COMPARATIVE STUDY ON PHYSICO-CHEMICAL AND MICROBIOLOGICAL PROPERTIES OF SOFT CHEESE PREPARED BY USING CRUDE AANK (*Calotropis gigantea*) AND JACKFRUIT (*Artocarpus heterophyllus*) PROTEASES

by

Ashish Koirala

Department of Food Technology

Central Campus of Technology

Institute of Science and Technology

Tribhuvan University, Nepal

2021

Comparative Study on Physico-Chemical and Microbiological Properties of Soft Cheese Prepared by Using Crude Aank (*Calotropis gigantea)* and Jackfruit (*Artocarpus heterophyllus*) Proteases

A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfilment of the requirements for the degree of B. Tech. in Food Technology

by

Ashish Koirala

Department of Food Technology

Central Campus of Technology

Institute of Science and Technology

Tribhuvan University, Nepal

2021

Tribhuvan University

Institute of Science and Technology

Department of Food Technology

Central Campus of Technology, Dharan

Approval Letter

This ***dissertation*** entitled ***Comparative Study on Physico-Chemical and Microbiological Properties of Soft Cheese Prepared by Using Crude Aank (Calotropis gigantea) and Jackfruit (Artocarpus heterophyllus) Proteases*** presented by **Ashish Koirala** has been accepted as the partial fulfilment of the requirement for the **B. Tech. degree** in **Food Technology.**

Dissertation Committee

1. **Head of Department** _____

   (Mr. Basanta Kumar Rai, Prof.)

2. **External Examiner** _____

   (Dr. Dhan Bahadur Karki, Prof.)

3. **Supervisor** _____

   (Mr. Bunty Maskey, Asst. Prof.)

4. **Internal Examiner** _____

   (Mr. Ram Sovit Yadav, Asst. Prof.)

2021

Acknowledgements

I would like to thank my supervisor, Asst. Prof. Mr. Bunty Maskey whose, support, guidance, encouragement, recommendations and expertise were invaluable in formulating the research questions and methodology and pushing me to sharpen my thinking and bringing this work to a higher level.

I would like to express gratitude to Assoc. Prof. Dr. Dil Kumar Limbu (Campus Chief, Central Campus of Technology) and Prof. Basanta Kumar Rai (Chairperson, Department of Food Technology, Central Campus of Technology) for providing me with all the facilities that I needed to successfully complete my dissertation.

I would like to acknowledge my senior Mr. Nabindra Kumar Shrestha from whom I have received a great deal of support and assistance throughout the work. Special thanks to my friends; Bibek Shrestha, Subash Sigdel, Diwash Acharya, Abhash Gaire, Shagar Parajuli, Sumitra Shrestha, Shusma Paudel and Sharad Bhattarai and my beloved juniors; Chiranjivi Belbase, Rajiv Guragain, Anup Paudel, Trilochan Pandey, Kshitiz Luitel, Mohit Khadka, Milan Bolakhe, and Prakash Sapkota from Central Campus of Technology for their help and support during the completion of this work. I would also like to thank all those who have helped me directly or indirectly throughout the course of the work.

I would also like to express my sincere thanks to my teachers, staffs of library and laboratory for their direct and indirect co-operation and suggestion.

Finally, my deep and sincere gratitude to my family for their continuous and unparalleled love, help and support. I am grateful to my sister for always being there for me as a friend. I am forever indebted to my parents for giving me the opportunities and experiences that have made me who I am.

Date of submission: March, 2021

(Ashish Koirala)

Abstract

The main objective of this dissertation work was to perform comparative study on the physico-chemical and microbiological properties of soft cheese prepared by using crude aank (*Calotropis gigantea*) and jackfruit (*Artocarpus heterophyllus)* proteases. Milk clotting activity (MCA), proteolytic activity (PA) and protein content of the extracted proteases were measured. Optimum conditions of temperature and pH of milk for maximum MCA and minimum time of coagulation (TOC) were determined by response surface methodology. The crude plant proteases were used as milk coagulants in soft cheese preparation at optimized conditions. The physicochemical and microbiological properties of prepared cheeses were compared with rennet cheese.

Numerical optimization study revealed that the optimum condition for milk clotting was found to be 45°C and pH 6.5 for crude aank protease, as well as 65°C and pH 6.5 for crude jackfruit protease. The optimized TOC and MCA for crude aank protease were found to be 40 s and 300 units respectively, while; TOC and MCA for crude jackfruit protease were found to be 108 s and 111.11 units respectively. The physico-chemical analysis showed non-significant ($P>0.05$) differences in protein content among all samples. Significantly ($P<0.05$) higher level of moisture and ash content, and lower level of fat and calcium content were observed in cheese prepared by crude aank and jackfruit proteases when compared to rennet cheese. Acidity of cheese made using crude jackfruit protease was significantly different ($P<0.05$) with rennet cheese, while no significant difference ($P<0.05$) in acidity was observed between cheeses made using jackfruit protease and rennet. Microbiological analysis revealed significantly ($P<0.05$) higher level of total plate count in cheese made using crude aank protease while significantly ($P<0.05$) lower level of TPC was seen in cheese made using jackfruit protease when compared to rennet cheese. There was no significant difference ($P>0.05$) in yeast and mold count among all the cheese samples.

Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
BSE	Bovine spongiform Encephalopathy
CBS	Customized Brewing Solutions
CCP	Colloidal Calcium Phosphate
DANIDA	Danish International Development Agency
DDC	Dairy Development Corporation
DNA	*Deoxyribonucleic acid*
FAO	Food and Agriculture Organization
FDB	Fat on Dry Basis
FDM	Fat on Dry Matter
HTST	High Temperature Short Time
IDF	International Dairy Federation

Abbreviation	Full form
LSD	Least Significant Difference
LTLT	Low Temperature Long Time
MCA	Milk Clotting Activity
MFFB	Moisture on Fat Free Basis
MFFS	Moisture in Fat Free Substance
MT	Metric Tonnes
NDDB	Nepal Dairy Development Board
NSLAB	Non-Starter Lactic Acid Bacteria
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PPM	Parts Per Million
RCT	Rennet Coagulation Time
RO	Reverse Osmosis
SCN	Thiocyanate

Abbreviation	Full form
TOC	Time of Coagulation
TPC	Total Plate Count
UF	Ultrafiltration
VRBA	Violet Red Bile Agar
WFFS	Water in Fat Free Substance
WHO	World Health Organization

Part I

Introduction

1.1 General introduction

Cheese is a fermented milk based solid dairy product which is produced by coagulating milk with the help of calf rennet or suitable coagulating agents and partly draining whey. It features a variety of flavours and is produced all over the world (McSweeney *et al.*, 2017). Cheese can be prepared from whole milk, partially skimmed milk or skimmed milk, all of which can be obtained from cow, buffalo, camel, goat, reindeer, horse and donkey milk in addition with milk powder (Fox and McSweeney,2017). Cheese is a highly nutritious fermented product. When compared to whole milk (3-4% protein and fat), it contains 30-40% protein and fat along with other minerals and fat-soluble vitamins. Fewer varieties of cheese are available in Nepal compared to the European and American regions. Due to increased awareness of the health benefits of cheese and expansion of cattle farming in Nepal, the demand and supply of cheese is in increasing trend. Cheese manufacture can be an interesting method to overcome the milk holiday in the surplus season (Sharma Khanal *et al.*, 2019).

Proteolytic enzymes that are used in the coagulation of milk can be obtained from different sources, such as different animal species (e.g., pig, cow, and chicken pepsin), microbial proteases (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and plant proteases. Some plants reported to have proteases that yields good milk clotting activity are *Cucumis melo* (Uchikoba and Kaneda, 1996); *Oryza sativa* (Asakura *et al.*, 1997); *Lactuca sativa* (Lo Piero *et al.*, 2002); *Cynara humilis* (Esteves *et al.*, 2003); *Helianthus annus* (Egito *et al.*, 2007); *Ficus racemose* (Devaraj *et al*., 2008); *Solanum dubium* (Ahmed *et al.*, 2009); *Bromelia hieronymi* (Bruno *et al.*, 2010); *Ficus religiosa* (Kumari *et al.*, 2010); *Euphorbia neriifolia* (Yadav *et al.*, 2011); *Streblus asper* (Tripathi *et al.*, 2011); *Onopordum acanthium* (Brutti *et al.*, 2012); *Cirsium vulgare* (Lufrano *et al.*, 2012); *Moringa oleifera* (Pontual *et al.*, 2012); *Zingiber officinale* (Gagaoua *et al.*, 2015; Hashim *et al.*, 2011); *Withania coagulans* (Pezeshki *et al.*, 2011; Salehi *et al.*, 2017) and *Carica papaya* (Maskey and Shrestha, 2020).

Much research has been aimed at discovering a plant-based milk clotting enzyme that would satisfactorily replace calf rennet in cheese production (Duarte *et al.*, 2009). Hydrolysis of κ-casein can be achieved by plant extracts which leads to curd formation. Plant enzymes are also the main enzymes responsible for β-casein hydrolysis (Roseiro *et al.*, 2003). Plant proteases can be used as milk coagulants for producing cheeses aimed at lacto-vegetarian consumers and ecological markets. The use of these plant proteases as milk coagulants is interesting because of its natural source (Gomez *et al.*, 2001). They can also be used for the manufacture of Kosher and Halal products (Galan *et al.*, 2008). Recent publications on new proteolytic enzymes from vegetable origin for milk clotting (Lopes *et al.*, 1998) revealed that vegetable coagulants are a subject with growing interest for the dairy technology (Roseiro *et al.*, 2003).

1.2 Statement of the problem

Rennet is considered a famous exogenous enzyme used in dairy processing, and has been used for cheese production since 6000 B.C.(Abada, 2019). The two enzymes (chymosin and pepsin) present in the animal rennet breaks the Met_{105}-Phe_{106} bond of the κ-casein present on the surface of the casein micelles (Chazzara *et al.*, 2007). The cheese production increased by a factor of approximately 3.5 since 1961 but the rennet supply decreased due to the limited availability of ruminant stomachs (Jacob *et al.*, 2011). Plant proteases have been used as milk coagulants in cheesemaking for a long time either as crude extracts or in purified form and these coagulants can be used as an alternative to calf rennet (Shah *et al.*, 2014). Various factors such as high price of rennet, religious concerns, diet, or ban on recombinant calf rennet has encouraged the search for alternative milk-clotting sources (Roseiro *et al.*, 2003).

The increasing consumption of cheese and the decreasing number of calves slaughtered led to an increase in the price of calf rennet, to a shortage in rennet, and to a search for alternative milk coagulants (Fox and Cogan, 2004). This problem raised a question in the cheese making industry about whether they could find a substitute to the rennet. To solve this problem, a suitable cheap non-rennet coagulating agent has to be identified which could benefit cheese makers as well as consumers.

1.3 Objectives

The objectives of the research can be divided into two parts:

1.3.1 General objective

The general objective of the dissertation work is to perform comparative study on the physico-chemical and microbiological properties of soft cheese prepared by using crude aank (*Calotropis gigantea*) and jackfruit (*Artocarpus heterophyllus)* proteases.

1.3.2 Specific objectives

- To extract the crude proteolytic enzymes from latex of aank (*Calotropis gigantea*) and jackfruit (*Artocarpus heterophyllus*).
- To determine the milk clotting activity (MCA), proteolytic activity (PA) and protein content of the enzyme from plant proteases.
- To find out the optimized conditions of temperature and pH of the crude proteases for maximum MCA and minimum TOC.
- To prepare soft cheese by using the proteases as milk coagulants in pre-optimized conditions.
- To compare the physicochemical and microbiological properties of soft cheeses.

1.4 Significance of the study

Cheese is a nutritious and tasty milk product prepared from the coagulation of casein with fat by the action of proteolytic enzyme (Cavalli *et al.*, 2005). Traditionally, calf rennet has been the rennet of choice for the production of various cheese variety (Düsterhöft *et al.*, 2017). Increase in production of cheese with reduced supply of calf's stomach has caused inadequate supply of rennet for many years (Fox and Cogan, 2004). Steps involved in the extraction and purification of rennet from tissue of animal stomach are very complex. This causes rennet to be very expensive. Major causes for increase in demand for alternative sources of milk coagulants are

limited supply of rennet and calf diseases like bovine spongiform encephalopathy (BSE) (Cavalcanti *et al.*, 2004). Calf rennet, which consists primarily of chymosin, has traditionally been used in cheese making, but a growing cheese industry, combined with a limited supply of calf rennet, has led to the search for calf rennet substitute. Substitute for rennet can originate from animal sources (e.g. porcine pepsin, bovine pepsin and chicken pepsin), microbial sources or plant sources (Sousa *et al.*, 2001).

The present study is conducted to investigate the possible source of proteolytic enzymes from plant sources for the coagulation of milk. Proteases from *Calotropis gigantea* and *Artocarpus heterophyllus* possesses promising industrial application in terms of milk-clotting activity. Therefore, the present work will be solely based on the comparison of the plant-based enzymes in the preparation of cheese taking an account on the consumer acceptability of the product.

1.5 Limitations of the study

Some of the hurdles that came during the thesis are listed as follows:

- Purification of the crude enzyme was not performed.
- Texture evaluation of the product was not carried out.
- Casein hydrolysis pattern was not studied.

Part II

Literature review

2.1 Introduction to cheese technology

Cheese manufacture is essentially a process of dehydration and acidification where the fat and protein (casein) of milk are concentrated between 6 to12 folds, and the pH is reduced from about 6.6 in milk to between 4.6 to 5.4 in freshly made curd. While the manufacturing protocol differs markedly with variety, the basic manufacturing steps common to all varieties are coagulation, acidification, dehydration, moulding/shaping and salting. Once the curd has been through the five basic manufacturing steps, it is referred to as cheese. Cheeses may be consumed fresh, as in the case of fresh cheeses (e.g. Mozzarella, Quark, Cottage cheese), or may be stored and ripened for periods ranging from 1 week to 2 years depending on the variety (e.g. Cheddar, Gouda, Parmesan) (Guinee and O'Kennedy, 2007)

2.2 Cheese production

The production of cheese has been growing continuously in the world as a whole for more than twenty years (about 4% per annum) – just under 6 million metric tons in 1961, 7.6 million in 1970-71, 12.3 million in 1984 and 14.65 million in 1994 (Farkye, 2004). World cheese production in 2015, 2016, 2017, 2018, 2019 and 2020 were 19.52, 19.81, 20.44, 20.72, 20.98 and 21.22 million MT respectively. In 2020, the European Union was the top producer of cheese worldwide, with a production volume of around 10.35 million metric tons of cheese (Shahbandeh, 2021).

2.2.1 Cheese production- a Nepalese scenario

The production of cheese started in Nepal from around 1953/54 when a public-sector cheese factory was set up over the mountain range of Langtang. Warner Schulthess a Swiss nationale, senior specialist from FAO, and Gauri Pd. Sharma were the first persons to establish cheese industry in Nepal (Thapa, 2006).

The production of cheese has not significantly improved both qualitatively and quantitatively, despite its history of about 47 years. DANIDA has been playing a significant role in promoting the cheese industry in recent years. In the year 2000, Nepal produces some 350 MT of cheese. Nepalese cheese generated $525,000 in foreign exchange in 1994. It may not be very impressive, but the figures are nonetheless promising. Demand for cheese is growing gradually. The annual requirement for cheese in Nepal is around 800 MT, according to a rather conservative estimate. By importing them from foreign countries, Nepal has fulfilled the demand for cheese. These cheeses are commonly sold at rates that are between 5-10 times the price of Nepalese cheese. For Nepal, cheesemaking may also be a very significant economic practice (Acharya, 2010).

Six yak cheese factories are run in four districts (Ramechhap, Dolakha, Solukhumbu, and Rasuwa), four Kanchan Cheese factories in two districts (Illam and Panchthar), and one buffalo-milk cheese factories at Nagarkot (Kavrepalanchowk). All of these factories are operated by DDC. At least 10 private Yak cheese factories operated alongside the DDC cheese plants until 1995 (Thapa, 2006).

Cheese is not a very popular food item in the Nepalese in general. Many are not familiar to the flavor and taste of cheese. In Nepal's food market, it is a comparatively expensive dairy commodity. Increased understanding of health requirements and hygienic products has increased demand for pasteurized milk and milk products, including cheese in general. In the future, this market is bound to rise more (Pradhan, 2000).

2.3 Quality of milk in relation to cheese making

The quality of milk in cheesemaking can be considered good if it fulfills the requirements of manufacturer and consumer (Peri, 2006). High quality dairy products are produced from good quality raw milk (Lasztity, 2009). Milk quality includes, features such as safety, purity, sensory properties, chemical composition and physical properties. A cheese manufacture can measure these aspects by microbiological, chemical and physical analysis (Law and Tamine, 2010).

The quality of cheese is mainly affected by the quality of milk. The milk quality for cheese production is influenced by factors such as milk composition, the levels of indigenous contaminants, enzyme activity, somatic cell count and levels of chemical residues. Casein and fat content have a role in determining cheese yield potential of milk. The yield of cheese is directly related to the content of casein in milk. Fat influences texture flavour, mouthfeel and consistency of cheese whereas the casein fraction of cheese is responsible for curd firmness, syneresis rate and moisture retention rate of cheese. Milk with high composition of casein and fat, low levels of enzymes and low somatic cell count is generally good quality milk for cheese manufacturing (Nassar and Emirie, 2015). In the following groups, the quality criteria of milk that are relevant to cheesemaking are discussed.

1. Chemical composition of milk
2. Abnormal milk
3. Changes in milk after production.
4. Inhibitory substances and other residues.
5. Microbiological quality of milk.

2.3.1 Chemical composition of milk

Milk composition has considerable effect on yield and composition of the cheese. The milk may vary substantially in composition, especially if it originates from only a few cows, as in farmhouse cheesemaking, or with the season if most cows calve at about the same time. However, cheese of satisfactory quality can be made of almost all milk, providing that the manufacturing process is adjusted. In most cases, the milk is standardized so as to yield the desired fat content in the dry matter of the cheese (Walstra *et al.*, 2006).

