maximum colour intensity (browning) from maillard reactions.

5.2.2.2 Index of Proteolysis

Law *et al.* (1977) used the Hull method successfully to show a correlation between proteolysis and off flavour in milk. A similar correlation between tyrosine and off flavour development was noticed in this trial also. Sterilized milk ST1, which showed maximum tyrosine value at 15 days of storage exhibited the highest intensity of unclean flavour. Off flavour development as a function of proteolysis had been put forward by Mc Kellar (1981).

According to Mottar (1981), increase in NPN content of UHT milk during storage was due to proteolysis by indigenous enzymes or by enzymes of bacterial origin. Highly significant increase in NPN values of STI and ST2 on 7 days and 15 days of storage suggest higher proteolytic activity in these groups. Higher NPN values in ST1and ST2 when compared to control are due to the action of P12 and S4 in ST1 and ST2 respectively.

Findings of this trial are in consonance with the reports of Mitchell and Ewings (1985) who observed that bitter taste was experienced when NPN content increased from 0.03 per cent to 0.06 per cent. Bitterness could not be detected in any of the treatment groups on 0 day. However it became significant in ST1 and ST2 by seven days as the NPN value increased to 0.11 per cent and 0.06 per cent respectively. Deeth *et al.* (2002) linked bitterness related to proteolysis as a significant defect in high heat treated skim milk.

A linear correlation between proteolysis and shelf life in skim milk has been proposed by Janzen *et al.* (1982). A similar trend was evident in this trial also. The groups with higher NPN values and higher proteolytic changes were found to have lower overall quality rating. The negative impact of heat resistant proteases of psychrotrophs in sterilised milk had been reported by Adams *et al.* (1975).

5.2.2.3 Sensory Evaluation

Quantitative descriptive analysis approach has gained acceptance for sensory evaluation of dairy products. It helps to identify the determinant product attribute that influence the consumer choice within a product class.

Thermal processing results in unfolding of milk proteins such that sulphydril groups that are normally masked in native protein are exposed to outer surfaces, which in turn lead to the development of cooked flavour (Hoffman and VanMill, 1997). The same reason can be attributed to the cooked flavour observed in sterilized milk. The amino acids cysteine and methionine had been implicated in the development of cooked flavour (Swaisgood *et al.*, 1987). The HPLC profile of amino acids of skim milk and skim milk acted upon by P12 and S4 showed the presence of cysteine and methionine in latter two groups. Still cooked flavour was not much appreciated in ST1 and ST2. This could be because of masking of cooked flavour by other off flavours like unclean flavour. Within treatments, cooked flavour reduced in intensity with storage. Driessen (1983) has also reported that cooked flavour dissipated with time.

Development of unclean flavour in dairy products is linked to proteolysis (White and Marshall, 1973). In the control group this defect was negligible during the entire period of storage. In ST1 and ST2 unclean flavour became detectable after 7 days. Mac kellar (1981) reported off flavour in high heat treated milk as a function of proteolysis. The intensity of this defect was significantly higher in ST1 (inoculated with *Pseudomonas*) than ST2 on both 7 days and 15 days of storage. *Pseudomonas* proteases have been specifically implicated in unclean off flavour by White and Marshall, (1973). Reports of Mitchell and Ewings, (1985) also support this. This clearly points out the fact that sterilization temperature was not effective in inactivating the proteases of P12 and S4. Survivability of *Pseudomonas* proteases to sterilization temperature had been reported by Egziabher *et al.* (1980) and Patel *et al.* (1983). The detrimental effect of *Pseudomonas* proteases in refrigerated milk even after UHT treatment had been reported by Dunstall *et al.* (2005). A linear correlation between relative protease activity and flavour score had been reported by Janzen *et al.* (1982). Enright and Kelly (1999) opined that heat treatments severe enough to eliminate proteolytic activity would adversely affect milk protein functionality.

Bitter taste is associated with the undesirable proteolysis that leads to the accumulation of bitter peptides (Ma *et al.*, 2000). In the control group significant level of bitterness was not detected during 15 days of storage. Active heat resistant proteases of P12 and S4 in sterilized milk explain the development of bitter flavour in ST1 and ST2. In milk, better thermostability for proteases had been reported by Fox (1981) and Muir (1996). Bitterness associated with *Pseudomonas* proteases had been reported by Tye *et al.* (1988) and Mc Kellar (1989). Adams *et al.* (1975) reported that β and K casein fractions were not susceptible to the proteases liberated by Gram negative psychrotrophs. According to Lemieux and Simard (1992), hydrophobic peptide level was highly related with hydrolysis of β-casein and extend of hydrolysis of β-casein was positively correlated with bitterness. Significantly higher bitterness in ST2 could be due to greater susceptibility of β-casein to *Bacillus* proteases. Plasminogen activation by psychrotrophic proteases (Kohlmann *et al.*, 1991) could also be a reason for increased bitterness in ST1 and

ST2. Proteolysis progressed with storage. As a result bitterness increased in both ST1 and ST2 during storage. Adams *et al.* (1975) also observed increased susceptibility of milk proteins to protease, along with increased bitterness during storage.

Sourness was detected only in ST2 after seven days of storage. Spores of *Bacillus* that had survived heat treatment might have germinated and got metabolically active, producing acid and thereby increasing sourness. Acid production by *Bacillus* had been reported by Vassiliadou (1992). Further, in this work acid production was confirmed by litmus milk reactions. Acidification can also result from maillard reactions (Gaucher *et al.*, 2008). Formic acid production and subsequent increase in acidity had been confirmed by lactose degradation (Kelly and Foley, 1997). Highest colour intensity by way of maillard reaction in ST2 as observed in this trial is also in consonance with the above finding.

Though viscosity did not differ significantly between treatments on 0 day, viscosity increased significantly for ST2 after seven days and decreased significantly for ST1 after 15 days when compared to control. Increase in viscosity with time is suggestive of progressive protein denaturation and unfolding of protein which in turn can be considered as an indication of age gelation. The proteolytic enzymes of S4 in ST2 too resulted in progressive protein denaturation and subsequently an increase in viscosity.

Datta and Deeth (2003) reported that proteolysis could lead to an increase in viscosity. Inspite of absence of added proleotytic organism, viscosity of control at 15 days was significantly higher than at 0 and seven days. Celestino *et al.* (1997) reported that certain samples gelled even when they had no detectable protease activity. Increase in viscosity of control could be because of a physicochemical effect resulting in structural changes of casein micelle (Gaucher *et al.*, 2008). Viscosity had a decreasing trend for ST1 with storage when compared to ST2 which exhibited an opposite trend. This difference could be attributed to the differences in the specificities of enzymes released by P12 and S4 in ST1 and ST2 respectively. The different amino acid profile obtained after the

action of P12 and S4 on skim milk is in supportive of this difference in the specificity of enzyme. Datta and Deeth (2001) reported that in age gelation, there could be an initial stage characterized by a short period of thinning followed by a longer period of with comparatively lesser changes in viscosity. Reduction in viscosity of ST1 could be a result of thinning, arising from the action of protease enzyme of P12.