2.3.1.1 Milk protein (casein) and its effect

In typical bovine milk there is approximately 3.6g of crude protein per 100 g of milk, of which about 95% is true protein (78% casein and 17% whey proteins) and the remainder is non-protein nitrogen compounds (Davies and Law, 1983). Casein is a phosphoprotein and accounts for about

80% of cow milk protein. Casein consists of four distinct protein types; α_{s1}-, α_{s2}-, β- and κ-casein which account for 40, 11, 37 and 125 of total casein (KC and Rai, 2007). The proteins remaining in milk serum after the caseins have been removed by acidification to pH 4.6 are termed whey proteins (Patel *et al.*, 1990). Whey protein also known as serum protein accounts for 20% milk protein and contains β-lactoglobulin (8.5%), lactalbumin (5.1%), immunoglobulins (1.7%), and serum albumins (KC and Rai, 2007).

Casein is the most important protein fraction for cheesemaking and accounts for nearly 80% of total milk protein (Thomas, 1980). In traditional cheddar cheesemaking only milk casein incorporates into the cheese curd while the other proteins are lost in the whey. Cheese yield is directly related to the total casein and gat concentrations of milk (Amenu, 2004). According to Guinee *et al.* (2001), a 0.1% reduction in the total casein concentration reduces cheddar cheese yield potential by 0.5 kg/100 kg milk.

2.3.1.2 Milk fat

The fat content of the milk and the fatty acid composition of the lipids can vary considerably as a result of changes in factors like breed of cow, diet, and stage of lactation. The fat content can vary from about 3% to 6%, but typically is in the range 3.5 to 4.7%. Milk lipids are very important as they confer distinctive nutritional, textural and organoleptic properties in dairy products, such as cream, butter, whole milk powder and cheese (MacGibbon and Taylor, 2006).

The level of fat influences several aspects of cheese, including composition, biochemistry, microstructure, yield, rheological and textural properties and cooking properties. For a given fat content, the type of fat and the state of the fat has a major impact on the rheology, flavor and cooking properties of the cheese. Fat also contributes to flavor directly and indirectly through lipolysis (Guinee and McSweeney, 2006). Milk fat contributes directly and indirectly to cheese yield. The direct contribution of fat to cheese yield is clearly reflected by prediction equations, which relate cheese yield to the concentrations, and recoveries, of milk fat and protein (Melili *et al.*, 2002).

2.3.1.3 Milk salt

The common name for milk salts is ‘ash’. A significant proportion of metallic and non-metallic components are included. For example, calcium (Ca^{++}) is a very important component of ash in cheesemaking (Kindstedt and Kosikowski, 1985). Calcium exists in milk in soluble and colloidal forms. The equilibrium between the soluble and colloidal forms is especially dependent on pH, temperature and ionic strength (Wolfschoon-Pombo, 1997). The addition of calcium increases the ionic calcium concentration in milk and reduces the rennet clotting time, enhances the retention of this mineral in the cheeses, improves dry weight retention rate of protein and fat (Solorza and Bell, 1998).

Milk calcium concentration is described to influence cheese texture, which is an important rheological property of cheese quality. The total calcium content of several cheese varieties varies mostly due to differences in the manufacturing pH values (Lee *et al.*, 2005). The calcium content of milk greatly influences the rennet coagulation time, firmness if the curd and together with phosphate is important for the drainage of whey (Kindstedt and Kosikowski, 1985).

2.3.2 Abnormal milk

The general term used to describe any form of milk that varies significantly from normal milk, is abnormal milk. It normally includes mastitis milk, colostrum and late lactation milk. Due to factors or conditions resulting in ‘slow starter’, slow coagulation with rennet and formation of weak curd, abnormal milk is not appropriate for cheesemaking. Abnormal enzyme content may affect both ripening and developing of flavor (De, 2000).

2.3.2.1 Mastitis milk

Due to the altered chemical composition, the technological properties of milk produced by animals with sub-clinical and clinical mastitis are vastly different from those of normal milk. Mastitis milk has alkaline pH, high somatic cell count, presence of antibiotic residue and low calcium and casein contents which slows the growth of starter, delays the renneting action, weakens curd firmness and reduces the cheese yield (De, 2000).

2.3.2.2 Colostrum

Colostrum milk also known as foremilk is the first milk secreted at the time of parturition. It differs from the milk secreted later, by containing more lactalbumin and lactoprotein, and also being rich in antibodies that confer passive immunity to the newborn (Godhia and Patel, 2013). Colostrum milk is undesirable for cheesemaking as the curd lacks elasticity, and shrinkability, retains more moisture and its drying is difficult. It encourages the develoopment of undesirable organism. The presence of somatic cells and natural inhibitors in milk of this type may interfere with the activity of the starter (Bylund, 1995).

2.3.2.3 Late lactation milk

Due to its large sodium and potassium content, late lactated milk is poor for cheese making, resulting in more protein hydrated than normal milk (De, 2000).

2.3.3 Changes in the milk after production

During the period between milk production and the start of cheesemaking, milk is subjected to physical, chemical, bacteriological and organoleptic alterations. Milk may have taints after production, such as feed flavor, weed flavor, etc. that are inherently present in the milk. Thus, milk may show off-flavor development, acid production, lipolysis and proteolysis, changes in casein micelles and salt balance and oxidation after production (Upadhyay, 2003).

2.3.3.1 Changes in casein micelles and salt equilibrium

The practice of long-term retention of cooled raw milk in bulk tanks not only increases the possibility of psychrotrophic growth, but also changes the physicochemical status of milk (Upadhyay, 2003).

Changes in concentration of casein, calcium and phosphorus in the soluble phase of bulk milk stored at 4^{o}C up to 7 days has been shown in the Table 2.1.

Table 2.1 Changes in concentration of casein, calcium and phosphorus in the soluble phase of milk stored at 4°C storage

Days	Casein fraction (mg/ml)			Minerals (ppm)	
	αs_1	β	γ	Ca (ppm)	P (ppm)
0	1.66	1.32	0.87	430	395
1	1.73	1.56	0.96	485	407
2	1.80	1.81	0.98	528	426
3	1.68	1.51	0.96	506	418
4	1.71	1.67	0.90	519	420
5	1.66	1.53	0.88	489	401
6	1.74	1.59	0.93	492	405
7	1.72	1.56	0.86	487	401

Source: Upadhyay (2003)

2.3.4 Inhibitory substances

Raw milk is known to contain inhibitory systems that are natural. Furthermore, residual antibiotics, detergents and sanitizers may also contain milk. The effect on cheese production of the presence of such inhibitory systems is set out in the following sections (Walstra *et al*., 2006).

2.3.4.1 Natural inhibitory systems in milk

Immunoglobulin, lactoferrin, lysozyme and the lactoperoxidase-thiocyanate-hydrogen peroxide system are the natural inhibitory system in raw milk. The presence of such inhibitory systems can influence cheese production, especially when cheese is made from raw milk, as these systems have been found to be active against lactic streptococci, affecting their multiplication and acid production. Under aerobic conditions, certain strains of lactic acid bacteria can produce sufficient hydrogen peroxide and self-inhibitory effects can be observed during cheesemaking (FAO, 1999).

2.3.4.2 Antibiotic residues

Antibiotics may gain access to milk as a result of treatment of animal, usually for mastitis. According to Farkye (2004), the presence of residual antibiotics in milk is highly unsatisfactory from public health point of view. Their presence can also give rise to partial or complete inhibition of cheese starter, which in turn can lead to several faults, slow whey drainage, high moisture in cheese, early and late blowing, weak and pasty body, different types of taints, cracks, open texture and sponginess.

2.3.4.3 Residues of detergents and sanitizers

Production farm, chilling center and dairy factory use detergents and sanitizers for a wide range of applications. When applied by 'Good practice' they cause no residue problems in milk. However, their misuse possesses major problem (Walstra *et al.*, 2006).

2.3.5 Microbiological quality of milk

Raw milk secreted by healthy animal is free of microorganism. Most to the bacteria present in raw milk are contaminants of the outside and gain entrance into the milk from various sources including soil, bedding, manure, feed and milking equipment. Improvement of handling and processing of milk such as developments in closed milking systems, use of bulk tanks to store and transport raw milk and changes in refrigeration systems have resulted in shifts in the microflora and predominantly Gram-positive, acid-producing bacteria to Gram-negative, psychrotrophic micro-organism, mainly Pseudomonas species (Giffel and Wells-Bennik, 2010).

For cheesemaking, a low count milk, free from fault producing microorganism such as the coliforms, yeast, clostridia and certain species of lactobacilli, *Propionibacterium* and Micrococci capable of growing in milk acid condition and producing faults like gassiness and discoloration in cheese, is highly desirable. In addition, milk must be free from pathogens and also from organism which produces toxins involved in food poisoning (Upadhyay, 2003).

2.4 Pretreatments of milk for cheesemaking

Pretreatments have profound impact on production schedule of cheese, the effectiveness of cheese production, physico-chemical, microbiological and organoleptic characteristics of cheese and shelf life (Walstra *et al.*, 2006).

The various treatments employed are

- Chilling and cold storage
- Lactoperoxidase/thiocyanate/H_2O_2 treatment
- Thermization
- Bactofugation
- Microfiltration
- Pasteurization
- Standardization
- Homogenization
- Lactose hydrolysis
- Concentration

2.4.1 Chilling and cold storage

In advanced milk countries, most raw milk is cooled to 4°C and stored on the farm in refrigeration bulk tanks and in insulated or refrigerated storage tanks in cheese plants prior to its conversion into cheese. Raw milk tends to be older and colder for a much longer period of time than when milk is handled in cans. Such a long-term holding practice of cooled raw and/or processed milk not only increases the likelihood of psychrotrophic development, but also changes the physico-chemical status of milk components, particularly casein and minerals. In

fact, this has been shown to change milk behaviour during subsequent cheesemaking (Upadhyay, 2003).

2.4.2 Lactoperoxidase treatment

Lactoperoxidase is an enzyme that occurs in raw milk, colostrum, saliva and other biological secretion. Bovine milk naturally contains 10 to 60 mg/l of milk. The enzyme reacts with thiocyanate (SCN^-) in the presence of hydrogen peroxide and forms antimicrobial compounds. This is termed as the lactoperoxidase system (LPS). Fresh milk contains 1 to 10 mg of thiocyanate per liter, which is not sufficient to activate the LPS. Hydrogen peroxide, the third component of the LPS, is not present in fresh milk due to the action of natural catalase, peroxidase or superoxide dismutase. Approximately, 8 to 10 mg hydrogen peroxide per liter is required for LPS. In the LPS reaction, thiocyanate is oxidized to the antimicrobial hypothiocyanite ($OSCN^-$) which exist in equilibrium with hypothiocyanous acid (Davidson and Zivanovic, 2003).

The lactoperoxidase method can be used for the preservation of raw milk as an alternative to chilling and to solve the problem posed by psychrotrophs in stored milk. Unless lactoperoxidase is inactivated by appropriate heat treatment (e.g. 78°C/15 s), the ability of starter organisms to produce H_2O_2 results in the reactivation of the lactoperoxidase system. However, such extreme heat treatment is not widely used for cheese milk, so it is expected that lactoperoxidase is still present in milk treated with lactoperoxidase. There is therefore a potential for the reactivation of the lactoperoxidase system, leading to problems in the production of cheese due to the suppression of starter activity (FAO, 1999).

2.4.3 Thermization

The thermization process is a subpasteurization heat treatment of milk at 62-65ºC for 10-20 s, followed by refrigeration. It is used as a prepasteurization treatment of raw milk to safeguard milk quality during prolonged storage in insulated silos. The process is also used as a postpasteurization treatment of dairy products. Research has shown that this process effectively reduces both the total and psychrotrophic bacterial counts, enabling thermized milk to be stored

for up to 3 days longer at 8ºC. In this way, detectable sensory changes and the concomitant decrease on shelf-life are limited (Swart *et al.*, 2003)

2.4.4 Bactofugation

Bactofugation is a process which is usually applied to remove spores from products that are low pastrurized. This may involve removal of spores of *Bacillus* spp. or *Clostridium* and related species from cheese milk. Spores are quite small, but the density difference with plasma is larger than that of bacteria, and at separation temperatures of 60 to 65ºC, a substantial proportion can be removed, generally 90 to 95%. By using two bactofuges in series, a reduction by over 99% can generally be attained. It should be noted that bactofugation is by no means equivalent to sterilization: the product still contains heat-resistance bacteria and a small number of spores. The process may serve to give pasteurized beverage milk a longer shelf life or to obtain cheese that is not subject to the defect of late blowing without inducing unfavorable changer in the milk (Walstra *et al*., 2006).

2.4.5 Microfiltration

Without the application of heat, microfiltration effectively removes the indigenous microorganisms from milk. Microfiltration seems to offer the ideal approach for evaluating the contribution of non-starter lactic acid bacteria (NSLAB) to cheese maturation without complicating changes in milk protein or enzymes induced by heat, except those on the membrane of fat globules. Microfiltration was introduced some years ago in the so-called 'Bactocatch' process as an alternative to bactofugation (Upadhyay, 2003).

2.4.6 Standardization

Standardization of cheese milk is necessary due to variation in the composition of milk and to ensure that the final product meets the legal requirements. Fat and protein are the two components that constitute the main body of cheese. Therefore, it is reasonable to standardize the milk at the ratio of fat/protein, which gives good quality of cheese and even quality of production during all seasons. The adjustment of the casein/fat ratio (C/F) in cheese milk in the 0.69:1 to 0.70:1 range produces a cheese with better body-texture characteristics (Scott, 1986).

2.4.7 Pasteurization

Pasteurization in cheese milk serves to kill pathogenic and harmful organisms. A more intense pasteurization causes part of the serum proteins to become insoluble, leading to an increase in cheese yield, it decreases the renetability and the syneresis, it inactivates xanthine oxidase, thereby increasing the risk of bacterial spoilage (Walstra *et al.*, 2006).

Pasteurization temperature-time combination is dependent on the cheese variety and mode of pasteurization employed. According to Scott (1986) three systems of pasteurization practiced in most counties are:

1. Flash heating (no holding) to temperature of 75-95℃
2. HTST: 71-75℃ /14-40 s
3. LTLT: 61-65℃ / 20-40 min.

Salient effects of heat treatment of cheese milk outlined are:

1. This allows the cheese manufacturer to maintain higher quality and uniformity with the guaranteed safety of public health.
2. As a consequence of eliminating the antagonistic effect of contaminant organisms, it allows better growth of starter bacteria.
3. Results of increased cheese yield due to higher retention of moisture and higher total recovery of solids due to denaturation of whey proteins with improved concomitant incorporation in the curd matrix along with fat (De, 2000).

The rennet coagulation time increases with increasing intensity of heat treatment, producing soft curd, losing elasticity and coherence due to the heat mediated complex of β-lactoglobulin and κ-casein, altered casein micelle charges and surface properties, and change in the ionic and colloidal milk salt system. To overcome the adverse effect, acidification, a slightly higher setting temperature and calcium chloride addition can be used (Barbano, 1999).

However, overheating of cheese milk might cause higher retention of milk coagulant in the cheese curd which contribute to bitterness defect in cheese. Particles of bacteriophage, antibiotics, lysozyme present in raw milk are not destroyed, but after pasteurization this affects the growth of the starter during cheese making (Upadhyay, 2003).

2.4.8 Homogenization

In manufacturing most of the cheese verities such as Cream, Blue and Soft varieties, homogenization is not generally practiced. Homogenization of milk is done for:

- Reducing the loss of fat in whey (incorporation of small fat globules in the curd) and thus increasing the yield of cheese.
- Improving the texture of cheeses by making the curd smoother and finer, ensuring greater retention of moisture.
- Producing 'white cheese' from cows' milk similar to that of goat milk cheese (Banks, 1992).

2.4.9 Lactose hydrolysis

It has been reported that the use of lactose hydrolyzed milk for cheesemaking has the following effects on production:

- Faster production of acid as growth of starter is stimulated.
- Before renneting and cheddaring, milk ripening time could be reduced by 25-30% and 20-25%, respectively.
- Increase the moisture content of cheese slightly.
- Whey obtained in the process of cheese making could be used for syrup production as it contains increased glucose and galactose levels (Upadhyay, 2003).

2.4.10 Concentration

The production of cheese is essentially a controlled dewatering process in which the milk fat and casein are concentrated around 6-12 times, depending on the variety of cheese produced. The concentration of milk for cheesemaking can be achieved by:

- Thermal evaporation under vacuum.
- Membrane processing (UF/RO).
- Addition of milk powder, condensed whey or dried whey (Bylund, 1995).

Ultrafiltration is the most commonly used concentration process in the production of soft cheese varieties in general. Three direct benefits of ultrafiltration in cheese milk, along with the reduction in production time, labor, transport and storage, are:

- Increased cheese production by integrating the whey protein into the cheese.
- Increased capacity of the plant due to the reduction in the amount of fluid to be processed.
- Reduced utilization of rennet salt and color (Kosikowski, 1982).

2.5 Additives in cheese milk

The starter culture and rennet are the essential additives of the cheese making method. Other components, such as calcium chloride ($CaCl_2$) and saltpeter and acidulants, may sometimes need to be provided (Bylund, 1995).

2.5.1 Calcium salt

In the secondary phase of rennet action, calcium plays an important role. Therefore, for successful coagulation, the calcium balance between soluble, colloidal and complex is very important. The lack of calcium balance and disturbance is due to chilling and long-term cold

storage at 4-5 ° C, causing β-casein dissociation, severe milk heating during pasteurization, and water-based milk dilution (Lucey, 1993).

In various forms, such as calcium chloride ($CaCl_2$), calcium can be added to milk up to 0.02 percent, dibasic calcium phosphate is recommended for use with pepsin rennet (<0.01 percent), lime water and calcium lactate (Upadhyay, 2003).

It is believed that the beneficial effects of $CaCl_2$ addition on Rennet Coagulation Time (RCT) and gel strength are due to an increase in Ca^{++}, an increase in Colloidal Calcium Phosphate (CCP) and a decrease in pH (De, 2000). In improving the melt and other associated functional properties of mozzarella cheese, micellar calcium plays an important role (Joshi *et al.,* 2004).

2.5.2 Cheese colour

In order to give cheese an attractive and appetizing appearance, it is common practice to add extra color to pale colored milk. Two important colors are found in milk but lost in whey are riboflavin and carotenoids. The color of annatto cheese of vegetable origin is widely used at a rate of 88 g/1000 kg (Kosikowski, 1982).