Colour intensity was almost similar for all the treatments at 0 day. Intensity increased significantly for ST2 from 7 days. For control and ST1 this parameter rarely showed significant difference with storage. Color intensity changes were relevant and significant for ST2. This might be because maillard reactions proceeded much faster under the influence of proteases liberated by S4. Polymerisation initiated by maillard type reaction increased viscosity (Andrews, 1975). Maillard reactions could lead to increase in acidity (Gaucher *et al.*, 2008). In consonance with this report, in this trial also ST2 that showed maximum browning had significantly higher acidity and viscosity than the other two treatment groups.

Overall quality rating was the highest for control on all tested days. In the present trial, bitterness and unclean flavour was found to have direct influence on overall quality rating. Chapman *et al.* (2001) observed that perception of bitterness had a dramatic effect on the overall quality perception. Unclean flavour imparted by the protease of P12 in ST1 (Mitchell and Ewings, 1985) was also found to have a similar negative impact on overall quality. From the results observed it had to be inferred that P12 and S4 produced enzymes that could withstand sterilization temperature. Renner (1988) had reported of enzyme activity of base milk adversely affecting functionality of milk powder. He reported that a Psychrotrophic population as low as 10^4 cfu/ml in raw milk kept under refrigeration for 72 h, contained thermoresistant proteases that significantly limited the shelf life after UHT treatment. Psychrotrophic proteases that remained active in ST1 and ST2 account for their low overall quality rating. Muir (1996) also noticed proteases unlikely to be destroyed during processing. The increased stability of proteases

could be attributed to the protective effect of Ca^{2+} ions in skim milk (Daniel *et al.*, 1995).

Sterilization is intended to make the medium free of viable cells. From the observation made in this work, it can be inferred that as long as microbial enzymes remain active, the term 'sterile' cannot conotate shelf stability.

5.3 ALKALINE PROTEASES

Generally, proteases produced from microorganisms are constitutive or partially inducible in nature and, under most culture conditions, *Bacillus* species produce extracellular proteases during post exponential and stationary phases. Extracellular protease production is strongly influenced by media components: variation in carbon to nitrogen ratio, presence of easily metabolisable sugars, metal ions and rapidly metabolisable nitrogen sources like amino acids. Physical factors like aeration, inoculum density, pH, temperature and incubation period affect many enzymatic processes and transportation of compounds across the cell membrane. Factors effecting production of alkaline protease by the isolate S4 were assessed to optimize growth conditions and maximize the enzyme yield.

5.3.1 Environmental Factors

5.3.1.1 Temperature and Period of Incubation

Under the experimental conditions, the maximum protease activity of 137.88 ± 1.26 was attained after 72 h of incubation at 42°C. However the culture attained 82.8 per cent of this maximum activity after 24 h at 37°C. Haque and Russel (2005) reported strains of *Bacillus cereus* to have a wide optimum temperature range of 37 to 47^{0}C. Observations in this trial are indicative of temperature being a critical factor controlling the metabolism of *Bacillus*.This findings is in accordance with the reports of Frankeena *et al.*, 1986. Anticipating the extra energy requirement during the scaling up process incubation conditions chosen for further work was 37°C for 24 h.

The results of this trial are in agreement with Chantawannakul *et al.* (2002) who suggested an incubation temperature of 37°C for 24 h to harvest the protease enzyme from *B. subtilis*, when grown in nutrient broth. However different authors suggested different time temperature combinations of incubation for harvesting protease enzyme of different *Bacillus* species 35°C for 3 days (Shimogaki *et al.*, 1991), 50°C for 15 h (Johnvesly *et al.*, 2001); 37°C for 48 h (Joo *et al.*, 2003); 37°C for 48 h (Shafee *et al.*, 2005) and 30°C for 72 h (Banik and Prakash, 2006). The differences could be attributed to the differences in species and strain used, media composition and culture conditions.

Chantawannakul *et al.* (2002) reported that protease production reached the highest during exponential phase and remained constant when spores were formed. In the present trial rate of enzyme production was very high in the first 24 h beyond which the rate of increase was not that remarkable. In the culture conditions provided in this trial, organism would have completed the logarithmic phase and entered stationary phase (sporulation) by 24 h. Frankeena *et al.* (1986) reported a link between enzyme synthesis and energy metabolism in *Bacillus*. Observations made in this trial also support this.

5.3.1.2 pH of the Medium

In this trial maximum production of protease was observed, when pH of the fermentation medium was adjusted to 11.0. Results suggest that with increasing pH, organism performed better in terms of production of protease enzyme. Just by increasing the pH from 8 to 11 and from 9 to 11 protease production could be enhanced by 47.5 per cent and 24.8 per cent respectively. Less intense stress symptoms by induction of a better buffering system at pH above 10.0 for *Bacillus* spp had been reported by Lindsay *et al.* (2002). The same reason could be accounted for the better performance at pH above 10.0. This could be because pH of the medium strongly affected enzyme processes and transportation of compounds across the membrane (Puri *et al.*, 2002). Importance of pH had been put forward by Chu *et al.* (1992).

Naidu and Devi (2005) found pH 9 to be optimum whereas Olajuyigbe and Ajele (2005) reported pH 8 to be the best for the production of protease enzyme by *Bacillus* species. In this trial maximum yield was attained at pH 11.0. The results are also suggestive of the enzyme to be an alkaline protease. Prakasham *et al.* (2005) also have identified pH to be a significant factor during the optimization of alkaline protease production by *Bacillus* species.

5.3.1.3 Aeration

Bacillus is an obligate aerobe that prefers aeration for their multiplication. However, results of the present study showed that aeration had a negative impact on the production of alkaline protease. Moon and Parulekar (1991) opined that culture conditions that promoted cell growth and that which favored production of protease could be different. They also reported that the rate of agitation affected the aeration level in fermentation medium and also the nutrient availability. Anderson (1980) also reported a similar observation.

Agitation was found to improve the yield of protease from *Bacillus* species (Joo *et al.*, 2003; Banik and Prakash, 2006). However, findings of this trial are not in agreement with these observations. In the present study, protease activity in the culture maintained with aeration was significantly lower than that without aeration. Probably rate of agitation was not sufficient enough to positively influence the isolate under test conditions. Strain difference of the test organism also could be a reason. Excessive aeration and agitation might have led to cell lysis (Darah and Ibrahim, 1996) and denaturation of enzyme (Lee *et al.*, 2002), such that enzyme activity got reduced.

5.3.1.4 Rate of Inoculum

Rate of inoculation can affect protease production. According to the results depicted in Table 7d, maximum protease production was achieved when inoculation was done at a level of three per cent. The enzyme activity obtained at a level of three per cent was significantly higher than that obtained at two percent. Activity obtained at a level of two percent was high compared to that obtained at a

level of one per cent. Such increase in production of protease enzyme with increase in size of inoculum has been reported by Kumar (1997). But when inoculation rate was increased to four per cent, enzyme activity reduced significantly. Sinha and Satyanarayana (1991) also have reported similar observations. This could be because higher inoculum sizes resulted in reduced dissolved oxygen and increased competition for nutrients. Lower inoculum sizes facilitated higher surface area to volume ratio. This reason can be attributed to the better performance at a level of three per cent when compared to four per cent (Shafee *et al.*, 2005). Under shake flask conditions, maximum yield for *Streptomyces diastaticus* was recorded with three per cent inoculum (Chaphalker and Dey, 1994). In this work also maximum performance was observed at the same rate of inoculation. The significant influence of combined effect of inoculum level and salt solution concentration on protease production had been reported by Prakasham *et al.* (2005).

5.3.2 Nutritional Factors

The medium components have been predicted to play a significant role in enhancing the production of alkaline proteases. In commercial practice, optimization of medium composition is done to balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. When the substrate for fermentation is made up of cheaper constituents, the possibility of making enzyme production commercially viable is still more. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen sources on the yield of enzyme. In this work, basal fermentation medium comprised of 0.5 per cent whey powder and 0.5 per cent skim milk powder with pH adjusted to 10.0.

5.3.2.1 Nitrogen Sources

Alkaline proteases comprise of 15.6 per cent nitrogen and its production is very much dependant on the availability of carbon and nitrogen sources in the medium (Kole *et al.*, 1988). Statistical analysis revealed that differences in the

enzyme yield were highly significant between all organic nitrogen sources except that between peptone and yeast extract.

According to the results depicted in Table 8a, the isolate used in this trial exhibited maximum protease activity when tryptone was added at a level of 0.5 per cent to basal fermentation medium comprising of 0.5 per cent each whey powder and skim milk powder. Kumar (1997) had also reported the superior performance of tryptone in terms of protease production when added at a level of 0.5 per cent to whey based basal fermentation media. Phadatare *et al.* (1993) found that tryptone at a level of two per cent was an excellent source of nitrogen for protease production.

When compared to control, incorporation of beef extract yeast extract peptone and tryptone enhanced the protease production by 14 per cent, 132.4 per cent, 139.9 per cent and 178.7 per cent respectively. The supportive role of tryptone and yeast extract in production of protease by *Bacillus* species had been reported by Naidu and Devi (2005). In this trial, performance of beef extract was poor when compared to other organic nitrogen sources. In contrast to this observation, Naidu and Devi (2005) found maximum protease yield for *Bacillus* species with beef extract. According to Shafee *et al.* (2005) peptone enhanced growth of *B. cereus* but repressed protease production. However in this trial peptone at a level of 0.5 per cent enhanced protease yield by 139.35 per cent. This variation might be due to the differences in the composition of basal fermentation medium and the strain of isolate used in this work.

Addition of inorganic nitrogen sources to the basal fermentation medium enhanced the yield of protease significantly. Of the various sources tested, ammonium sulphate gave the maximum yield (244.09 ± 2.51) followed by ammonium chloride, ammonium nitrate, potassium nitrate and ammonium carbonate. Maximum increase in the protease production by *Bacillus* after the addition of ammonium sulphate had been reported by Sinha and Satyanarayana (1991). Moderate to good level of enzyme activity from *Bacillus* species with ammonium chloride and potassium nitrate hds been reported by Nascimento and

Martins (2004). They reported maximum yield when ammonium nitrate was used. In this trial also ammonium nitrate was found to enhance the enzyme activity by 69.24 per cent when compared to control. Increase in enzyme yield by 97.75 per cent was attained with the incorporation of ammonium chloride. This finding is in line with the reports of Mehrotra *et al.* (1999) who reported ammonium chloride as the best nitrogen source for maximum production of alkaline protease by a *Bacillus* species.

Performance of ammonium carbonate was poor when compared to other sources of nitrogen. It could enhance the protease yield only by 23.5 per cent. In contrast to this finding Shafee *et al.* (2005) reported that when ammonium carbonate was used the inorganic nitrogen source *B. cereus* strain 146 exhibited maximum protease activity at 24 h which got repressed at 48 h and 72 h. Strain variation and differences in culture condition could account for the conflicting results.

5.3.2.2 Carbon Sources

The studies carried out to evaluate the influence of different carbon sources on the enzyme production in the modified medium revealed that trisodium citrate at 0.5 per cent concentration could significantly enhance the protease yield. Nascimento and Martins (2004) suggested trisodium citrate as one of the best carbon sources for protease production by *Bacillus* spp. Takii *et al.*, (1990) opined that neutralization of salts of organic acids for production of alkaline protease was interesting in view of their economy and ability to control pH variations. Nascimento and Martins (2004) also suggested trisodium citrate as the best carbon source for protease production by a thermophilic *Bacillus* species. Ferrero *et al.*(1996) suggested the use of $MgSO_4$, $CaCl_2$, $MnSO_4$ and $ZnSO_4$ along with trisodium citrate for protease production by *Bacillus licheniformis* MIR 29. Kumar (1997) also suggested the combined use of sodium citrate and $MgSO_4$ for enhanced production of protease. Same opinion has been put forward by Johnvesly and Nailk (2002). According to Mahmood *et al.*(2000) carbon sources enhanced protease production, when this was used poorly for growth purposes. Lesser utilization of

trisodium citrate for the growth by S4 accounts for its better performance in the yield of proteases. Shafee *et al.* (2005) reported that glucose induced maximum protease production by *Bacillus cereus* strain 146. However in this trial glucose was not found to have any significant effect on enzyme yield. The results of present trial suggest that starch either adversely affected the protease yield (corn starch) or did not have any influence on enzyme production (potato starch). The poor performance of starch and maltose observed in this trial is supported by the reports of Shafee *et al.*(2005). However, reports of Nascimento and Martins (2004) are contradictory to the observations made in this trial.

Disaccharides like sucrose and maltose were found to have a negative effect on the synthesis of alkaline protease. This could be due to catabolite repression as reported by Kumar (1997). In this trial sucrose was found to have no significant influence on enzyme yield. This finding is contradictory to the reports of Phadatare *et al.*, (1993) who observed that sucrose at a level of three per cent resulted in maximum enzyme production. According to the observations in this trial, incorporation of carbon sources did not enhance protease production in general. Probably the carbon sources provided in the medium was used by the isolate for growth purposes rather than for enzyme production (Mahmood *et al.*, 2000).

5.3.3 Partial Characterization of Enzyme

5.3.3.1 pH Stability

Results of the present trial points out the fact that to remain stable, the crude enzyme used in this trial prefers an alkaline medium. Assessment of enzyme activity on casein substrate after a preincubation at different pH for 24 h revealed that with increasing pH, enzyme showed a gradual increase in activity. Residual activity obtained was significantly different between treatments except that between pH 8 and 9 and pH 9 and 10. A similar gradual increase in protease activity above pH 7.0 till pH 10 had been reported by Son and Kim (2002). Takami *et al.* (1990) reported that pH was important in deciding the binding function

between enzyme and substrate. Better activity at alkaline pH might be due to the better binding of enzyme to the substrate at this pH. The present observation of maximum activity at pH 11 is in agreement with the reports of Joo *et al.* (2003) and Johnvesly and Nailk (2002).