2.5.3 Inhibitory salts

"Swiss inhibitory salts (Saltpeter) are applied to milk in the manufacture of less acidic cheese such as Edam, Gouda, to avoid the development of gas-producing species such as coliform/aerogenes classes of bacteria responsible for "early blowing" defect in cheese and butyric acid bacteria responsible for "late blowing" defect in cheese. To inhibit the growth of spores, a concentration of 10 to 100 ppm of nitrite or 2 to 5 ppm of nitrate is sufficient. The key drawback of the use of nitrate in cheesemaking is color defects and potentially carcinogenic effects. Lysozyme has been introduced to suppress clostridia species as a substitute for saltpeter (Farkye, 2004).

2.5.4 Starters

For cheesemaking, different starter types are used. The starters used in cheese starter cultures perform functions such as lactose mechanism, producing lactic acid, which is central to

conventional cheese manufacture and also minimizes the growth of spoilage and pathogenic organisms in the cheese; production of a wide range of enzymes and metabolic products that play an active role in generation a flavor profile and enhance organoleptic properties during maturation of the cheese; production of other antimicrobial substances that reduce the risk of survival and proliferation of pathogens and enhancement of the health promoting properties of the cheese. These properties have a significant impact on cheesemaking and flavor development. For instance, the rate and amount of lactic acid produced during cheesemaking will determine the amount of moisture lost, the final pH, and residual lactose in the curd, which in turn will have a strong influence on the maturation rate and the final flavor profile of the cheese (Broome *et al.*, 2011).

Streptococci, lactococci, leuconostocs and lactobacilli are lactic acid bacteria used as starters in cheesemaking. As combined cultures, or as single strain cultures, or as mixtures of single strain cultures, selected species of these genera are used. For the production of high-temperature cooked cheese varieties (e.g. Swiss and Parmesan), thermophilic starters (optimum temperature 37-45°C) are used where the starter must be able to withstand a high cooking temperature of 45°C and grow at relatively high temperatures (Cogan *et al*., 1997).

2.5.5 Salt

Salt in cheese serves two major roles: preservation and contributes directly to flavour and quality. The preservation action of NaCl is due to its depressing effect on the water activity of the cheese and the increment of osmotic pressure of aqueous phase causing dehydration of bacterial cells, killing them or, at least, preventing their growth. NaCl contributes directly saltiness in cheese, a flavour that is genereally highly appreciated. The flavour if salt-free cheese is insipid and watery. NaCl contributes indirectly to flavour of cheese by controlling influence on microbial and enzymatic activities, which in turn, influence lactose metabolism, cheese pH, degradation of fats, and casein, and the formation of flavour compounds such as peptides, free amino acids and free fatty acids (Guinee, 2007).

2.5.6 Rennet

Animal rennet is a milk clotting enzyme isolated from calf stomachs. The major component of rennet is chymosin but in commercial preparations of rennet other proteases, typically bovine pepsin, are found in varying concentrations. The process of rennet isolation and formulation was commercialized more than a century ago, but even today animal rennet is still an important enzyme in the dairy industry. This is despite the fact that animal rennet prices swing a lot as a result of the swing in raw material prices and are considerably higher than alternative milk clotting enzymes (Hellmuth and Van Den Brink, 2013). Rennet is added to milk shortly after the point when the starter culture is added or more commonly during the fermentation process as long as the pH is not too low (Lucey, 2004) .

The activity of the rennet is defined as the number of milliliters of milk that can be coagulated at a temperature of 35°C by one milliliter of liquid rennet within 40 min. Calf rennet stability is maximum at pH, 5.5-5.9 and photo-oxidation results in chymosin inactivation (Upadhyay, 2003).

2.6 Retention figures

At the beginning, the three-dimensional casein network which is formed during coagulation encloses all the other milk constituents. When the coagulum contracts, water and the constituents dissolved in the water are squeezed out, whereas fat globules and bacteria retained in the fine-meshed casein network (Acharya, 2010). The retention figures of some milk constituents in cheese are shown in Table 2.2.

Table 2.2 Retention figures of some milk constituents in cheese

Milk Constituents	Retention Figure
Protein	~75% (not higher than 88%)
Fat	~88-95%
lactose	~3-5%
Ash	~30-40%
Citric acid	~10%
Bacteria	~90% concentrated in curd grains

Source: Acharya (2010)

2.7 Plant proteases as rennet substitute

Proteases are enzymes that hydrolyze protein molecules into peptides and amino acids. These form a very diverse and complex group of enzymes. The specificity of these enzymes is governed by the type of the amino acid and other functional groups close to the bond being hydrolyzed (Alagarsamy *et al.*, 2006).

Proteases are the most commercially important enzymes because of their multiple applications in food and other industries. In recent decades, interest in plant proteases has been increased rapidly. The number of industrially employed enzymes of plant origin is still small but growing fast (González-Rábade *et al.*, 2011). Plants are an important source of proteases as plants require proteases throughout their life cycle (Schaller, 2005) .The most widely used plant proteases are papain, bromelain, ficin, actinidin, zingibain, and cardosins. Plant proteases are

being used in dairy processing, meat tenderization, bioactive peptide production, and baking industry (Shah and Mir, 2019).

Plant proteases have the ability to coagulate milk proteins and thus have been utilized as milk clotting enzymes in cheesemaking for centuries. These proteases are used as crude or in purified form; they are a substitute to the calf rennet. Plant proteases are a better alternative to calf rennet because of the limited availability and high price of calf rennet, religious factors, diet, or ban on recombinant calf rennet in some countries. Most of the proteases used as milk clotting enzymes are aspartic proteases, but proteases belonging to cysteine and serine groups have also been reported to have milk clotting activity under certain conditions (Shah *et al.*, 2014).

2.7.1 Production of plant proteases

Proteases are required by plants for various physiological and developmental processes from beginning up to death of a plant. They are present in all kinds of plant tissues and, thus, can be extracted from their natural sources or can be prepared using in vitro techniques (González-Rábade *et al.*, 2011)

2.7.1.1 Production from natural sources

Plant proteases can be extracted from natural sources by aqueous maceration of various plant organs such as flowers, seeds, roots, and leaves. The crude extract thus obtained may be further purified to obtain partially purified enzyme or pure enzyme depending upon the degree of purification. Precipitation with ammonium sulfate is an effective way to produce substantial amounts of active proteases(Shah *et al.*, 2014). Certain plants reported to possess promising amount of enzymes are extracted and purified are *Balanites aegyptiaca* fruit pulp (Beka *et al.*, 2014), *Zingiber offcinale* rhizomes (Gagaoua *et al.*, 2015) and tamarillo fruit (*Cyphomandra betacea* Cav.) (Li *et al.*, 2018).

2.7.1.2 In vitro production

The plants cells are totipotent and thus are able to produce the same chemical compounds in vitro and in vivo. The yield of enzymes differs between the two processes (Tamer and Mavituna,

1996). Generally, the yield of plant proteases obtained in vitro is lower than in vivo conditions. (González-Rábade *et al.*, 2011). Different plant organs produce proteases with different activities (Pérez *et al.*, 2013). Different in vitro techniques such as callus and cell suspension cultures have been used to produce proteases. For example, proteases produced from *Mirabilis jalapa* culture (Tamer and Mavituna, 1997), cell suspension culture of *Centaurea calcitrapa* (Raposo and Domingos, 2008), and callus culture of *Silybum marianum* (Cimino *et al.*, 2006) and *Cynara cardunculus* (Oliveira *et al.*, 2010).

In vitro techniques offer many advantages. Production of plant proteases by in vitro techniques leads to higher enzyme yields and minimizes the extraction procedures used in extraction from natural sources. Furthermore, these techniques reduce the effects of climate and seasonal changes and also the heterogeneity of enzymes produced from different parts of plant (González-Rábade *et al.*, 2011)

2.7.2 Classification of proteases

Proteases can be classified into many ways. Based on the site of action, they can be grouped into exoproteases and endoproteases. Exoproteases can cleave N- or C- terminal peptide bonds, while endoproteases cleave internal peptide bond (Palma *et al.*, 2002). Exoproteases can be further divided into aminopeptidases and carboxypeptidases based on the ability to cleave the N-terminal and C-terminal peptide bond. Endoproteases are classified on the basis of their catalytic mechanism, i.e., enzyme active site. (Rawlings *et al.*, 2010). The main catalytic types are aspartate, serine, cysteine, and metalloproteases (Bah *et al.*, 2006), but the plant proteases used as milk coagulants have been reported only from first three types and none from metalloproteases. Serine and cysteine proteases are catalytically very different from aspartic and metalloproteases in that the nucleophile of the catalytic site is part of an amino acid, whereas it is an activated water molecule in the other two groups (Bruno *et al.*, 2002).

2.7.2.1 Aspartic protease

Aspartic proteases are commonly known as acidic proteases (Rao *et al.*, 1998). They are peptidases that exhibit various activities and specificities. They are found in animals, plants,

fungi, and viruses. Aspartic proteases have been connected to a wide range of physiological functions, including mammalian digestion nutrients, defence against pathogen, yeast virulence, metastatis of breast cancer, pollen-pistil interactions, degradation of haemoglobin by parasites, and maturation of HIV proteins (Claverie-Martin and Vega-Hernandez, 2007). Aspartic proteases are endopeptidases having two aspartic residues within their active site that are vital for their catalytic activities (Yegin *et al.*, 2011). They are most active at acidic pH (3-4) (Rao *et al.*, 1998) and exhibit specificity against aromatic or bulky amino acid residues on both side of their peptide bond (Yegin *et al.*, 2011).

2.7.2.2 Cysteine protease

Cysteine proteases, also known as thiol proteases, are widely distributed among living organisms, found in both prokaryotes and eukaryotes. The catalytic mechanism of these enzymes involves a cysteine group in the active site (Turk *et al.*, 1997). In plants, cysteine proteases participate in both anabolic and catabolic processes and are involved in signalling pathways as well as protein maturation, degradation and protein rebuilt in response to different external stimuli (Grudkowska and Zagdanska, 2004). Cysteine proteases have great potential in the food, biotechnology, and pharmaceutical industries owing to their property of being active over a wide range of temperature and pH. Plants offer an attractive alternative for the production of cysteine proteases as they occur naturally in different tissues, in some cases in excessive amount (González-Rábade *et. al.*, 2011).

2.7.2.3 Serine protease

Serine proteases are one of the largest groups of proteolytic enzymes, found in eukaryotes and prokaryotes. They exhibit different types of activities including exopeptidases, endopeptidase, oligopeptidase and omegapeptidase (Rawlings and Barrett, 2004). Serine proteases possess a serine residue in their active site and share a number of biochemical and physiological features. In humans, they are divided in major groups, including the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase groups (Borgono *et al.*, 2007). In plants, they are widespread among taxonomic groups, from trees and crops to legumes and herbs and present

in almost all plant parts, but most abundant in fruits. Serine proteases from cucurbits, cereals, and trees are usually classified together (Rawlings and Barrett, 2004).

2.7.3 Aank (*Calotropis gigantea*) protease

The plant belongs to kingdom: plantae, Order: Gentianales, Family: Apocynaceae, Subfamily: Asclepiadoideae, Genus: Calotropis, Species*: C. gigantea*. Aank is a common wasteland weed and commonly known as giant milk weed. This plant is a native of Bangladesh, China, India, Indonesia, Malaysia, Pakistan, Philippines, Thailand and Sri Lanka. The plant has oval, light green leaves, milky stem and clusters of way flowers that are either white or lavender in colour. Plants are usually found scattered in dry coastal areas, on beaches, along roadsides, and in disturbed urban lots (Kumar *et al.*, 2011).

A number of proteolytic enzymes are obtained from the latex of the aank plant, *C. gigantea*. Among these are the calotrotpains FI and FII (Abraham and Joshi, 1979) and the Calotropins DI and DII (Sengupta *et al*., 1984). All these enzymes contain a free sulfhydryl group on which the activity depends, and therefore they are classified as plant sulfhydryl protease. (Heinemann *et al.*, 1982). Crude proteases from latex of aank plant has promising milk clotting activity and protease activity and bears the potentiality to replace calf rennet as milk coagulant in cheesemaking process. The crude protease showed optimum activity at 37ºC and pH of 5.5 (Rajagopalan *et al.*, 2014).

2.7.4 Jackfruit (*Artocarpus heterophyllus*) protease

Jackfruit (*Artocarpus heterophyllus*) belongs to the Moraceae family, native to India and seen abundant in Western Ghats, a biodiversity spot of India. Besides India, jackfruit is commonly grown in home gardens of tropical and subtropical countries especially in Sri Lanka, Bangladesh, Burma, Philippines, Indonesia, Thailand, Malaysia, and Brazil. It is considered to be the “poor man’s food”. It is a medium-size tree typically reaching 28-80 ft. in height that is easily assessible for its fruit. The fruit is borne on side branches and main branches of the tree. The average weight of a fruit is 3.5-10 kg, and sometimes a fruit may reach up to 25 kg. the ripe fruits consisted 29% pulp, 12% seeds, and 54% rind (Swami and Kalse, 2018).

Konkalmatt and Virupaksha (1990) purified a 79.5-kDa protein from jackfruit latex and characterized as serine-centered protease. A 48-kDa protease (AMP48) can be isolated and purified from crude latex of jackfruit tree. The protein inhibits bacterial and fungal growths. AMP48 contains protease activities by gelatinolysis and caseiolysis. The intact molecular mass of AMP48 is 48427.885 Da (Siritapetawee *et al.*, 2012). According to Tanboly (2003), the crude proteases showed optimum activity at 55ºC and pH of 7.5.

2.7.5 Evaluation of enzymatic activities of plant protease

Milk clotting activity (MCA) is the most important property of proteases used in cheese production. It is the ability of enzyme to hydrolyse specifically the κ-casein from milk (Jacob *et al.*, 2011). Concerning cheese production using plant rennet, the initial goal was always the production of coagulants with a maximum specific coagulant activity. This activity depends on several factors, such as the plant source, the part in the plant, as well as the type and the concentration of protease (Silvestre *et al.*, 2012). In order to detect the optimum of activity, different comparison studies were carried out between extracts of different parts in the same plant. Results of Anusha *et al* revealed that the highest clotting activity was observed in the extract of latex followed by extracts of stems, leaves, and flowers of *Calotropis gigantea*, in descending order. In fact, aqueous extracts from flowers have been widely used as substitutes of animal rennet (AR) in some artisanal Italian Spanish and Portuguese cheeses (Aquilanti *et al.*, 2011).

Protease is an enzyme that catalyzes proteolysis, breakdown of protein in smaller polypeptides or single amino acids by cleaving the peptide bonds that connect two amino acids. They follow a hydrolytic reaction mechanism. This potential is known as protease activity (PA) (Dhillon *et al.*, 2016).

Within the frame of research for a suitable substitute for calf chymosin, comparative studies of coagulant activities and sometimes MCA/PA ratios of plant extracts (or purified proteases) were established. For example, specific activities of crude and concentrated extracts obtained from seeds of *Helianthus annus* and *Albizia lebbeck* were evaluated, showing very low values

of 156×10^{-3} and 591×10^{-3} U/mg for *A. lebbeck* extracts (crude and concentrated) and 5.8×10^{-3} and 39×10^{-3} U/mg for *H. annus* extracts (Egito *et al.*, 2007).

2.7.5.1 MCA/PA

Mazorra-Manzano *et al.* (2013) compared the MCA of three crude extracts obtained from kiwi (*Actinidia chinensis*), ginger (*Zingiber officinale*), and melon (*Cucumis melo*). Highly significant differences were observed, which were attributed to different types of proteases (actinidin, ginger, and cucumisin) found in extracts of kiwi, melon, and ginger. The assessment of MCA/PA ratio showed that the chymosin had a value 67, 95, and 500 times higher than those obtained, respectively, for the three extracts, when using casein as substrate. To overcome reduced MCA/PA ratios found in plant extracts, Ben Amira *et al.* (2017) have varied the pH of C. cardunculus rennet from 3 to 6 and evaluated enzymatic activities of the crude extracts. They demonstrated that MCA/PA ratio increased with pH drop and reached a maximum value of 28.71 for extract at pH 3, which exceeded that of chymosin (23.59). The lowest ratio attributed to the extract at pH 6 was mainly related to its high PA as well as to its low MCA. The effect of increasing pH buffer on rennet activity could be explained by the fact that, at high pH, the extraction level of several other compounds, including non-proteolytic enzymes, is more important. These compounds may promote the development of extraneous reactions and interfere with enzyme tests, thus causing underestimation of MCA. In addition, the high content of phenolic compounds involves their swift oxidation to form pigments, which may attach to native enzymes, thus leading to their inactivation (Barros *et al.*, 2001).

MCA of the partially purified extract (30–50%) from Withania coagulans fruits was clearly higher than its PA. This index was sufficiently high to justify the use of this enzyme extract as an appropriate substitute of calf rennet (Kazemipour *et al.*, 2017). According to a recent study on plant rennets (Freitas *et al.*, 2016) *Calotropis procera* and *Cryptostegia grandiflora* latex fractions were found to be good sources of milk-clotting proteases. Both coagulant fractions were able to produce similar profiles of the κ-casein peptides, to those obtained for commercial chymosin. These biochemical findings were confirmed later by technological analysis of the

cheeses obtained, which showed closer yields and soluble proteins content to those of chymosin cheese.

2.8 Basic principles of cheese making

The basic cheese making principles are concentration, preservation and ripening (Nielsen and Ullum, 1989):

1. Concentration: Coagulation, whey exudation (in cheese vat cutting, cooking, stirring, during pressing and during salting), evaporation during storage.

2. Preservation: hygiene, pasteurization, concentration, acidification, salting, addition of saltpeter, surface treatment, cooling.

3. Ripening: changes in solids (protein, lactose, fat).

2.9 Basic manufacturing steps involved in cheese

The principle of cheese manufacturing is same for all types of cheese with slight differences according to the cheese type (Fox *et al.*, 2000). The general steps involved in cheese making are:

1. Milk reception
2. Milk preparation
3. Inoculation
4. Coagulation
5. Cutting
6. Cooking and Whey drainage
7. Salting
8. Pressing
9. Ripening or Ageing

2.9.1 Milk reception

After receiving the milk, it is analysed to find out the composition and microbial status. Presence of inhibiting factors for the growth of lactic acid bacteria, such as antibiotics and lacto peroxidase systems can slow down the rate of lactic acid production (Fox, 2011). Some microorganisms such as Coliform and thermoduric bacteria can also inhibit the growth of lactic acid bacteria (Panthi *et al.*, 2017). Clean and fresh milk with sound quality is desirable for the production of preferred quality cheese (Scott *et al.*, 1998).