Enzyme retained 92.6 per cent of the original activity after 24 h of exposure to pH 12. Stability of protease enzyme from *Bacillus* species over a pH ranging from 7- 12 had been reported earlier by Fujiwara *et al.* (1993); Joo *et al.*(2003) and Aftab *et al.* (2006). Such good stability at pH values up to 12 had been reported by Nogueira *et al.*(2006). This distinctive feature of high pH optimum is a common feature of all alkaline proteases. Johnvesly and Nailk (2002) reported 84 per cent residual activity after exposure to pH 12 for 2 h. The results of this trial are suggestive of the enzyme to be an alkaline protease. Stability at high pH indicates the enzyme to be a potential candidate for many industrial purposes.

5.3.3.2 Temperature Stability

Assessment of thermostability of enzyme showed that protease retained more than 60 per cent activity after 90 minutes of exposure to all the selected temperatures of 20°C, 40°C, 60°C and 80°C. Comparable thermostability and high optimum temperature for *Bacillus* protease had been reported by Shimogaki *et al.* (1991); Fujiwara *et al.* (1993); Sookheo *et al.* (2000) and Johnvesly *et al.* (2001). Koka and Weimer (2001) reported that thermostability of enzyme varied significantly with strain, growth medium and incubation period. At 20°C, 40°C and 60°C protease activity tended to decrease with increase in time of exposure. But the activity profile at 80°C showed a different pattern. After 30 minutes of exposure at 80°C, there was a sharp decline in activity to 46.4 per cent. This could be because enzyme was not capable of getting adapted to high temperature within 30 minutes. As the exposure period advanced, enzyme would have got adapted to the high temperature environment. This was evident from the increase in enzyme activity to 74.7 per cent. Such high thermostability of *Bacillus* protease was reported by Chopra and Mathur (1985). Inactivation of protease inhibitors at high temperature would have contributed to the enhanced enzyme activity. Maximum

residual activity after 60 min of exposure was for the sample kept at 80°C. High thermal resistance at 80°C owing to inactivation of protease inhibitors at higher temperatures had been reported by Poffee and Mertens (1988). Increase in activity could also be due to the release of intracellular protease from the cells at high temperatures (Rajmohan *et al.*, 2002). Reduction in protease activity on further exposure to temperature could be due to the thermal denaturation of enzyme resulting from prolonged exposure to high temperature.

Storage stability of the enzyme at 10°C and -10°C were assessed at 10 days interval for forty days. Stability of *Bacillus* protease at temperatures below 20^0C had been reported by Son and Kim (2002). For 20 days protease activity increased gradually such that activity increased to 145.4 percent and 129.3 percent at 10°C and -10°C respectively. This could be probably because of the inhibition of protease inhibitors at low temperature. On all tested days, frozen samples exhibited lesser activity than the corresponding samples at 10°C. Ice crystal formation at -10°C might have affected active conformation of enzyme thereby initiating enzyme denaturation and loss of activity. Even after 40 days of storage, samples at 10°C and -10°C exhibited a residual activity of 77.4 percent and 69.5 percent respectively. Adaptation of enzyme to low temperatures could be because enzyme was produced by a psychrotolerant strain of *Bacillus*. Results of this trial conclusively suggest the stability of enzyme to a very wide temperature range of -10 to 80°C.

5.3.3.3 Stability in the Presence of Metal Ions

Findings from this work suggest that all the metal ions used in this trial either enhanced or stabilized the enzyme activity. Ca^{++}, Mg^{++} and Zn^{++} were found to enhance the activity to 134.3 per cent, 134 per cent and 111.4 per cent respectively. Supportive role of Ca^{++} and Mg^{++} have been observed by Paliwal *et al.* (1994), Adinarayana *et al.* (2003) and Shafee *et al.* (2005). Conflicting reports are available with the role of zinc ions on enzyme activity (Nascimento and Martins, 2004). In this trial, influence of zinc ions on enzyme activity is in accordance with the findings of Kumar (1997) and Aftab *et al.* (2006). Improved

stability of enzyme in the presence of Na^+ could be because of the protective effect of Na^+ against autolysis (Banerji *et al.*, 1999). According to Shafee *et al.* (2005) Mn^{++} supported protease production maximally at 24 h of incubation. But in this trial, when compared to calcium ions, manganese ions stabilized the enzyme activity to a lesser extend. This finding is in accordance with the reports of Aftab *et al.* (2006). As per the results of this work, cobalt ions did not interfere with enzyme activity. A similar effect on *Bacillus* protease had been reported by Shimogaki *et al.*(1991). Nascimento and Martins (2004) reported that mercury ions inhibited the protease of *Bacillus* species completely. But in this work mercury ions improved the activity. Stimulatory effect of mercury ions have been noticed by Aftab *et al.* (2006) also. Mercury ions are known to cause enzyme inhibition by the hydrolytic degradation of disulphide bonds in the enzyme structure (Kumar, 1997). Absence of inhibition might be due to the absence of disulphide bonds. Enhancement of activity noted in this trial could be because of the improved stability by way of its role in maintaining the active conformation of enzyme.

5.3.3.4 Effect of Inhibitors

After preincubation with PMSF, protease activity reduced to 38.5 per cent of the control. Phenyl methyl sulphonyl fluride sulfonates the essential serine residue in the active site. The significant reduction in activity after exposure to PMSF suggests the presence of a serine group in the active site and thereby the possibility of crude protease preparation to have a serine protease. Serine proteases from *Bacillus* species has been reported by Shimogaki *et al.* (1991); Johnvesly *et al.* (2001); Banik and Prakash (2006).

Inhibition studies can give an insight into the nature of enzyme, its cofactor requirement and nature of active centre. When treated with EDTA 35.5 per cent reduction in activity was observed. This result is in close proximity with the findings of Kumar (1997). The present observation suggests the possibility of presence of metal ions in the active site of enzyme. Chen *et al.* (2004) reported the production of serine and metallo proteases by *Bacillus* species. A related work in this line has shown that metal ions stabilized enzyme activity, especially at high

temperature, by maintaining the active confirmation of enzyme (Beg *et al.*, 2003). Results of this trial also show the supportive role of metal ions in improving enzyme stability. Calcium ions were found to enhance the activity by 134.3 per cent; may be metal ions would have satisfied the cofactor requirements. Ethylene diamine tetra acetate, a chelator of divalent cations reduced the activity by removing the calcium ions that stabilized the tertiary structure and therefore lose of activity was due to enzyme denaturation rather than by inhibition (Freeman, 1993). Occurrence of metallo and serine proteases for *Bacillus* species have been reported by Chopra and Mathur (1985); Chen *et al.* (2004).

Exposure to β-mercaptoethanol under experimental conditions resulted in a residual activity of 93.1 per cent. This observation is in close proximity with Banik and Prakash (2006) who reported 96.7 per cent residual activity with β-mercaptoethanol, a cysteine type inhibitor. The finding that β-mercaptoethanol did not have a marked inhibitory effect suggest that the protease enzyme lacked a cysteine residue or an essential thiol group in the active site. The possibility of absence of an active thiol group is consistent with the earlier observation that mercury ions, a disulphide bond inhibitor did not interfere with enzyme activity.