2.9.2 Milk preparation

Different steps for treatment of milk for cheese manufacturing are as follows:

1. Filtration/centrifugation

This is done to remove dirt or suspended particles. Bactofugation can also be done to reduce the number of spores of *Clostridium tyrobutyricum* (Banks, 2011; Legg *et al.*, 2017).

2. Pasteurization

The main purpose of pasteurization is to kill all pathogenic and harmful microorganisms. Pasteurization also inactivates phosphatase and xanthine oxidase enzymes present in the milk. Yield of cheese can also be increased by pasteurization as it insolubilizes part of serum protein (Banks, 2011). High temperature and short time treatment at 72ºC for 15 s is commonly used for continuous pasteurization (Ong *et al.*, 2017).

3. Standarization

Standardization of milk with high fat content is done by separation the cream and again adding the required amount of cream to the skim milk (Fox. 2011). Before ripening the milk, additives including colour and salts (calcium chloride and sodium nitrate) must be homogenously mixed in the milk as solution or sometimes in the form of dry salt (Scott *et al*., 1998).

2.9.3 Inoculation

The treated milk is warmed up to 30ºC and starter culture is added at the rate of 0.05 to 4%. The milk is left for 45 min to 1.5 h for ripening. Temperature, types of cheese and quantity of the starter used are the factors that vary the duration of ripening (Fox, 2011). The ripening period becomes shorter if the quantity of the starter culture is greater and vice versa (Banks, 2011).

2.9.4 Coagulation

Ph controls the nature and speed at which the coagulum is formed. Thus, rennet must be added at optimum pH (Ong *et al.*, 2017). The range of pH used for renneting to prepare most cheeses is 6.5 to 6.35. Curd firmness is affected by the ratio of fat to protein and the quantity of whey proteins in the curd. Brief, cost-effective, and well-regulated coagulation process is desirable and the coagulation should not cause excessive loss of curd and fat in whey (Scott *et al.*, 1998). Generally, one part of rennet can clot 10,000 parts (0.01% v/v) of milk. Thus, rennet is diluted with water before addition (Legg *et al.*, 2017).

2.9.5 Cutting

Cutting time is very important during cheesemaking as it directly influences the yield and composition of cheese (Lawrence *et al.*, 2004). Quality of cheese is affected by factors like temperature, size of curds, speed of agitation and pH, all of which vary according to cheese type (Fox and McSweeney, 2017). Cutting time of coagulum varies between 25 min and 2 h according to the process and recipe. Cutting time if often calculated by multiplying the time taken to flocculate by three (Scott *et al.*, 1998).

2.9.6 Cooking and whey drainage

Cutting is followed by cooking during cheese manufacturing. Gentle stirring of the curd is done during cooking to prevent excessive crushing and fat loss. Increment in stirring rate is done when the outer covering of the curd looks like membrane. Cooking aids in more discharge of whey from the curd due to contraction and pressure exerted on the curd grains (Fagan *et al.*, 2017). Increase in cooking temperature also triggers the metabolism of the starter bacteria

(present in the curd) which in turn increases the production of lactic acid and drops pH (Ong *et al.*, 2017). During cheesemaking, the decrease in level of lactose has a huge effect on the growth of lactic acid bacteria. Therefore, different techniques are imposed to adjust the level of lactose in the curd (Düsterhöft *et al.*, 2017). The first method involves production of lactic acid to lower the pH and shrink the curd by heat, the second method is addition of water in whey so that increment in osmotic pressure across the curd membrane extracts lactose from the curd to whey (Scott *et al.*, 1998). The whey is drained when the pH reaches 6.1-6.5 depending upon cheese variety (Tunick, 2014).

2.9.7 Salting

Variation of salt concentration in cheese is due to the method of application and the types of cheese. Immersion in brine solution and dry salting are the common practice for untextured cheese. Further ripening or ageing and proteolysis of the cheese are governed by salting (Ong *et al.*, 2017). Salting method varies according to the type of cheese. Salting on the surface of moulded curd is done for blue veined cheese, brine salting is done for Edam, cheese is directly immersed in brine for Gouda cheese, and dry salting is done for cheddar and cottage cheeses. Cheese salted by brine are held to make the curd into a compact mass of appropriate size so as to handle it easily (Guinee and Fox, 2017).

2.9.8 Ripening or Ageing

Ripening is concerned with the production of desirable texture and aroma in cheese and is governed by the proteolysis and other different biochemical reactions in cheese (Fox, 2011). Ripening can be separated into two stages. The first stage is from 7 to 14 days, in which reduction in the rubbery texture of cheese is brought by the residual coagulant enzymes by rapidly hydrolysing α_{s1}-caseins to α_{s2}-caseins degradation products (Lawrence *et al.*, 1987). During the residual ripening period proteolysis is continued by coagulant enzymes, native milk protease and enzymes produced by starter bacteria and secondary micro-flora (Fox and McSweeney, 2017). For cheese which is not vacuum packed, the temperature and relative humidity ranges from 5 to 12ºC and 87 to 95% respectively (Hort and Le Grys, 2001).

2.10 Soft cheese manufacturing method

The methodology for soft cheese manufacturing given by (Jain *et al.*, 2019) has been shown in Fig. 2.1.

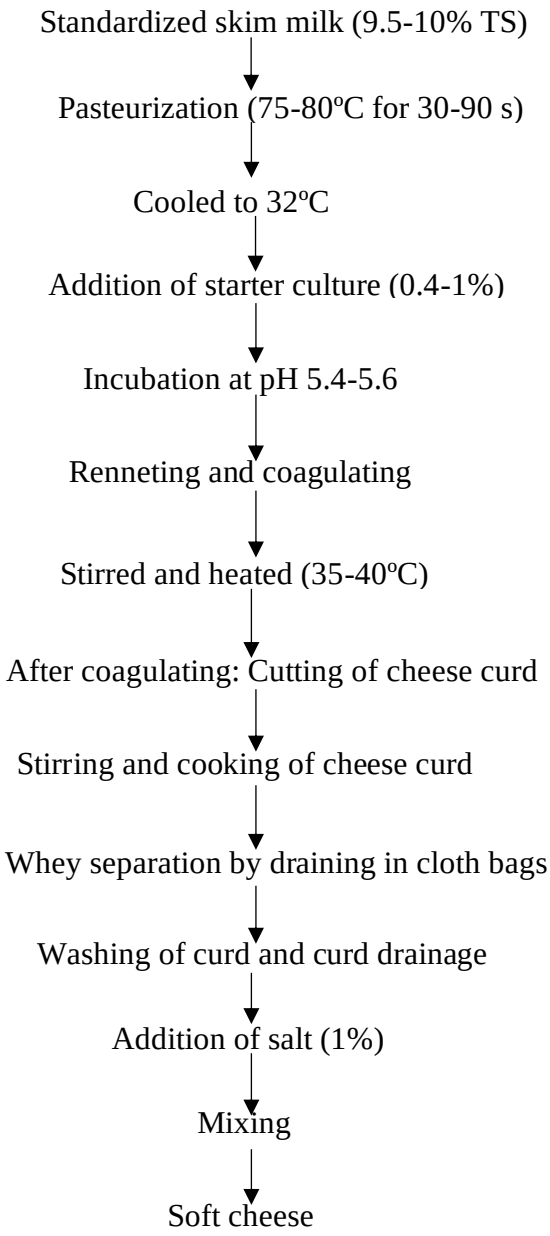

Fig. 2.1 Processing steps for soft cheese making (Jain *et al.*, 2019).

Part III

Materials and methods

3.1 Materials

3.1.1 Milk

Raw cow milk of local breed was purchased from local farm of Hattisar, Dharan-14. Fresh milk (6 l) was collected directly from farmers immediately after milking of healthy cows with no recent medication history. The collected milk was transported in a container and was stored in refrigerated condition at 4ºC until further use.

3.1.2 Rennet

Rennet (CHR.HANSEN, Denmark) was purchased from Trishuli Traders, Kathmandu.

3.1.3 Plants latex

The latex of aank plant (*Calotropis gigantea*) was collected from Dharan-11 and the latex of jackfruit plant (*Artocarpus heterophyllus*) were collected from Dharan-14.

3.2 Methods

3.2.1 Extraction of crude plant proteases

3.2.1.1 Preparation of crude aank proteases

50 ml latex oozing out from mature plant was collected through giving V shaped incision on the branches of the plant (Murugan, 2012). Collected latex was mixed with 0.05 M sodium acetate buffer (pH 5.5) at the ratio of 1:5. The extracts were filtered through muslin cloth and the filtrates were centrifuged at 4000 rpm for 20 min. The supernatants obtained were used as the crude proteases. Crude proteases were stored in deep freezer (-20ºC) until further analysis (Oseni and Ekperigin, 2013).

3.2.1.2 Extraction of crude jackfruit proteases

Latex was obtained from plant trunk. For this purpose, sharp incisions were made on tree trunk to open the latex vessels situated in the bark (Buranov and Elmuradov, 2010). The freshly collected latex (50 ml) was diluted using 0.05 M phosphate buffer (five volumes). The diluted latex was centrifuged for 20 min at about 10000 rpm in high-speed centrifuge. After that clear and colorless supernatant that is crude form of enzyme extract was collected and stored for further experiments (Somavarapu *et al.,* 2017).

3.2.1.3 Milk clotting activity

The milk-clotting activity was determined following the procedure described by IDF (1992). 60 g of skimmed milk powder was reconstituted in 500 ml of 0.01 mol/L $CaCl_2$ (pH 6.5) and the mixture was stored at 4°C. The extract was added at a proportion of 0.2 ml per 1.0 ml of milk (0.2:1 v/v). The clotting point was estimated during the manual shaking of the test tube, at very short time intervals (5-10 s). The coagulation time was documented when separate particles were noticeable. One milk-clotting unit was defined as the amount of enzyme that clots 10 ml of substrate within 40 min (2400 s) at 37°C (Berridge, 1952).

$$\text{MCA (U/ml)} = (2400/T) \times (S/E)$$

Where, T = time necessary for the micellar formation (seconds); S = volume of the milk (ml); E = volume of the enzyme extract (ml).

3.2.1.4 Protease activity

Protease activity was determined using protocol given by Cupp-Enyard and Aldrich (2008). This uses casein as protease substrate. 0.05 ml of enzyme extract was dissolved in 5 ml sodium acetate buffer 10 mM (pH 7.5) and 5 ml calcium acetate buffer 10 mM (pH 7.5). For each sample 455 µL Casein 65%(w/v) were preheated in a thermal bath at 60, 65 and 70ºC for 10 min and then 20 µL of these were added. After 10 min of reaction, the reactions were stropped by the addition of 455 µL trichloroacetic acid 110 mM. And were kept in the thermal bath for another

30 min. Each reaction has its negative control, which did not have enzyme during preincubation, but it was added after the trichloroacetic acid addition.

Aliquots of 625 µL enzyme solution were added to 1570 µL sodium carbonate 500 mM and 250 µL of Folin and Ciocalteus Phenol or Folin's reagent. The protease activity was detected spectrophotometrically since the released tyrosine developed a blue coloration. Each sample was read in a spectrophotometer at 660 nm and compared with a calibration curve. One protease unit was defined as the amount of casein hydrolyzed to produce color equivalent to 1.0 µM (181 µg) of tyrosine per min at pH 7.5 and 37°C (color by Folin's reagent) and was calculated by the following reaction:

$$\text{Protease activity (µMoles Tyrosine)} = \frac{(\text{mol Tyrosine}) \times V_T}{V_E \times t \times V_C}$$

where V_T is the total assay volume in ml, V_E is the volume of the enzyme used ml, t is the reaction time in min, and V_C is the volume used in the colorimetric reaction in ml.

3.2.1.5 Protein concentration

Protein content of the crude plant latex was determined using the procedure given by Lowry *et al.* (1951). For this, 1 ml of enzyme extract was mixed with 5 ml of alkaline copper solution in a test tube. Alkaline copper solution was prepared by mixing 50 ml of reagent A (2% sodium carbonate in 0.1 N NaOH) and 1 ml of reagent B (0.5% copper sulphate in 1% sodium potassium tartarate). The contents of the tubes were allowed to stand for 10 min and 0.5 ml of diluted Folin's reagent (1:1 mixture of Folin's reagent and 0.1 N NaOH) was added rapidly with immediate mixing and the tube was incubated at room temperature in dark for 30 min. The contents of the tube were read in a spectrophotometer at 660 nm and compared with a calibration.

For calibration curve; 0.2, 04, 0.6, 0.8 and 1 ml of working standard solution (200 µg/ml of Bovine Serum Albumin) was pipetted out in labeled test tubes. A tube with 1 ml of distilled water serves as blank. The volume in each test tube was made up to 1 ml with distilled water. 5 ml of alkaline copper solution was added to all the test tubes including the blank. The contents

of the tubes were mixed by shaking/vortexing the tubes and were allowed to stand for 10 min. 0.5 ml of diluted Folin's reagent was added rapidly with immediate mixing and the tubes were incubated at room temperature in dark for 30 min. The absorbance of all the tubes were measured in a spectrophotometer at 660 nm and a standard curve was plotted by taking concentration of BSA along X-axis and absorbance at 660 nm along Y-axis.

3.2.1.6 Experimental design

The experimental design, data analysis and model building were performed using "Design Expert" software (Version 12, Stat-Ease Inc., USA). The non-rennet soft cheese was prepared with variations in: (a) pH and (b) temperature of milk during enzyme addition, as shown in Table 3.1 and Table 3.2. The independent variables and their levels were selected on the basis of literature and preliminary experiments. Crude aank proteases shows activity between pH range of 4.5-6.5 (Rajagopalan *et al.*, 2014) and thermal stability between 37-50ºC (Bindhu and Singh, 2014). According to Sayed and Tanboly (2003) the temperature and pH range for crude jackfruit protease is 35-75ºC and 6.5-8 respectively. A two-factor central composite design was employed. The response variables were Time of Coagulation (TOC) and Milk Clotting Activity (MCA) of the plant enzyme.

Table 3.1 Different constraints for optimization for crude aank enzyme.

Name	Goal	Range
Temperature of milk	To be in range	30-50℃
pH of milk	Target = 6.5	4.5-6.5
Time of Coagulation (TOC)	To be minimized	To be determined
Milk Clotting Activity (MCA)	To be maximized	To be determined

Table 3.2 Different constraints of optimization for crude jackfruit enzyme

Name	Goal	Range
Temperature of milk	To be in range	35-75℃
pH of milk	Target = 6.5	6.5-8
Time of Coagulation (TOC)	To be minimized	To be determined
Milk Clotting Activity (MCA)	To be maximized	To be determined

The responses TOC and MCA for different experimental combinations were related to the coded variables (X_i, i = 1, and 2) by a second-degree polynomial equation

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1.X_2 + \varepsilon$$

The coefficients of the polynomial were represented by β_0 (constant), β_1, β_2, (linear effects); β_{12}, (interaction effects); β_{11}, β_{22} (quadratic effects); and ε (random error). Data were modelled by multiple regression analysis and the statistical significance of the terms were examined by analysis of variance for each.

3.2.1.7 Analysis of data

The independent process variables were correlated using a second order quadratic model. Multiple regression analysis with Design Expert® software was used to determine the second order polynomial coefficient for each term of the equation. The statistical significance of the terms was investigated using analysis of variance for each response after the data were fitted to the selected models. R^2 (coefficient of determination the amount of variation around the mean explained by the model), adjusted R^2 (a measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model), predicted R^2 (a measure of good the model predicts a model value) and Fisher's F test were used to test the adequacy of the model. Coefficient of determination R^2 is a measure of degree of fit as it is the ratio of explained variation to the total variation. A better empirical model fits the actual data when R^2

approaches unity. The smaller the value of R^2, the less relevance the dependent variables in the model have in explaining the behavior variation. Then the effect of predictors on the response was interpreted using the models.

The analysis of variance (ANOVA) tables was generated and the significance of all terms in the polynomial equation was judged statistically by computing the F-value at 5% level of significance.

3.2.2 Preparation of cheese

Cheeses were prepared using three variations. A general process for the preparation of all three samples is shown in Fig 3.1. The cheese prepared using calf rennet by direct acidification was labelled as cheese A. Cheese sample prepared using crude aank proteases was labelled as cheese B. Similarly, cheese sample prepared using crude jackfruit proteases was labelled as cheese C.

Briefly, milk having 3.7% fat and 8.5% SNF content was heated until it reached the temperature of 75ºC followed by stirring for 1 min. For cheese A, the initial pH (6.5) of milk was reduced to 5.6 by the addition of 2% citric acid solution and the enzyme rennet was added at a concentration of 0.1% and temperature of 37ºC for optimum activity. The curd was then cut by a stainless-steel knife to separate the whey and it was further cooked at 38ºC for 15 min. The curds were drained using cheese cloth and mixed with 2% common salt. The cheese was stored in refrigerator at below 5ºC. Similar procedure was followed for cheeses B and C after determining the optimum conditions by RSM (Jain *et al.*, 2019).

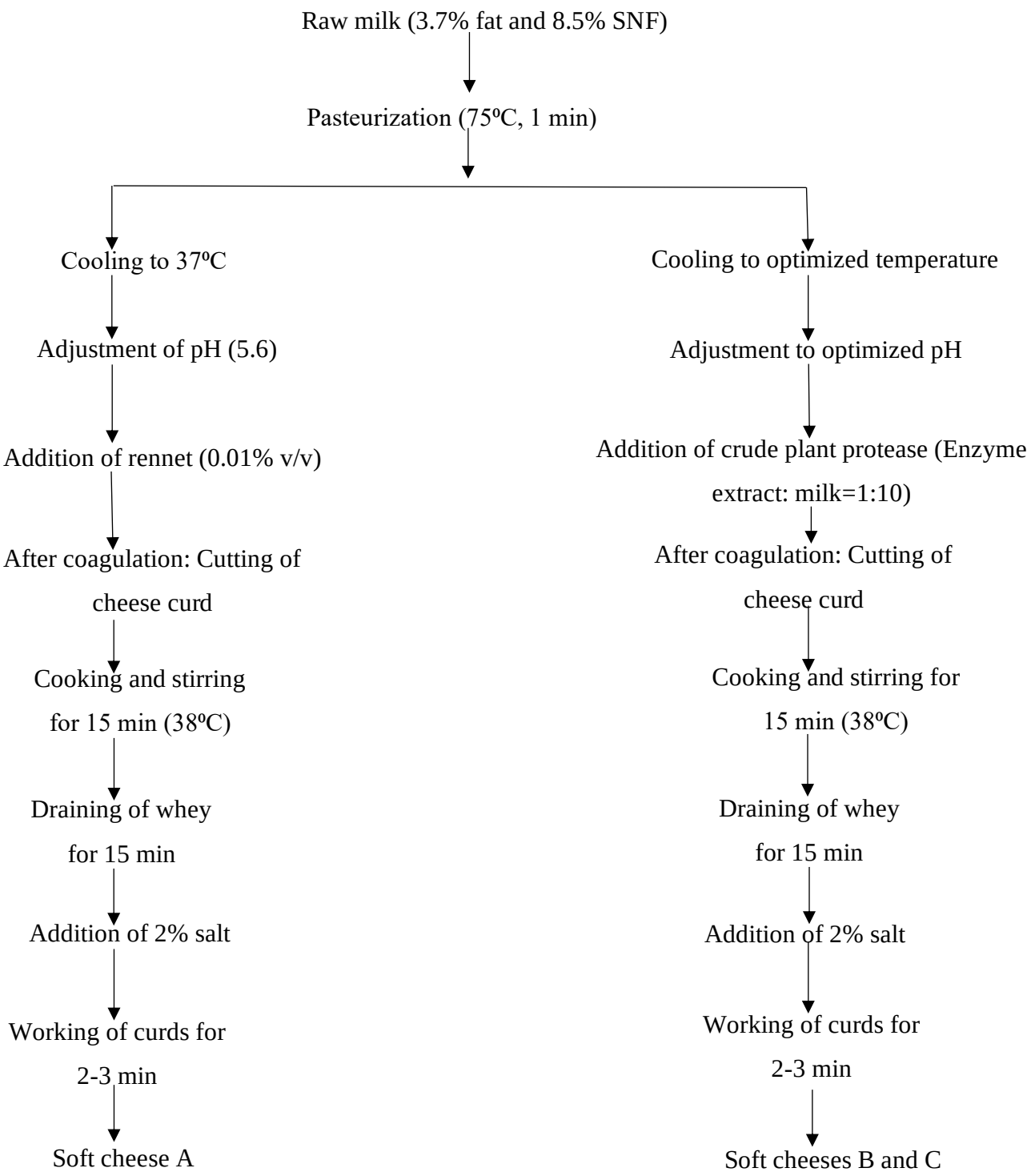

Fig. 3.1 Preparation steps of soft cheese with slight modification (Jain *et al.*, 2019)

3.2.3 Physicochemical analysis of milk and cheese

3.2.3.1 Determination of fat in milk and cheese

Fat in milk and cheese was determined by Gerber method (AOAC, 2005).

3.2.3.2 Determination of SNF in milk

SNF in milk was determined by Lactometer test (AOAC, 2005).

3.2.3.3 Determination of pH in milk and cheese

The pH in milk and cheese was determined by pH meter (AOAC, 2005).

3.2.3.4 Determination of protein in milk and cheese

Protein content in milk was determined by formal titration method whereas protein in cheese was determined by the Kjeldahl method (AOAC, 2005).

3.2.3.5 Determination of acidity in milk and cheese

The acidity of milk and cheese were determined by titrimetric method (AOAC, 2005).

3.2.3.6 Determination of calcium in cheese

Calcium content of the cheese was determined by volumetric method (AOAC, 2005).

3.2.3.7 Determination of moisture in cheese

Moisture in cheese was determined by the oven-drying method (AOAC, 2005).

3.2.3.8 Determination of ash in cheese

The ash content in cheese was determined by dry ashing method (AOAC, 2005).

3.2.3.9 Theoretical yield and actual yield

Theoretical yield was calculated using the modification on Van Slyke equation given by Mullan (2008).

$$Y = \frac{(0.93 \times \%\text{M fat}) + (\%\text{M casein} - 0.1) \times 109}{100 - \text{moisture in cheese}}$$

where, % M fat = % fat in milk and % M casein = % casein in milk.

The 0.93×milk fat assumes that some 93% of milk fat is retained in the cheese. The value for casein – 0.1 approximates to a theoretical loss of 4% casein and casein retention of approximately 96%. The 109 is a 'constant' to allow milk salts retention of whey protein and lactose

Actual yield is defined as the kg of cheese per 100 kg of milk as described by Abd El-Gawad and Ahmed (2011) . The percentage of cheese yield was calculated as follow:

$$\text{Cheese yield}(\%) = \frac{\text{Weight of cheese(kg)}}{\text{Weight of milk(kg)}} \times 100$$

3.2.4 Microbiological analysis of cheese

3.2.4.1 Coliform count

Total Coliform of cheese was determined by pour plate technique on Violet Red Bile Agar (VRBA) medium (AOAC, 2005).

3.2.4.2 TPC of cheese

Total Plate Count (TPC) was determined by pour plate technique on Plate Count Agar (PCA) medium (AOAC, 2005).

3.2.4.3 Yeast and molds count

Yeasts and Molds count were determined by pour plate technique on Potato Dextrose Agar (PDA) medium (AOAC, 2005).

3.2.5 Statistical analysis

Data was statistically processed by GenStat (12^{th} edition) developed by VSN International Limited for Analysis of Variance (ANOVA). Means of the data was separated whether they are significant or not by using Least Significant Difference (LSD) method at 5% level of significance.

Part IV

Results and discussion

In this research work, crude plant proteases were extracted from latex of aank (*Calotropis gigantea*) and jackfruit (*Artocarpus heterophyllus*) plants. The impact of pH and temperature of milk on time of coagulation (TOC) and milk coagulating activity (MCA) were analyzed by response surface methodology. The cheeses thus prepared from rennet (A), crude aank enzyme (B) and crude jackfruit enzyme (C) were analyzed for physico-chemical and microbiological properties.

4.1 Numerical optimization for time of coagulation and milk clotting activity (TOC) and milk clotting activity (MCA)

The measured expansion of TOC and MCA for crude aank proteases varied from 40-315 s and 38.09-300 units respectively (Appendix A) whereas the TOC and MCA for crude jackfruit protease varied from 108-10800 s and 1.11-111.11 units respectively. Table B.1 and B.2 show the coefficient of the model and other statistical attributes TOC whereas Table B.3 and B.4 show that of MCA for the crude aank protease. Similarly, Table B.5 and B.6 show the coefficients of the model and other statistical attributes of TOC whereas Table B.7 and B.8 show that of MCA for the crude jackfruit protease.

Coded equations for crude aank protease,

$$TOC = 182.94 + 3.54A - 58.25B + 0.63AB - 109.69A^2 + 67.50B^2 \quad \text{....4.1}$$

$$MCA = 82.99 + 12.21A + 51.69B + 26.80AB + 104.48A^2 - 44.93B^2 \quad \text{....4.2}$$

Coded equations for crude jackfruit protease,

$$TOC = 29.49 + 2085.21A - 2312.15B - 2785.65AB + 1782.79A^2 + 1462.64B^2 \quad \text{....4.3}$$

$$MCA = 49.27 - 27.02A + 34.61B - 18.69AB - 4.07A^2 - 12.80B^2 \quad \text{....4.4}$$

Where A and B are the coded values of pH of milk and temperature of the milk. A, B, A^2, B^2 and AB are model terms.

In the quadratic equation 4.1, for the crude aank protease, TOC had non-significant ($P>0.05$) positive effect of pH of milk but significant ($P<0.05$) negative effect of temperature of milk (B) at 95% level of confidence. The quadratic term of pH of milk had highly significant ($P<0.05$) negative effect and the quadratic term of temperature of milk had highly significant ($P<0.05$) positive effect on TOC as given in Table B.2. The interaction term of pH of milk and temperature of milk (AB) had non-significant ($P>0.05$) positive effect on TOC.

Similarly, the quadratic equation 4.2 of MCA of crude aank protease, shows that pH of milk (A) had non-significant ($P>0.05$) positive effect but temperature of milk (B) had significant ($P<0.05$) positive effect on MCA at 95% level of confidence. The quadratic term of pH of milk had highly significant ($P<0.05$) positive effect but the quadratic term of temperature of milk had non-significant ($P>0.05$) negative effect on MCA as given by Table B.4. The interaction term of pH of milk and temperature of milk (AB) had non-significant ($P>0.05$) positive effect on MCA.

In the quadratic equation 4.3, TOC of crude jackfruit protease had significant ($P<0.05$) positive effect of pH of milk (A) and significant ($P<0.05$) negative effect of temperature of milk (B) at 95% confidence level. The quadratic term of pH of milk had significant ($P<0.05$) positive effect on TOC whereas the quadratic term of temperature of milk had non-significant ($P>0.05$) positive effect TOC as given in Table B.6. The interaction term of pH of milk and temperature of milk (AB) had significant ($P<0.05$) negative effect on TOC.

Similarly, the quadratic equation 4.4 of MCA of crude jackfruit showed that pH of milk (A) had significant ($P<0.05$) negative effect on MCA and temperature of milk (B) had significant ($P<0.05$) positive effect on MCA at 95% confidence level. The quadratic term of pH of milk had non-significant ($P>0.05$) negative effect and the quadratic term of temperature of milk had significant ($P<0.05$) positive effect on MCA at as given by Table B.8. The interaction term of pH of milk and temperature of milk (AB) had significant ($P<0.05$) negative effect on MCA.

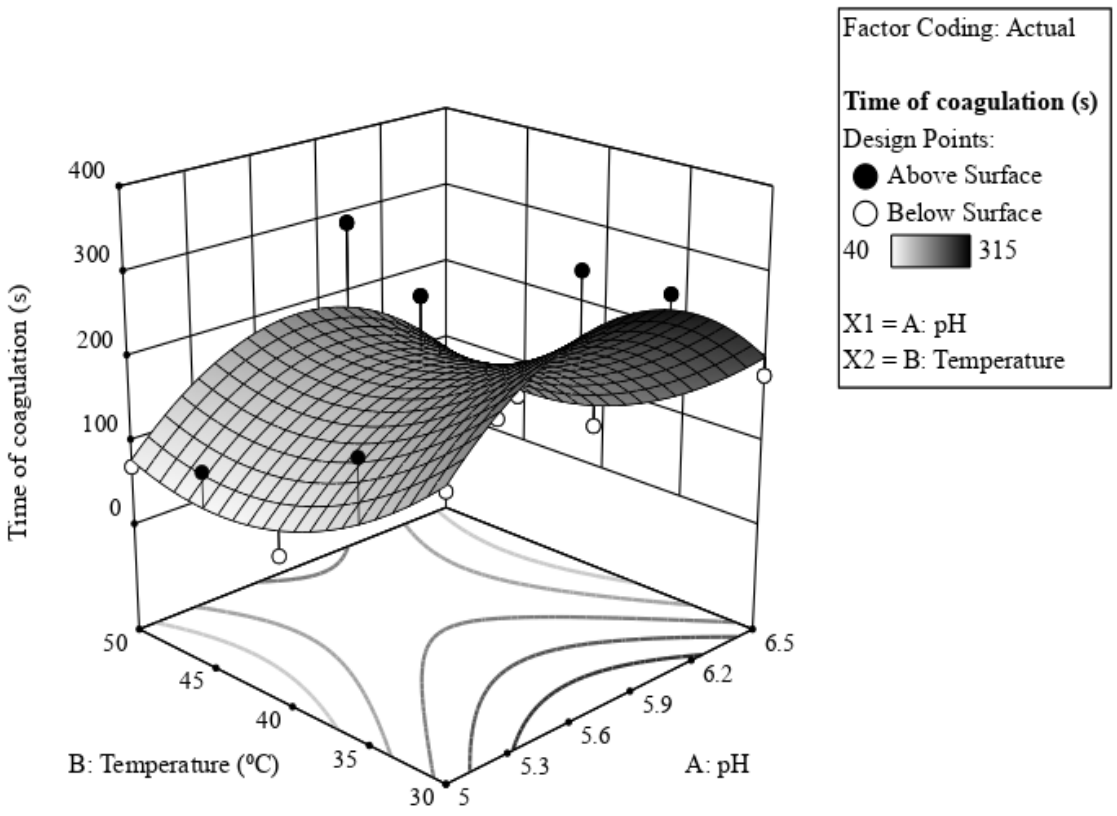

Fig. 4.1 Response surface plot of TOC of crude aank protease as a function of pH and temperature of milk.

The combined increase of pH and temperature of milk caused nonlinear increase in TOC of crude aank protease as it increased up to a maximum point and further increase led to decline in TOC (Fig. 4.1).

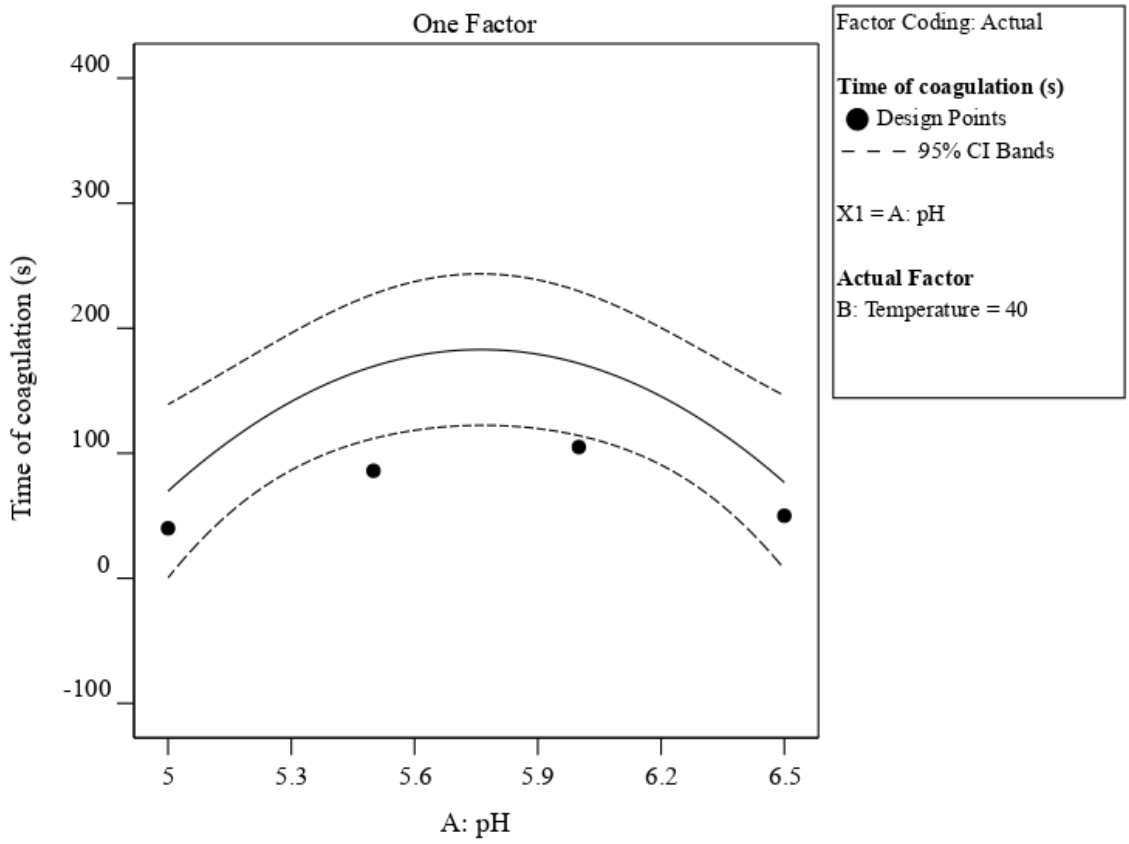

Fig. 4.2 Single factor interaction graph of TOC of crude aank protease for individual factor A: pH

From Fig. 4.2, it can be concluded that increase in pH led to the increase of TOC of crude aank protease up to a certain point and further increase in pH resulted in the decline of TOC.

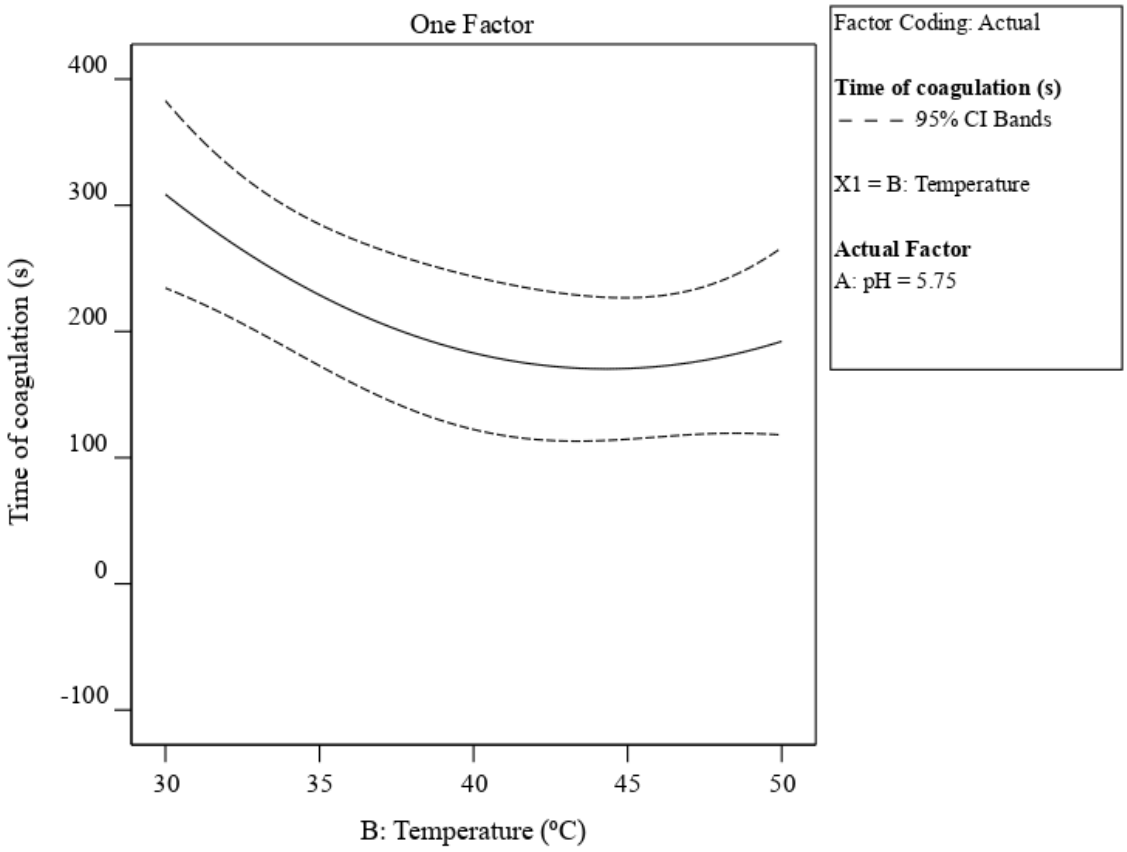

Fig. 4.3 Single factor interaction graph of TOC of crude aank protease for individual factor B: temperature

From Fig. 4.3, it can be concluded that increase in temperature led to the decrease in TOC of crude aank protease but the effect was not linear.