Compatibility with surfactants and oxidizing agents are advantageous for enzymes to be used as detergent additives. As per the results, protease activity enhanced significantly in the presence of surfactants. The anionic surfactant, SDS acted as an activator such that activity increased by 2.2 times for the enzyme tested under experimental conditions. This is in accordance with the findings of Nogueira *et al.* (2006) who found that SDS enhanced the protease activity of *Teredinobacter turnirae* by 3.7 times. The protease enzyme exhibited a better performance in the presence of nonionic surfactant Tween 80. Enzyme activity increased to 111.2 per cent when compared to control. Similar effect of nonionic surfactant has been reported by Joo *et al.* (2003) for the alkaline protease of *B.clausii*.

Stability with oxidizing agent is beneficial as it facilitates better performance in bleach based formulations. Reduction in enzyme activity observed

on treatment with H_2O_2 was insignificant when compared to control. Stability of *Bacillus* protease to H_2O_2 is in corroboration with the findings of Joo *et al.* (2003) and Nilegaonkar *et al.* (2007). The results of present trial confirms the stability of protease in the presence of oxidising agent and surfactant suggesting that protease preparation used in this trial is an ideal candidate to be used as a detergent additive.

5.3.4 Assessment of Cleaning Eefficiency of Inbuilt Detergents

5.3.4.1 Milk Soil

Cleaning system followed in any process depends on the type of soil and nature of surface. According to Moore *et al.* (1951) soil accumulates on glass and stainless steel surface to a similar degree. In this study, soil was made artificially on glass Petri dishes using pasteurized whole milk as this was the most commonly encountered soil. According to Twomey (1975) protein contributes to more than 50 per cent of soil. Hence the crude protease enzyme obtained from isolate S4 was assessed for its effect in improving the efficiency of cleaning formulations.

When water alone was used, cleaning was not at all effective. Usage of crude enzyme preparation alone, also did not give satisfactory result. Whittaker *et al.* (1984) opined that enzyme alone could not act as satisfactory cleansing agents. They proposed a combination of chelator and surfactant for better results. When labolene was used as per manufacturers' recommendations, the protein residues persisted, especially in the periphery, clearly indicating the lack of ability of cleaning solution to dislodge the dried soil. When enzyme based formulation was used, obviously, dislodgement and clearing of plates was far better than the control. Varghese (2002) also gave similar reports. To rule out the influence of detergent components other than crude enzyme, a cleaning formulation comprising of a chelator and surfactant along with sodium hydroxide was also checked for cleaning efficiency. Performance of this solution was very poor and the plates were characterized by a 'foggy' appearance, probably because of fat residue. From the results, it can be concluded that crude enzyme did possess a complementary effect and that the inbuilt formulation comprising of cell free supernatant sodium

hydroxide, EDTA (Chelator) and Tween 80 (surfactant) fared the best with regard to efficiency of cleaning.

5.3.4.2 Wash test for removal of proteinaceous stain

Proteases exhibiting activity in high alkaline range are recognized as potential detergent additives in stain removing solutions (Anwar and Saleemuddin, 1998). When casein was used as a substrate the crude protease enzyme used in this work had an optimum pH 11. It retained even 98.6 per cent of native activity after exposure to pH 12 for 24 h. Stability at an alkaline pH make the enzyme used in this work, a suitable candidate as detergent additive. For an enzyme to be used as a detergent additive it should be stable and active in the presence of typical detergent ingredients like surfactants, builders, bleaching agents and other formulation aids at moderately high temperatures. The anionic surfactant sodium dodecyl sulphate was found to increase the activity of the crude protease preparation by 2.2 times. The results obtained are also indicative of its compatibility with the non ionic surfactant Tween 80. Nogueira *et al.* (2006) opined that proteases compatible and complementary with anionic detergent sodium dodecyl sulphate were preferred as detergent additives. Joo *et al.* (2003) found that protease enzyme of *Bacillus* species was compatible with the anionic detergent, Tween 80. Better performance of the crude enzyme used in this trial in the presence of sodium dodecyl sulphate and Tween 80 has already been confirmed. Hydrogen peroxide, the commonly used bleaching agent was found to have no significant adverse affect on the crude enzyme. According to the earlier observation, the crude protease from S4 retained more than 60 per cent activity after exposure to 60°C and 80°C for 90min. Hence it could be expected that protease remained active during the wash cycle which was carried out at 55°C. Stability of enzyme at low temperature further indicated its potential to be used in cold wash surface which is of relevance due to the energy costs involved in heating water.

According to the results got in the wash trial, the best performance was observed when the stained cloth pieces were washed with the inbuilt detergent formulation comprising of a commercially available detergent, crude enzyme and

H_2O_2. Earlier observations made in this work indicate that, after exposure to pH 9.0 and pH 10 for 24 h activity increased by 18 per cent and 25 per cent respectively. Hence it could be inferred that protease remained active in the formulation used in this experiment (pH-10). The enzyme assisted in the removal of blood stain. The results of the wash test conclusively proved that incorporation of enzyme improved the performance of the detergent solution. Detergent compatibility of the enzyme is distinctly established by the wash test. The results of this experiment confirm the potential of enzyme to be used as a detergent additive, specifically to tackle proteinaceous stain.

5.3.5 Purification of Enzyme

The removal of cells, solids and colloids from the fermentation broth is the primary step in recovery of extracellular enzymes. Anustrup (1980) recommended the use of ammonium sulphate for inorganic salting out of proteins. In this work also, first step in recovery of enzymes comprised of inorganic salting out with ammonium sulphate. For the recovery of protease enzyme from *Bacillus* species Chopra and Mathur (1985) used 40-70 per cent precipitate, and Adinarayana *et al.* (2003) used precipitate at 50-70 per cent saturation. Banik and Prakash (2006) resorted to extractive fermentation of the 80 per cent ammonium sulphate for recovery of protease from *Bacillus cereus.* Preliminary trials in the present work indicated that precipitate at 40-80 per cent fraction gave maximum activity. The CFS possessed protein content 0.121 mg/ml and an activity of 92.6 µmoles of tyrosine/ml/min. The precipitate obtained at 40-80 per cent saturation was dissolved in minimum quantity of Tris Hcl buffer (pH 11) and dialysed against the same buffer for 24h. Dialysis of ammonium sulphate pellet to remove the contaminating proteins had been suggested by Chopra and Mathur (1985). The protease activity of the dialysate increased to 412.08µmoles of tyrosine/ml/min whereas the protein content decreased to 0.022 mg/ml, indicating that dialysis was effective in removing unwanted extraneous protein. Son and Kim (2002) also supported use of dialysis in purifying enzymes. The increase in specific activity of dialysate to 18730.91 suggested that the enzyme could be purified to an extent of 24.48 fold.