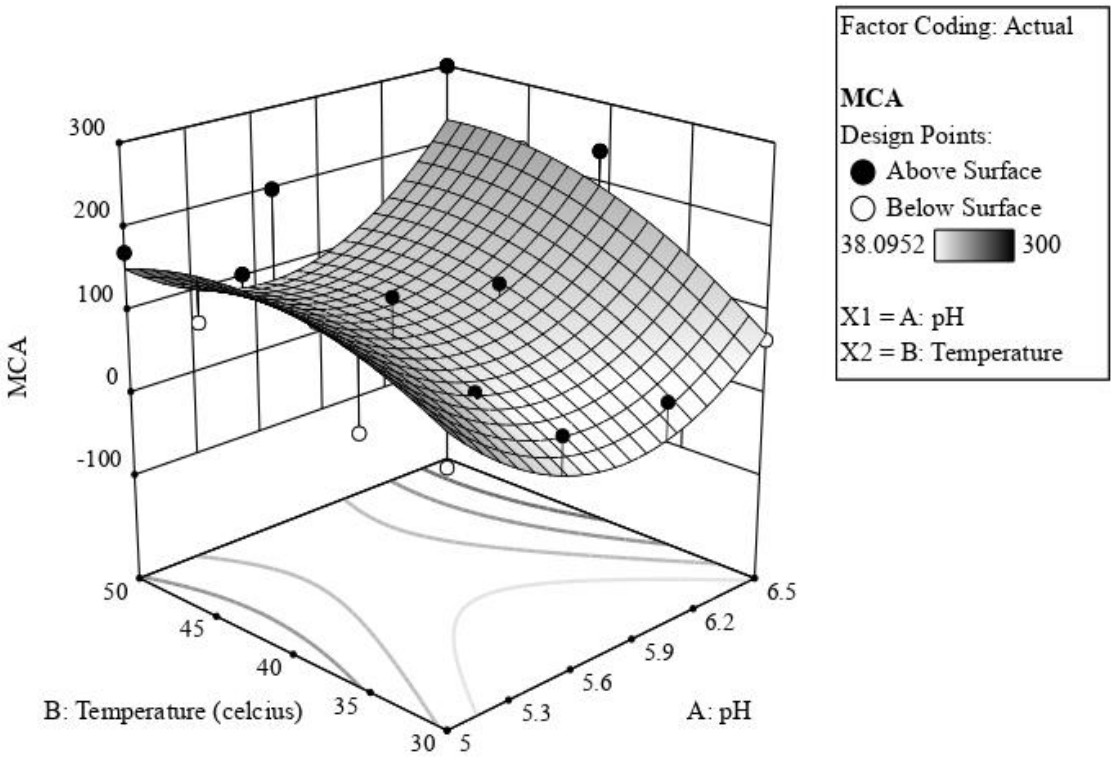

Fig. 4.4 Response surface plot of MCA of crude aank protease as a function of pH and temperature of milk

With the combined increase of pH and temperature of milk, MCA of crude aank protease decreased and reached minimum until further increase led to the increase in MCA (Fig. 4.4)

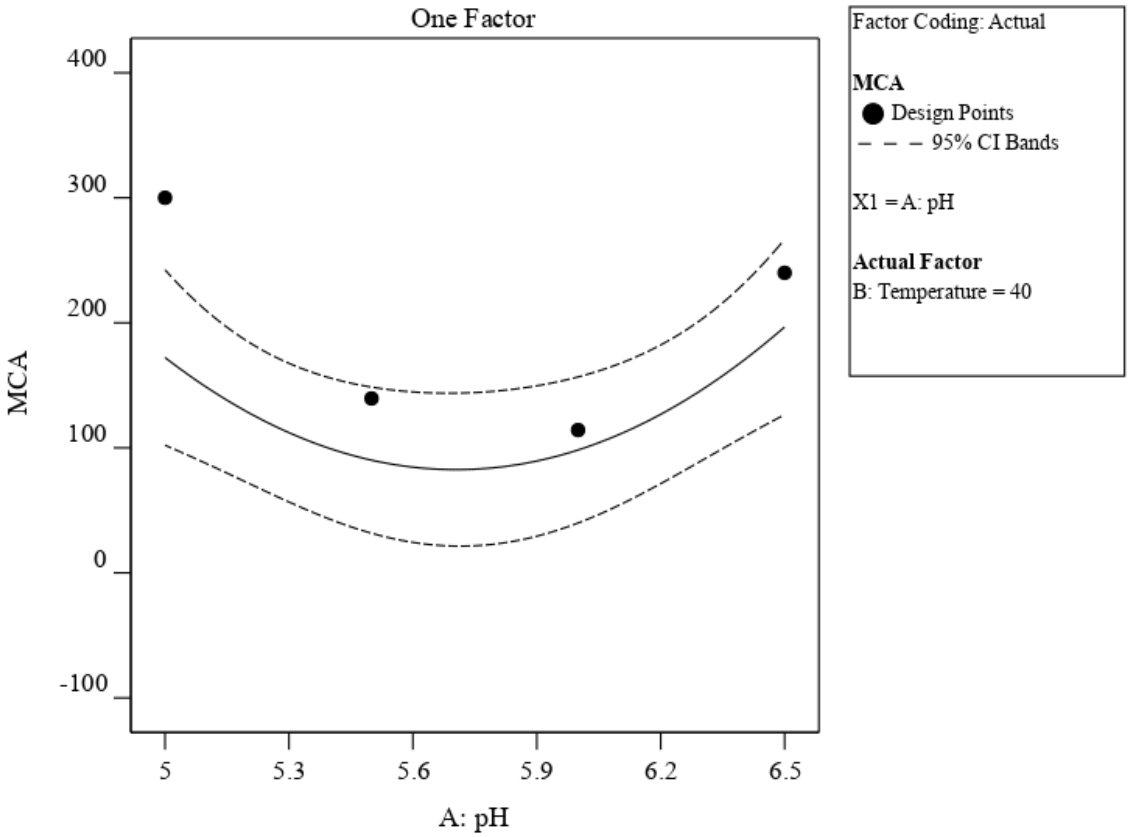

Fig. 4.5 Single factor interaction graph of MCA of crude aank protease for individual factor A: pH

Fig. 4.5 revealed that MCA of crude aank protease decreased with the increase in pH upto a certain point. Further increase in pH beyond that point led to increase in MCA.

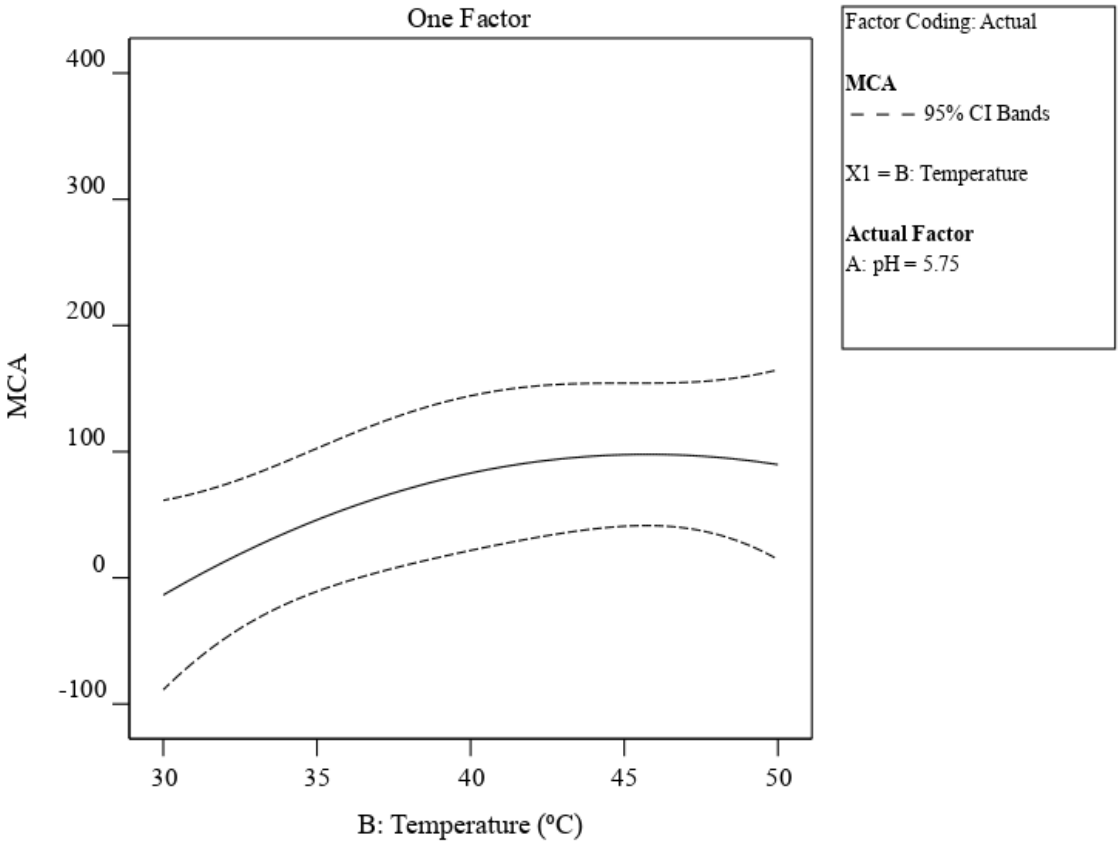

Fig. 4.6 Single factor interaction graph of MCA of crude aank protease for individual factor B: temperature

Fig. 4.6 showed that MCA of crude aank protease increased slightly with increase in temperature. The increase was nonlinear.

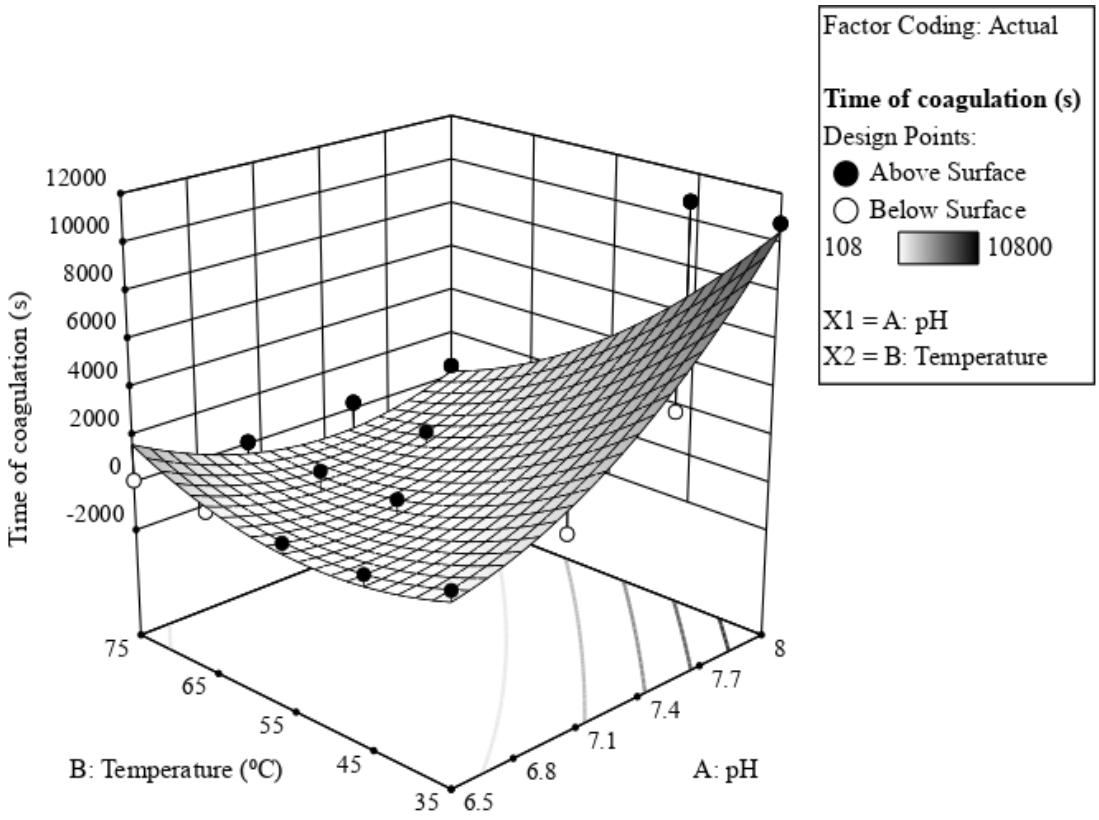

Fig. 4.7 Response surface plot of TOC of crude jackfruit protease as a function of pH and temperature of milk

From Fig. 4.7, it can be concluded that the combined increase of pH and temperature of milk led to a slight decrease in TOC of crude jackfruit protease.

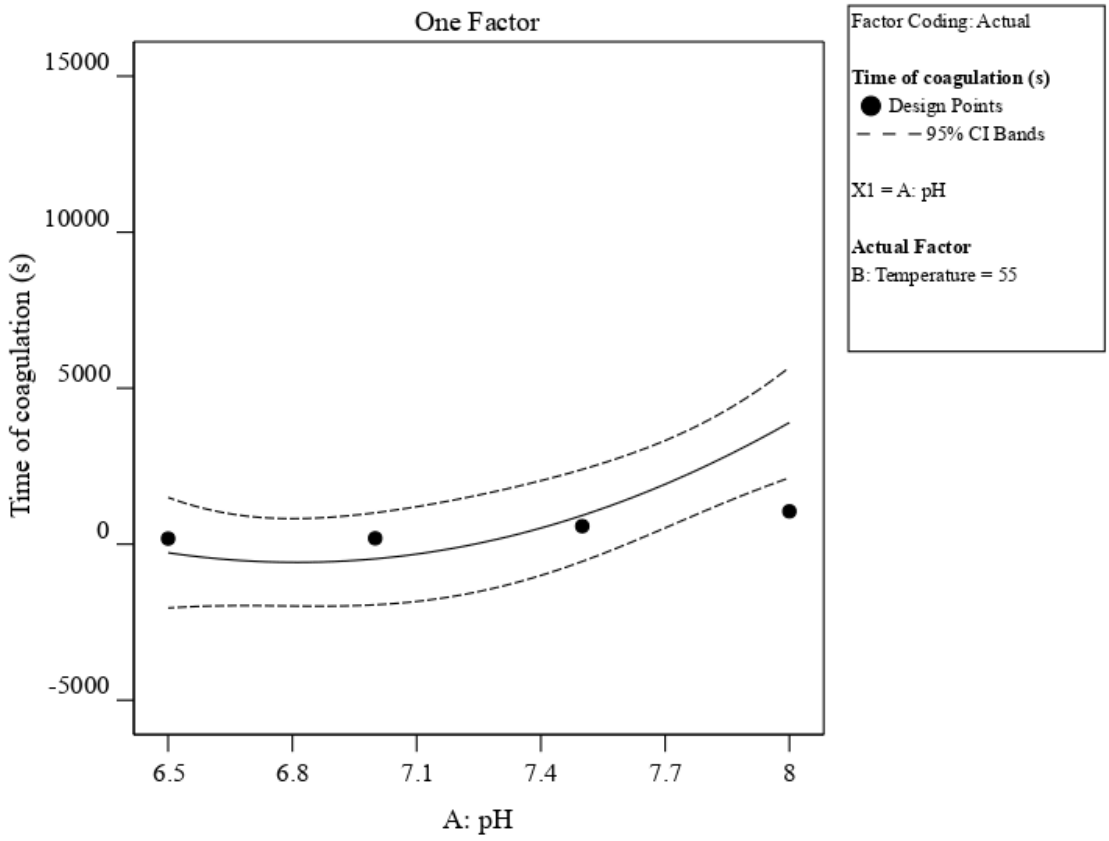

Fig. 4.8 Single factor interaction graph of TOC of crude jackfruit protease for individual factor A: pH

Fig. 4.8 revealed nonlinear increase of TOC of crude jackfruit protease with increase in pH of milk.