Gel filtration chromatography/size exclusion chromatography is a technique for separation and purification of biologically active substances like enzymes. Gel filtration had been used successfully for the purification of alkaline proteases by *Bacillus* species (Chopra and Mathur, 1985; Kumar, 1997; Son and Kim, 2002; Adinarayana *et al.*, 2003; Banik and Prakash, 2006). Of the 70 fractions collected after the passage of dialysate through seralose 4B, 19^{th,} 26^{h} 27th and 38^{h} fraction showed the presence of protein. Tyrosine value of 27th fraction was found to increase to 765.3. Protein content of the fraction reduced to 0.008 mg/ml. The 27th fraction showed a specific activity of 95672.5, indicating that protease enzyme was purified by 125.06 fold. When the 27th fraction was passed through the column of Seralose 6B, having a fractionation range of 10 kDa - 5000 kDA, many protein peaks were obtained. The specific activity of all these fractions was determined. The 39th fraction that showed maximum specific activity (102428.2) was rechromatographed through Seralose 6B. Screening of the fractions so obtained revealed that protein was present only in the 9th fraction. Tyrosine value of this fraction was found to increase to 864.8 whereas protein value remained at 0.008 mg/ml. Thus specific activity of this fraction further increased to 108100 demonstrating that enzyme was purified by 141.31 fold. Vidyasagar *et al.* (2006) also could achieve a purification fold of 116 by gel permeation chromatography.

The homogenity of the purified protease from the isolate S4 was ascertained by SDS polyacrylamide gel electrophoresis under reducing conditions. Lanes 2 and 3 which contained the sample gave a single band, confirming the homogenity of enzyme preparation. By comparing the position of protein band of the sample band with that of molecular marker, that enzyme was found to have a molecular weight between 43 kDA and 66 kDa. Alkaline proteases of lower molecular weight have been reported from *Bacillus* species previously (Shimogaki *et al.*, 1991; Kim et al., 2001; Prakash and Banik, 2005). Based on Mf values, molecular weight of enzyme obtained in this study could be approximated as 50.5 kDa. Sookheo *et al.* (2000) succeeded in characterising a thermostable protease of 71 kDa from *Bacillus* species. Chopra and Mathur (1985) also characterized RM-

67 protease with a molecular mass of 67.6 kDa from *Bacillus* species Morihara (1974) opined that Mf values for bacterial alkaline proteases was highly variable. Purification methods required for enzyme purification depend on the type of microorganism and the nature of enzyme.

The psychrotrophic spore former *B.cereus* was found to have the potential to produce alkaline protease that was durable to temperature as high as 80 °C and as low as -10°C. .Compatibility with surfactants and oxidizing agent confirms the potential of protease to be used in cleaning formulations.Ammonium sulphate precipitation; dialysis and gel filtration was effective in achieving a purification fold of 141.31.The study indicated the potential of protease enzyme produced by a spoilage organism to be used in dairy plant sanitation.

Summary

6. SUMMARY

Food industries have undergone progressive refinement and processing sophistication during recent years. Still bacterial spoilage mediated by enzymes continue to cause significant economic losses for food industry. Thermodurics and psychrotrophs are gaining importance in milk due to adoption of heat treatment and strengthening of cold chain. Most of these organisms are capable of producing thermostable enzymes in raw milk, that can adversely affect the shelf life of products prepared from it. Some enzymes, especially the proteases produced by members belonging to the genera *Pseudomonas* and *Bacillus* are highly thermostable. If these enzymes are effective in alkaline pH range and compatible with other detergent components, they have got the immense potential to be used in cleaning formulations. This trial was taken up to study the impact of enzyme producers in milk on the shelf life of curd (dahi) and sterilised milk and to partially purify and characterise a protease enzyme suitable for cleaning formulations.

- A total of 71 isolates obtained from raw milk, processed milk, tap water and alkaline water washings were qualitatively assessed for its ability to produce the spoilage enzymes: proteases, lipases and lecithinases. Ninety per cent of *Bacillus* isolates and cent per cent of *Pseudomonas* isolates obtained in this trial showed protease activity. Lipase activity was evident in 72 per cent of Bacillus isolates and 100 per cent of *Pseudomonas* isolates. In general, lipase activity was more for *Pseudomonas* spp than for *Bacillus* spp. Lipase production was found to be more at 7°C than at 37°C. All the *Bacillus* isolates obtained from alkaline water washings showed strong lecithinase activity at 37°C.

- Pasteurised milk screened in this trial revealed a unique bacterial profile predominated by *P. aeruginosa* that exhibited remarkable protease and lipase activity along with mild lecithinase activity. A distinct shift to predominance of *Pseudomonas* spp. after processing of milk is suggestive of post processing contamination.

Screening of isolates for their potential to produce extracellular protease in alkaline medium revealed that isolate S4 showed maximum activity under alkaline conditions. Isolate S4 was obtained from sterilised skim milk that showed signs of spoilage after refrigerated storage for one week. The psychrotrophic spore former was identified as *Bacillus cereus*.

The impact of psychrotrophic spoilage enzyme on quality of curd (dahi) was done in terms of selected parameters. The treatment groups CT1 and CT2 made from milk precultured with P12 and S4 respectively showed significantly lower pH and higher titratable acidity on 0 day itself. The same trend continued till ten days of storage. The proteolysis initiated by P12 and S4 in base milk would have stimulated the starter culture. Hence a higher titratable acidity for the treatment group, when compared to control on all tested days of storage.

The syneresis values obtained in this trial clearly indicate that psychrotrophic spoilage enzymes can influence syneresis value significantly. Any variation in the protein quality of milk can contribute to variations in syneresis. The curd CT1 and CT2 prepared from milk containing psychrotrophic protease had lower water holding capacity and protein hydration and hence increased syneresis.

The firmness of control curd was significantly lower than CT1 and CT2 on all tested days of storage. The peptides and amino acids released by the proteolytic action of P12 and S4 stimulated curd starters, improved lactic acid production, increased curd contraction and syneresis thereby enhancing firmness. The denatured protein matrix in CT1 and CT2 also made a positive influence on firmness values. Viscosity values of CTI and CT2 were significantly lower than control probably due to the higher protein denaturation by way of action of P12 and S4. In general viscosity decreased with increase in firmness.

- The poorer performance of CTI and CT2 during sensory evaluation confirms the significant negative impact of psychrotrophic spoilage enzymes on the overall quality and shelf life of curd.

- For assessing the influence of spoilage enzymes especially proteases on sterilised milk, pH,tyrosine and NPN values were estimated on 0, 7 and 15 days of storage. The pH values showed a significant difference from control only at 15 days of storage. Highly significant increase in NPN values in ST1 and ST2 on 7 and 15 days of storage suggest a higher proteolytic activity in this group. Off-flavour development was found to be a function of proteolysis. A linear correlation was found to exist between proteolysis and off-flavour. The perception of bitterness was found to have a significant effect on the overall quality perception of the product. The results confirm the negative impact of psychrotrophic proteases in sterilised skim milk.

- As a part of optimisation of growth conditions for production of alkaline protease from isolate S4, the influence of various environmental factors and nutritional factors were assessed. Based on the results obtained, production of protease enzyme was carried out in a medium with pH – 11.0. Inoculation was done at a level of three per cent and harvesting of enzyme was done at 24 h of incubation at 37°C without aeration.