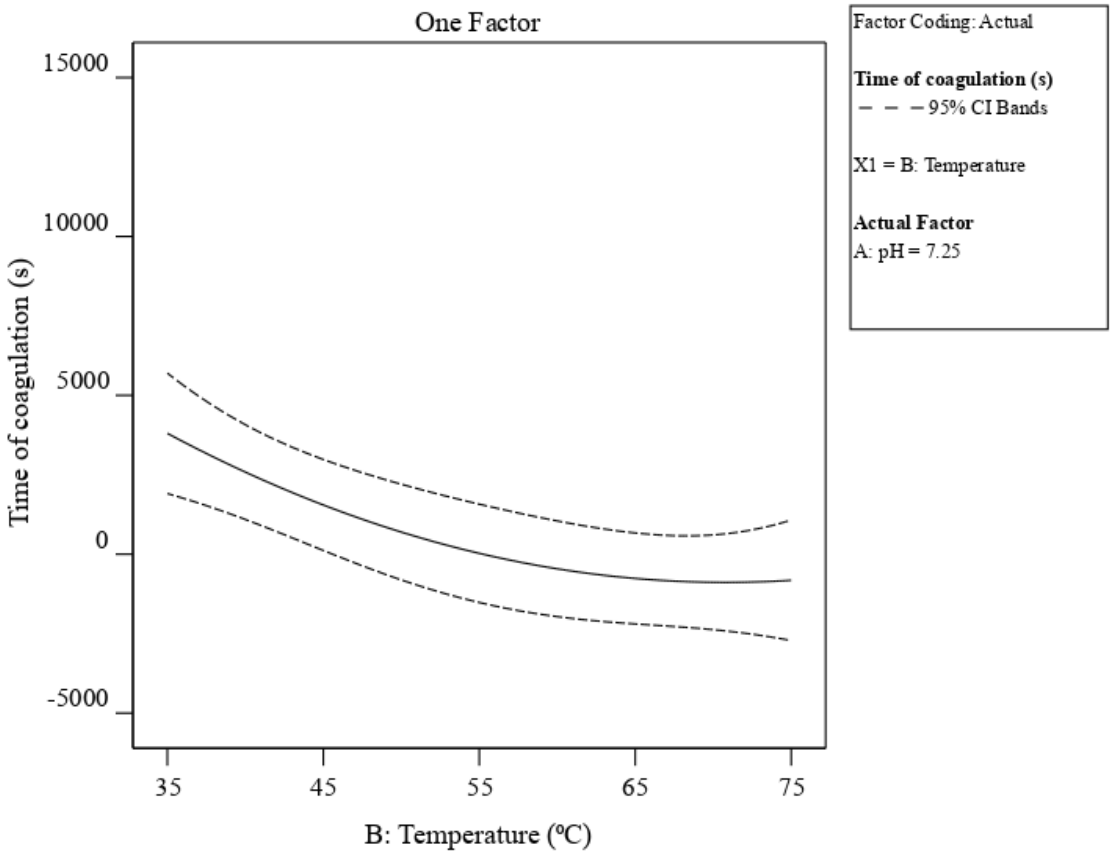

Fig. 4.9 Single factor interaction graph of TOC of crude jackfruit protease for individual factor B: temperature

From Fig. 4.9, it can be concluded that increase in milk temperature caused non linear drastic decline of TOC of crude jackfruit protease.

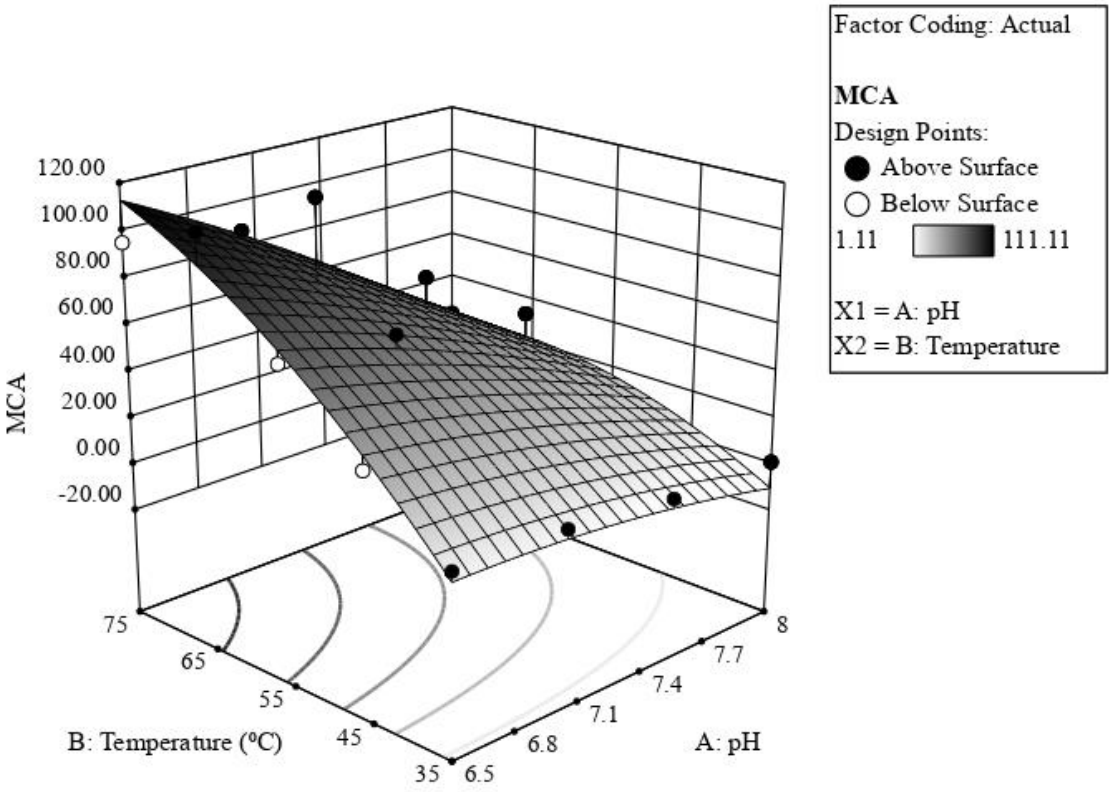

Fig 4.10 Response surface plot of MCA of crude jackfruit protease as a function of pH and temperature of milk

Fig. 4.10 showed that combined increase of pH and temperature of milk led to a slight increase in MCA of crude jackfruit protease.

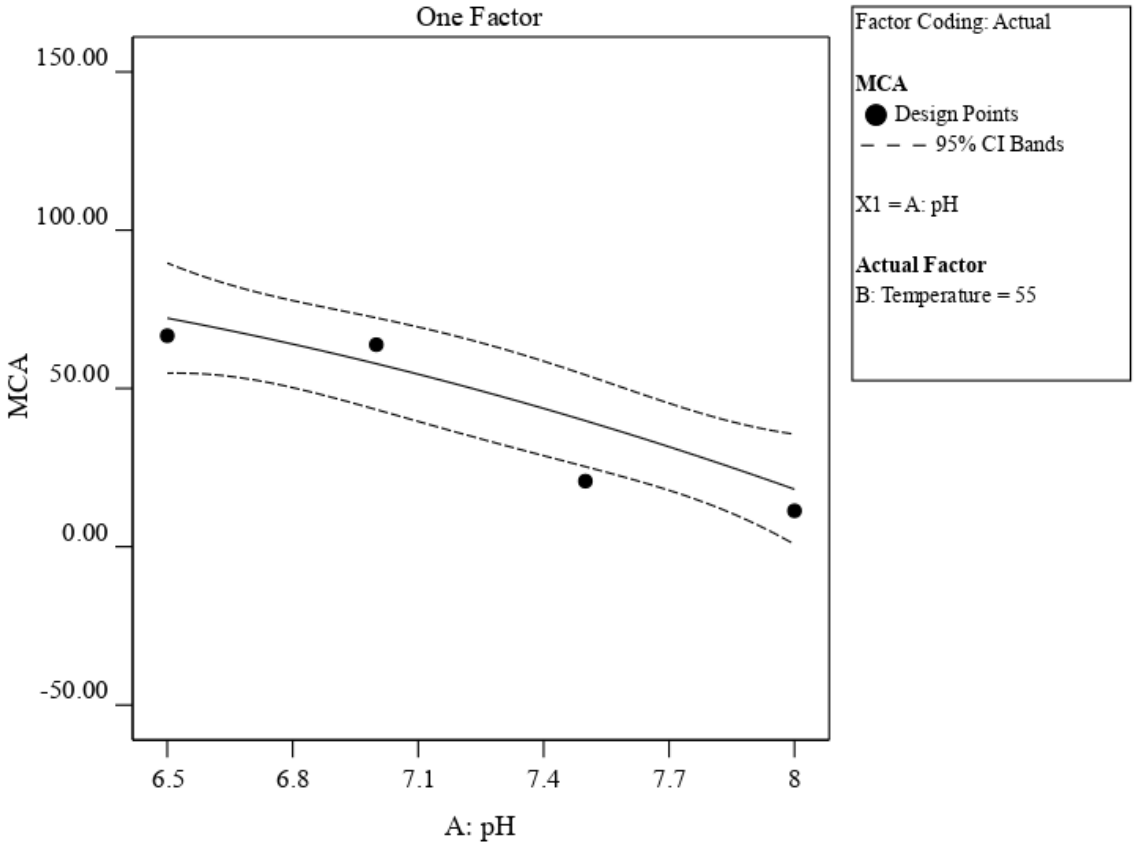

Fig. 4.11 Single factor interaction graph of MCA of crude jackfruit protease for individual factor A: pH

Fig. 4.11 revealed that increase in milk pH caused drastic decline in MCA of crude jackfruit protease.

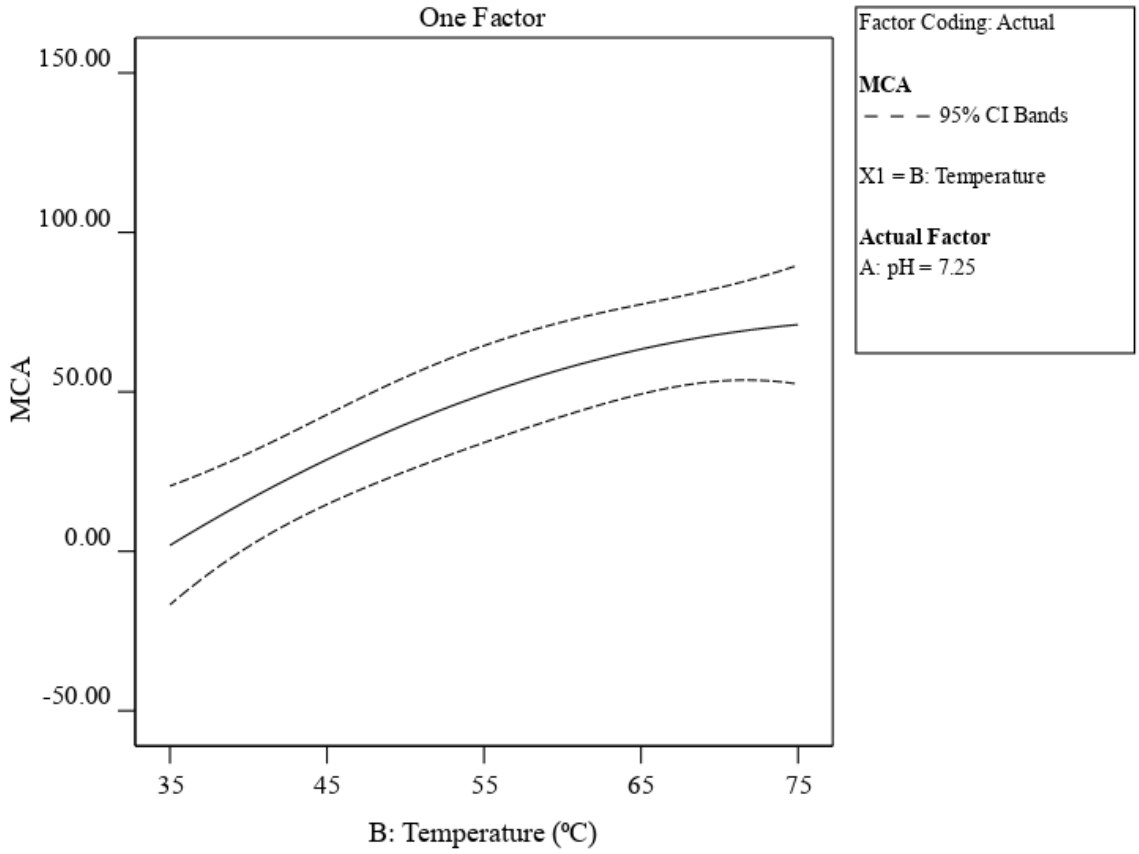

Fig. 4.12 Single factor interaction graph of MCA of crude aank protease for individual factor B: temperature

From Fig. 4.12, it can be concluded that increase in milk temperature resulted in drastic non-linear increase in MCA of crude jackfruit protease.

4.1.1 Optimization of crude enzymes

A numerical response optimization technique was applied to determine the optimum combination of temperature of milk and pH of milk for the minimum time of coagulation and maximum milk clotting activity, which is shown in Table 4.1 and Table 4.2.

Table 4.1 Different constraints for optimization of crude aank protease

Name	Goal	Lower limit	Upper limit
Temperature of milk (^{0}C)	is in range	30	50
pH of milk	is target 6.5	4.5	6.5
Time of coagulation (s)	Minimize	40	315
Milk clotting activity (U/ml)	Maximize	38.09	300

Under the assumptions by Design Expert (version 12), the optimum conditions for minimum TOC and MCA of crude aank proteases were found to be 6.5 pH and 45ºC of milk temperature. The response predicted by the software for these optimum conditions reported MCA of 300 units at 40 s of coagulation time.

Table 4.2 Different constraints of optimization for crude jackfruit protease

Name	Goal	Lower limit	Upper limit
Temperature of milk (^{0}C)	is in range	35	75
pH of milk	is target 6.5	6.5	8
Time of coagulation (s)	Minimize	108	10800
Milk clotting activity (U/ml)	Maximize	1.11	111.11

Under the assumptions by Design Expert (version 12), the optimum conditions for minimum time of coagulation and maximum milk clotting activity of crude jackfruit protease were found to be 6.5 pH and 65ºC of milk temperature. The response predicted by the software for these optimum conditions reported MCA of 111.11 units at 108 s of coagulation time.

4.1.2 Protease activity and protein concentration of crude enzymes

Crude proteases were subjected to protease activity determination at enzyme concentration 1%, pH 6.5 and incubation time 10 min. Crude proteases were also subjected to protein determination of different enzyme extracted from plant latex. The results for protease activity were calculated using equation deduced from standard curve (Fig. D.1) and the results for protein concentration were calculated using equation deduced from standard curve (Fig. D.2). The results of protease activity and protein concentration are tabulated in Table 4.3.

Table 4.3 Protease activity and protein concentration of crude enzymes

Parameters	Plant latex	
	Calotropis gigantea	*Artocarpus heterophyllus*
Protease activity (µMoles Tyrosine)	$0.544^{a} \pm 0.003$	$0.286^{b} \pm 0.001$
Protein concentration (µg/ml)	$0.106^{a} \pm 0.005$	$0.139^{b} \pm 0.004$

Note: Values are the mean of three determinations. Figures after ± are standard deviation.

From Table 4.3, protease activity of crude *Calotropis gigantea* protease was found to be 0.544 µmoles tyrosine which is in agreement with the data given by Bindhu and Singh (2014) and the proteolytic activity of crude *Artocarpus heterophyllus* protease was 0.286 umoles tyrosine which was slightly higher than the data given by Singh *et al.* (2015). Variation might be due to the differences in species of the plants producing serine proteases. The protease activity of crude *Calotropis gigantea* protease was significantly ($P<0.05$) higher than the protease activity of crude *Artocarpus heterophyllus* protease (Table D.1). The protein

concentration of crude *Artocarpus heterophyllus* protease is 1388 mg/ml which is higher than the results given by Siritapetawee *et al*. (2012) and the protein concentration of crude *Calotropis gigantea* is 1058 mg/ml which is higher than the results given by Freitas *et al.* (2007). The variation in data of protein content can be attributed to the difference in variety, climatic condition, soil condition, maturity stages of plants and experimental conditions. Protein concentration of crude Artocarpus heterophyllus protease was significantly ($P<0.05$) higher than the protein concentration of crude Calotropis gigantea protease as revealed by (Table D.2).

4.1.3 Verification of model

Within the scope of the variables studied in Central Composite Design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. The conditions and the results of the confirmatory experiments are presented in Table 4.4.

Table 4.4 Predicted and actual values of the responses at the optimized condition

Response	Conditions			Predicted value	Mean Observed value
	Crude Enzyme	Temperature of milk (ºC)	pH of milk		
TOC (s)	*Calotropis gigantea*	45	6.5	64.825	67.333
MCA (U/ml)	*Calotropis gigantea*	45	6.5	224.691	216.977
TOC (s)	*Artocarpus heterophyllus*	65	6.5	537.72	533.333
MCA (U/ml)	*Artocarpus heterophyllus*	65	6.5	99.60	101.12

4.2 Physicochemical properties

4.2.1 Chemical composition of raw milk

The proximate composition of raw cow milk is given in Table 4.5.

Table 4.5 Proximate composition of raw cow milk

Parameters	Cow milk
Moisture (%)	87.3±0.17
Fat (%)	3.7±0.05
Protein (%)	3.2±0.02
SNF (%)	8.57±0.14
Ash (%)	0.7±0.01
pH	6.5±0

Note: Values are the means of three determinations. Figures after ± are the standard deviation.

The resulted presented in Table 4.6 revealed that the moisture, fat, protein, SNF, ash and pH in cow milk were 87.3%, 3.7%, 3.2%, 8.57%, 0.7% and 6.5 respectively. The values are similar to those reported by Walstra *et al.* (2006) and any variation may be due to cow breed, milking conditions and milking time.

4.2.2 Chemical composition of soft cheese

The chemical composition of the cream cheeses made from rennet (A*), Calotropis gigantea* latex (B) and *Artocarpus heterophyllus* latex (C) has been shown in Table 4.6.

The moisture percentage is in the line with the findings of Johnson *et al.* (2001) and the findings of Nawaz *et al.* (2011) but higher than that of Rana *et al.* (2017). The results were

higher than the findings of Islam (2006), who reported moisture content of cows' milk cheese as 46.8%. The variation in moisture might be due to the difference in milk composition, activity of coagulant and processing techniques. Among the three cheeses the average moisture was more in C. Variation in moisture might be attributed due to the difficulty in whey drainage, resulting from clogging of the drainage screen by fine particles. Similar views were expressed by Hill *et al.* (1982). Significant differences ($P<0.05$) were observed between all three samples regarding moisture content (Table F.1). Moisture content in soft cheeses made using crude plant proteases were higher than that of rennet. Molecular forces involved in the coagulation of casein by crude plant proteases resulted into a greater water binding capacity of protein matrix of cheese which could be the reason of variation. Longer coagulation time for plant proteases results in more moisture retention in the final product which might be the reason for significant difference (Johnson *et al.*, 2001)

The fat level correlated with findings of Nawaz *et. al.* (2011). The results were similar to the findings of Ghosh and Singh (1990) and Islam (2006). Among three samples, the average fat was highest in A and lowest in C. The fat is one of the leading factors in determining the characteristic body, texture and flavor of cheese Abd El- Gawad *et al.* (2007). The lower value fat contents recorded in cheese made using plant latex might be due to the fact that it takes more time for coagulation as compared to rennet. This may be responsible for the retention of fat in the final product Khan and Masud (2013). Analysis of variance (Table F.2) regarding fat contents revealed that significant difference ($P<0.05$) was found between A and B, and A and C, while, non-significant difference ($P>0.05$) was recorded between B and C.