- Of the organic nitrogen sources tested, incorporation of 0.5 per cent tryptone into the basal fermentation medium (0.5 per cent each of skim milk powder and whey powder) enhanced the protease yield by 178.7 per cent. Among the inorganic nitrogen sources tested, ammonium sulphate gave the maximum yield. In general, supplementation of nitrogen sources in basal fermentation medium enhanced the yield of protease significantly. When different carbon sources were tested, only trisodium citrate enhanced the protease yield significantly. Poor performance by rest of carbon sources may be because these sources were used by the isolate for growth purpose rather than for enzyme production.

- For the characterisation of enzyme, stability of the crude protease to different pH, temperature and metal ions were assessed. Results of the trial points out the fact that to remain stable enzyme used in this trial prefer an alkaline medium. The enzyme exhibited maximum stability at pH 12.0. Assessment of thermostability of enzyme showed that protease retained more than 60 per cent activity after 90 min of exposure to selected temperature of 20°C, 40°C, 60°C and 80°C. High thermal resistance at 80°C owe to the inactivation of protease inhibitors. Comparable storage stability at 10°C and -10°C was also confirmed in this trial. Zn^{++}, Ca^{++}, Mg^{++}, Na^{+} and Hg^{++} enhanced the enzyme activity significantly. Mn^{++} and Co^{++} did not have significant effect on enzyme activity under test conditions. Improved performance in the presence of metal ions could be because of the improved stability by way of its role in maintaining the active conformation of enzyme.

- Inhibition of protease enzyme at a level of 61.5 per cent by phenyl methyl sulfonyl fluride suggest the presence of a serine group in the active site and thereby possibility of crude protease preparation to have a serine protease. Reduction in protease activity in the presence of EDTA is indicative of presence of metal ions in the active site of enzyme. Lack of marked inhibition by β-mercaptoethanol suggests that protease enzyme lack a cysteine residue in the active site. The anionic surfactant sodium dodecyl sulphate enhanced the protease activity by 2.2 times under experimental conditions. On exposure to nonionic surfactant Tween 80, the enzyme activity increased by 11.2 per cent. Reduction in enzyme activity observed on treatment with H_2O_2 was insignificant. The results of present trial confirmed the stability of protease in the presence of surfactants and oxidising agent suggesting protease preparation to be ideal as a detergent additive.

- Qualitative assessment of cleaning efficiency confirmed the superiority of enzyme based cleaning formulation.

- Ammonium sulphate precipitation, dialysis and gel filtration chromatography was effective in purifying the protease preparation by 141.31 fold. SDS PAGE showed that protease possessed a molecular weight of 50.5 kDa.

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*Originals not consulted.

APPENDIX I

SCORE CARD FOR SENSORY EVALUATION OF CURD

Date:

Code No.

ATTRIBUTES	MAXIMUM SCORE	SAMPLES		
		A	B	C
FLAVOUR	40			
BODY AND TEXTURE	35			
COLOUR & APPEARANCE	10			
PRODUCT ACIDITY	15			
TOTAL	100			

DESIRABLE CHARACTERISTICS

Curd (dahi) is a cultured milk product. It shall have a pleasing bouquet flavour resulting from the blend of a clean delicate somewhat aromatic odour and a pronounced though clean acid taste. It should be free from the following undesirable flavours like bitter, coarse due to over-ripening, flat (lack of flavour), off odour, metallic and yeasty. Body and texture should be firm, solid and uniform with negligible whey separation (Bureau of Indian Standards, 1980).

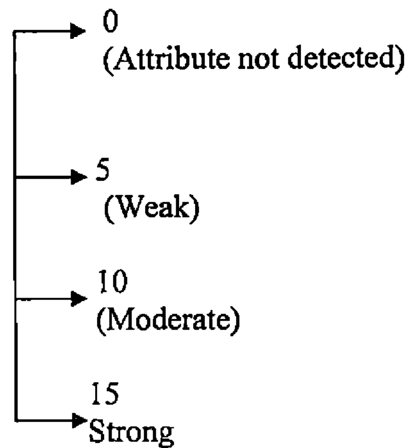
Panel member:

Signature

APPENDIX II

Modified Short Version Descriptive Analysis for Sterilized Milk

The product may be evaluated with respect to following attributes in the intensity scale value 0 to 15. Panelists shall write the actual number for the intensity of each attributes.



Reference standards used:

Attribute	Reference	Scale
Viscosity	2 per cent fat in milk	10
Color intensity	Pasteurized skim milk	5
Color intensity	Sweetened. Condensed milk	15
Sour taste	0.1 per cent citric acid	5
Bitter taste	0.5 per cent caffeine	5

Attribute	Intensity Scale		
	Sample A	Sample B	Sample C
Viscosity			
Colour intensity			
Cooked flavour			
Unclean falvour			
Bitter taste			
Sour taste			
After taste			
Overall quality rating <6 poor 6-10 fair 10-15 good			

Reference Standards provided.

Taster :

Signature:

APPENDIX-III

Assessment of spoilage potential of the isolates obtained from KAU Dairy plant

Isolate	Source	Genus	Ability to produce spoilage enzymes		
			Protease	Lipases	Lecithinase
R_1	Raw milk	*Escherichia*	-	-	-
R_2	Raw milk	*Pseudomonas*	++	++	-
R_3	Raw milk	*Enterobacter*	+	-	-
R_4	Raw milk	*Pseudomonas*	+++	++	-
R_5	Raw milk	*Pseudomonas*	++	+	-
R_6	Raw milk	*Staphylococcus*	-	+	-
R_7	Raw milk	*Pseudomonas*	++	++	-
R_8	Raw milk	*Micrococcus*	+	+	-
R_9	Raw milk	*Staphylococcus*	-	++	-
R_{10}	Raw milk	*Chromobacterium*	++	++	+
R_{11}	Raw milk	*Escherichia*	-	-	-
R_{12}	Raw milk	*Escherichia*	-	-	-
R_{13}	Raw milk	*Enterobacter*	-	-	-
R_{14}	Raw milk	*Lactobacillus*	+	-	-
R_{15}	Raw milk	*Micrococcus*	+	+	
R_{16}	Raw milk	*Pseudomonas*	++	++	+
R_{17}	Raw milk	*Enterobacter*	-	-	-
R_{18}	Raw milk	*Escherichia*	-	-	-
R_{19}	Raw milk	*Pseudomonas*	++	++	+
R_{20}	Raw milk	*Staphylococcus*	-	++	-
R_{21}	Raw milk	*Chromobacterium*	++	++	+
R_{22}	Raw milk	*Pseudomonas*	++	+	+
R_{23}	Raw milk	*Bacillus*	+++	++	+
R_{24}	Raw milk	*Bacillus*	+++	+	++

R_{25}	Raw milk	*Proteus*	-	-	-
R_{26}	Raw milk	*Escherichia*	-	-	-
P_1	Pasteurised Milk	*Pseudomonas*	+++	++	-
P_2	Pasteurised Milk	*Pseudomonas*	++	++	+
P_3	Pasteurised Milk	*Bacillus*	+++	+	+
P_4	Pasteurised Milk	*Bacillus*	++	+	-
P_5	Pasteurised Milk	*Pseudomonas*	++	+++	-
P_6	Pasteurised Milk	*E. coli*	-	-	-
P_7	Pasteurised Milk	*Pseudomonas*	++	++*	-
P_8	Pasteurised Milk	*Bacillus*	+++	+	+
P_9	Pasteurised Milk	*Micrococcus*	+	-	-
P_{10}	Pasteurised Milk	*Pseudomonas*	++	++	+
P_{11}	Pasteurised Milk	*Bacillus*	+++	+	-
P_{12}	Pasteurised Milk	*Pseudomonas*	++++	+++	++
P_{13}	Pasteurised Milk	*Staphylococcus*	-	++	-
P_{14}	Pasteurised Milk	*Proteus*	-	+	-
P_{15}	Pasteurised Milk	*Pseudomonas*	+++	++	+
P_{16}	Pasteurised Milk	*Enterobacter*	-	-	-
P_{17}	Pasteurised Milk	*Pseudomonas*	+++	++*	+
P_{18}	Pasteurised Milk	*Pseudomonas*	+++	++	-
S_1	Sterilized Skim Milk	*Bacillus*	+++	+	-
S_2	Sterilized Skim Milk	*Bacillus*	-	++	+
S_3	Sterilized Skim Milk	*Bacillus*	++	-	+++
S_4	Sterilized Skim Milk	*Bacillus*	+++	++	++
S_5	Sterilized Skim Milk	*Bacillus*	++	+*	+
S_6	Sterilized Skim Milk	*Bacillus*	++	+	-
S_7	Sterilized Skim Milk	*Bacillus*	++	+	-
S_8	Sterilized Skim Milk	*Bacillus*	++++	++	+
S_9	Sterilized Skim Milk	*Micrococcus*	+	-	-
S_{10}	Sterilized Skim Milk	*Bacillus*	+++	+	-
W_1	Tap Water	*Chromobacterium*	++	++	+
W_5	Tap Water	*Chromobacterium*	+++	++	+

W_2	Tap Water	*Pseudomonas*	++	+	+
W_3	Tap Water	*Pseudomonas*	++	+	+
W_4	Tap Water	*Pseudomonas*	++++	+++	++
W_6	Tap Water	*Flavobacterium*	+	+	-
W_7	Tap Water	*Bacillus*	-	+	-
W_8	Tap Water	*Micrococcus*	+	+	-
W_9	Tap Water	*Micrococcus*	-	+	-
W_{10}	Tap Water	*Bacillus*	+	+	-
W_{11}	Tap Water	*Escherichia*	-	-	-
W_{12}	Tap Water	*Pseudomonas*	++	++	-
A_1	Alkaline water washings	*Bacillus*	++	+	++++
A_2	Alkaline water washings	*Bacillus*	++	-	++++
A_3	Alkaline water washings	*Bacillus*	++	+	+++
A_4	Alkaline water washings	*Bacillus*	++	-	+++
A_5	Alkaline water washings	*Bacillus*	++	+	+

* The Isolate showed remarkable lipase activity within 24 h of incubation at 37°C.

APPENDIX IV

Morphological and biochemical characteristics of the isolates P12 and S4

Characteristics	Isolate P12	Isolate S4
Colony morphology	Small, circular, entire, smooth ,convex, shining, translucent colonies with a bluish tinge	Large, circular, rough, raised, dry and opaque colonies
Grams reaction	Negative	Positive
Microscopic appearance	Small thin rods	Bacilli in chains with central / subterminal ellipsoidal spores with no swelling of the body
Oxidase	+	-
Catalase	+	+
Motility	+	+
Pigment production	+ (bluish diffusible green)	-
Hugh leifsons media	Oxidation positive and Fermentation negative	Oxidation and fermentation positive
Growth at 5°C	+	+
Growth at 42°C	+	+
Glucose	+	+
Lactose	-	-
Salicin	-	+
Sucrose	-	+
ONPG	-	+
Nitrate reduction	+	+
Citrate	+	+

Urease	+	+
Indole	-	-
Voges Proskauer	-	+
Casein hydrolysis	+	+
Gelatin hydrolysis	+	-
Starch hydrolysis	-	+
Lecithinase enzyme	+	+

DETERGENT POTENTIAL OF ENZYMES OF DAIRY MICROFLORA AND THEIR EFFECT ON THE SHELF LIFE OF MILK PRODUCTS

BEENA. A. K.

Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy
in
Veterinary and Animal Sciences

Faculty of Veterinary and Animal Sciences
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ABSTRACT

A study was conducted to assess the detergent potential of a spoilage protease enzyme obtained from microflora of dairy plant environment. An attempt was also made to study the impact of selected enzyme producers on the shelf life of curd (dahi) and sterilised skim milk. A total of 71 bacterial isolates obtained from dairy environment were screened for their ability to produce spoilage enzymes like proteases lipases and lecithinases. From this *Pseudomonas aeruginosa* (P12) isolated from pasteurised milk and *Bacillus cereus* (S4) isolated from sterilized skim milk were selected for further work by taking into consideration their spoilage potential.

The influence of spoilage enzymes on selected physico-chemical characteristics of curd (dahi) and sterilized skim milk was evaluated by preparing the products from milk precultured with isolate P12 and S4. In general, proteolysis of milk was found to have an adverse effect on the quality of products. The stimulatory effect of proteolytic products of P12 and S4 on curd starters was evident from the higher values of acidity, firmness and syneresis in treated curd. The spoilage enzymes adversely affected the overall quality and shelf life of curd. In treated sterilised milk, tyrosine and NPN values were highly elevated. A linear correlation was found to exist between off-flavour and proteolysis. Curd and sterilised skim milk prepared from milk precultured with proteolytic organism were significantly different from that of control.

The possibility of exploiting an alkaline protease from spoilage organism in dairy plant sanitation was also looked into. Environmental conditions for the production of alkaline protease by a psychrotrophic strain of *Bacillus cereus* (S4) was optimised in whey based medium. The protease used in this trial preferred an alkaline medium to remain stable. The enzyme was found to be stable over a wide temperature range of -10°C to 80°C and a pH range of 7.0 to 12.0. The metal ions Ca^{++}, Mg^{++}, Zn^{++} and Hg^{++} enhanced the enzyme activity. Lack of

inhibition by Hg^{++} suggested lack of disulphide bonds in the active site of enzyme. Significant inhibition of activity by serine inhibitors indicated an essential serine residue in the active site of enzyme. The deleterious effect of EDTA on enzyme activity showed the supportive role of divalent cations. Marked residual activity on treatment with β-mercaptoethanol indicated the absence of cysteine residue for the enzyme. Enhancement of protease activity in the presence of surfactants and stability in the presence of H_2O_2 signified its potential to be used as detergent additive. Qualitative assessment of cleaning efficiency of inbuilt formulation substantiated the superiority of enzyme based formulations. Ammonium sulphate fractionation, dialysis and gel filtation using seralose 4B and Seralose 6B were effective in purifying the protease preparation by 141.31 fold. The purified protease was found to be a homogenous preparation of molecular weight of 50.5 kDa as determined by SDS PAGE.