The protein contents of all the cheeses are in line with the findings of Nawaz *et. al.* (2011). Proteolytic plant proteases are responsible for the formation of nitrogenous products of intermediate size, such as proteoses, peptones, polypeptides, peptides and free amino acids. Enzymes of microorganism act on these and other substances to form products like amino acids, amines, fatty acids, esters, aldehydes, alcohols and ketones Fox and McSweeney (2017). Among three samples, the average protein contents were more in B. Analysis of variance (Table F.3) regarding protein contents revealed that non-significant difference ($P>0.05$) was recorded

among all treatments. Disparity could be due to crude poroteases as it might contain proteinaceous material in it.

Table 4.6 Chemical composition of soft cheeses.

Parameters	Cheese samples		
	A	B	C
Moisture (%)	$48.6^{a}\pm0.07$	$50.29^{b}\pm0.22$	$54.32^{c}\pm0.07$
Fat (%)	$37.75^{a}\pm0.871$	$34.05^{b}\pm0.355$	$33.13^{b}\pm1.441$
Protein (%)	$25.16^{a}\pm0.454$	$25.36^{a}\pm1.499$	$25.51^{a}\pm0.246$
Ash (%)	$2.68^{a}\pm0.145$	$3.16^{b}\pm0.076$	$3.28^{b}\pm0.035$
pH	$5.63^{a}\pm0.005$	$6.1^{b}\pm0.01$	$6.11^{b}\pm0.005$
Acidity (% lactic acid)	$0.2^{a}\pm0.017$	$0.17^{ab}\pm0.01$	$0.15^{b}\pm0.017$
Calcium (mg/100 g)	$635.3^{a}\pm1.228$	$624.3^{b}\pm2.091$	$635.3^{c}\pm1.69$

Note: Values are the means of three determinations. Values of all parameters except moisture content and pH are expressed in terms of dry basis. Figures after ± sign are the standard deviation. Values in the row bearing similar superscript are not significantly different at 5% level of significance.

The ash contents of cream cheese are similar to the findings of Mijan *et al*. (2010). Khan and Masud (2013) and Patel and Gupta (1986), who had reported that the ash contents of cheese ranged from 2.50 to 3.20%. The possible reason for the less ash contents may be the seasonal variation in the composition of milk. Among three samples the average ash contents were more in C. The higher value was recorded in case of plant coagulants as compared to rennet, which is probably due to the remains of plant materials in the crude enzyme. Analysis of variance (Table

F.4) regarding ash contents revealed that significant difference (P>0.05) was found between A and B, and A and C, while, non-significant differences (P<0.05) was recorded between B and C.

pH is the most vital indicator of food quality and safety. pH of food such as milk and milk products are measured to ensure the quality of foodstuff (Razzaq, 2003). The functional properties of cheese are greatly influenced by the pH (Rowney *et al.*, 1999). Analysis of variance (Table F.6) regarding pH revealed that there were non-significant differences (P<0.05) was seen between B and C while significant difference (P>0.05) was found between A and B, and A and C. Among three samples the average pH was most in C and least in A. The possible variation may be due to the processing techniques, effect of coagulants, incubation and coagulation time and temperature. pH of the cheese may vary due to the strength of coagulants.

The titratable acidity of cheeses is similar to the findings of Nawaz *et al.* (2011). Table F.5 also revealed a non-significant (P<0.05) difference was found between sample A and B, and B and C, while, significant difference (P>0.05) was recorded between sample A and C. Slight increase in acidity was observed in cheese prepared with calf rennet which is in line with the findings of Nunez *et al.* (1991) who reported the higher acidity in cheese prepared with animal rennet due to higher whey retention and subsequent lactose fermentation. Analysis of variance (Table F.5) regarding titratable acidity revealed that non-significant difference (P>0.05) was seen between B and C, and A and B, while, significant difference (P<0.05) was found between A and C.

The calcium content of cheeses A, B and C range from 615-632 mg/100 g of cheese. Among three samples the calcium content was most in A. According to Keller *et al.* (1973), calcium content inversely correlates to the moisture content of the cheese. Similarly, Joshi *et al.* (2004) reported that caseins are more hydrated as the level of bound calcium decreases in milk. Analysis of variance (Table F.7) regarding calcium content revealed that significant difference (P>0.05) among all treatment.

4.2.3 Theoretical and actual yield

The theoretical and actual yield of cheeses A, B and C have been presented in Table E.1 and shown in Fig. 4.13.

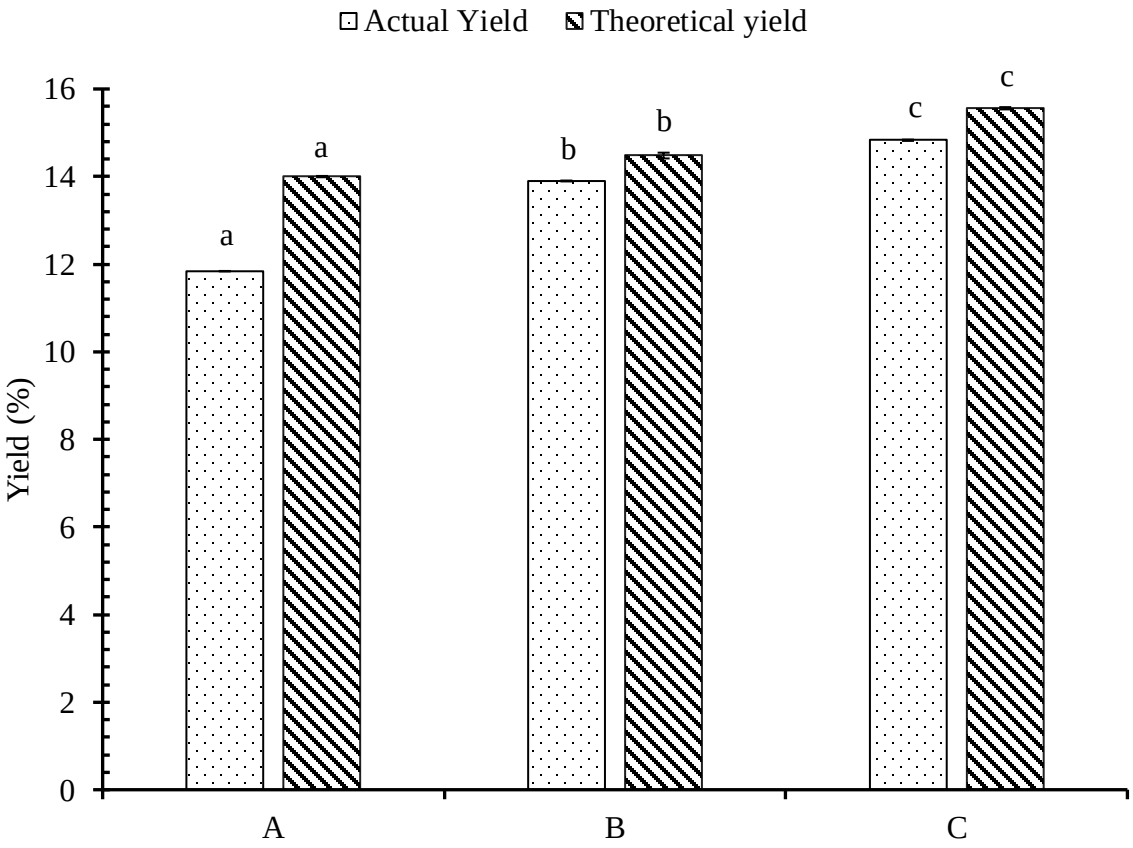

Fig. 4.13 Theoretical and Actual yield of cream cheeses

*bars with different alphabets are significantly different at $p<0.05$.

4.2.3.1 Theoretical yield

Theoretical yield of cheese can be estimated from the milk fat and casein or protein contents of milk by using Van Slyke Equation of cheese yield. Different earlier workers worked on the theoretical yield of specialty cheeses like Cheddar cheese (Barbano, 1999) and Mozzarella cheese (Rudan *et al.*, 1999). The theoretical yield of the cheeses is shown in Table F.1. Among them the average theoretical yield was more in C. The slight variation in theoretical yield might

be due to the moisture contents in final cheese (Melili *et al.*, 2002). Analysis of variance (Table F.8) regarding theoretical yield revealed that there was significant difference (P>0.05) among all treatments.

4.2.3.2 Actual yield

Optimum yield of cheese is of vital importance for cheese in cheese making operation. Emmons and Binns (1990) reported that accurate estimates of cheese yield were of great importance in establishing the relationship between composition of milk and yield of cheese and in assessing the efficiency of an operation in converting milk into cheese. The actual yield of soft cheeses is shown in Table E.1. Actual yield of all samples is lower than the findings of Mahajan and Chaudhari (2014). The variation may be due to difference in milk composition and processing technique. Actual yield is always lower than the theoretical yield. The yield reduction may be due to poor cheese making technique resulting in low casein and/or fat retention. Among three treatments the average actual yield was more in C. The higher yield in cheese prepared with plant protease may be attributed to the longer coagulation time resulting in more moisture contents which increased the yield. Analysis of variance (Table F.9) regarding actual yield revealed that there was significant difference (P>0.05) among all treatments.

4.3 Microbiological analysis

The result of microbiological analysis of soft cheese samples are presented in Fig. 4.14 and in Table E.2 (Appendix E). It showed the average value for TPC and yeasts and molds of sample.

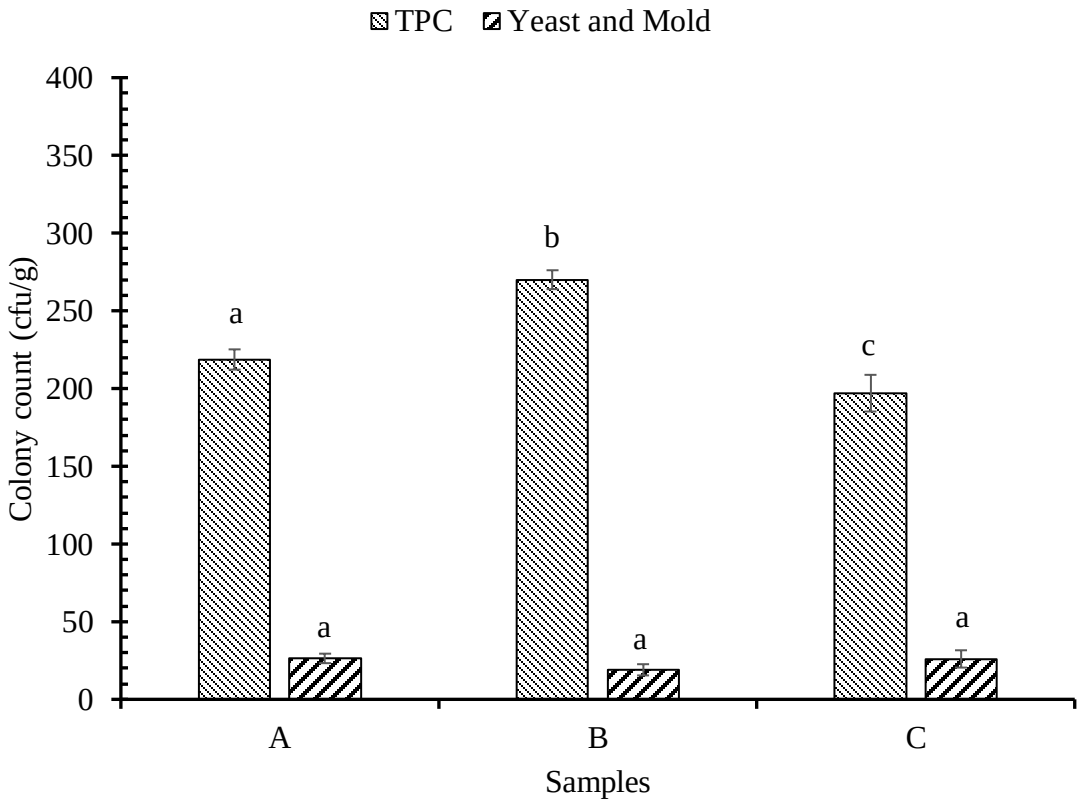

Fig. 4.14 Microbiology of Cream cheese

*Values are the means of two determinations. Values on top bars bearing similar superscript are not significantly different at 5% level of significance.

Significant difference (P>0.05) was found for TPC (Table F.10) but non-significant differences were found for yeast and molds count (Table F.11) of samples. For TPC, all the samples were significantly different from each other whereas for yeast and molds count, non-significant differences were found between all the samples at 5% level of significance.

Coliform were not detected in all cheese samples. It might be due to heat treatment during cheese making. However, the presence of TPC and yeasts and molds were detected which might be due to handling contamination or oxygen trapped in the package. According to Kosikowski and Fox (1968), heat treatment at 61.1°C for 16.5 s reduced the coliform count by 93.8%. Also,

low heat treatment at 57.2, 58.9, 60 and 61.1℃ for 16.5 s reduced the coliform count by 57.3, 75.0, 81.5 and 93.8%, respectively.

Part V

Conclusions and recommendations

5.1 Conclusions

On the basis of this research, the following conclusions can be drawn:

1. The optimized time of coagulation and milk clotting activity of crude aank protease were 300 units and 40 s respectively and for crude jackfruit protease it was 111.11 units and 108 s.
2. The protease activity of crude aank protease and crude jackfruit protease were found to be 0.544 units/ml of enzyme and 0.286 units/ml of enzyme respectively.
3. The protein concentration of prepared crude aank protease and crude jackfruit protease 1058 mg/ml and 1388 mg/ml respectively
4. Numerical optimization study revealed that the optimum condition for maximum milk clotting activity and minimum time of coagulation for crude aank protease were pH 6.5 and temperature 45ºC and for crude jackfruit protease the optimized conditions were pH 6.5 and temperature 67ºC.
5. The physico-chemical analysis, showed that there were no significant differences ($P>0.05$) in protein among cheese samples made using rennet and crude enzyme proteases while significant differences were observed in moisture, fat, ash, calcium, acidity, pH and cheese yield.
6. Coliforms were not detected in all samples. Microbiological analysis showed significant difference ($P>0.05$) in total plate count was observed in all three samples while no significant difference was found in the count of yeast and mold.

5.2 Recommendations

Based on the current study the following recommendations can be made:

1. Purification of crude aank and jackfruit proteases can be done.
2. Other plant extracts such as bromelain, ficin etc can be used as rennet substitute to prepare cheese.

Part VI

Summary

The main objective of this research work is to extract proteolytic enzymes from the latex of aank (*Calotropis gigantea*) and jackfruit (*Artocarpus heterophyllus*) plants to be used as milk coagulants in cheesemaking. The milk clotting activity (MCA), proteolytic activity (PA) and protein content of the crude plant proteases were determined and the optimized conditions of the crude proteases for maximum MCA and minimum TOC was determined using response surface methodology. Soft cheese samples were prepared using crude plant proteases. The physicochemical and microbiological properties of soft cheese prepared were compared with control which was prepared using calf rennet as milk coagulant.

Investigation was done on the impact of processing parameters namely pH of milk and temperature of milk on TOC and MCA by response surface methodology. An empirical quadratic model was applied to experimental data pertaining to the average enzymatic activity and equation describing the optimal conditions was obtained. For crude *Calotropis gigantea* protease, the optimized time of coagulation (40 s) and milk clotting activity (300 units) were obtained at pH of milk 6.5 and temperature of milk 45ºC. Similarly, for *Artocarpus heterophyllus* proteases, the optimized time of coagulation (108 s) and milk clotting activity (111.11 units) were obtained at pH of milk 6.5 and temperature 65ºC. Similarly, the protease activity of the enzyme was also determined using optimized condition which justified the use of those conditions. These optimized values were applied in the preparation of soft cheese. The physicochemical analysis showed that protein content of cheese samples was non-significantly different ($P>0.05$) but significant ($P<0.05$) difference was observed in number of parameters like moisture, fat, ash, pH, acidity and calcium. Microbiological analysis showed that coliforms were not detected in all cheese samples but TPC and yeast and molds were detected. The average TPC and yeast and molds count for crude *C. gigantea* cheese sample was 270 cfu/g and 197 cfu/g. Similarly, the average TPC and yeast and molds count for crude *A. heterophyllus* cheese sample was 19 cfu/g and 26 cfu/g respectively.

Therefore, it was observed that crude protease from *C. gigantea* and *A. heterophyllus* showed good results in the production of soft cheese. The quality of the cheese prepared from this easily available source can further be improved by initiating the process of purification of proteases used.

References

Abada, E. A. (2019). Application of microbial enzymes in the dairy industry. *In:* "Enzymes in Food Biotechnology". (M. Kuddus, Ed.). pp. 61-72. Academic Press. [ISBN 978-0-12-813280-7].

Abd El- Gawad, A. M. and Ahmed, N. (2011). Cheese yield as affected by some parameters Review. *Acta Sci. Pol., Technol. Aliment.* **10** (2), 131-153.

Abd El- Gawad, A. M., Sabek Reda, A. M. and Hegazi Nagla, A. (2007). Quality and properties of low-fat buffaloe`s Kashkaval cheese. *Int. J. Dairy Sci.* **2**, 244-251. [doi: 10.3923/ijds.2007.244.251].

Abraham, K. I. and Joshi, P. N. (1979). Studies on proteinases from *Calotropis gigantea* latex. I. Purification and some properties of two proteinases containing carbohydrate. *Biochimica Biophysica Acta*. **568** (1), 111-119. [doi: 10.1016/0005-2744(79)90278-x].

Acharya, P. P. (2010). "Textbook of Dairy Chemistry". Highland Publication (P) Ltd. Kathmandu, Nepal.

Ahmed, I. A. M., Morishima, I., Babiker, E. E. and Mori, N. (2009). Characterization of partially purified milk clotting enzyme from *Solanum dubium* fresen seeds. *Food Chem.* **116**, 395-400.

Alagarsamy, S., Larroche, C. and Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade proteases: A perspective. *Food Technol. Biotechnol.* **44**, 211-220.

Amenu, B. (2004). Environmental and dietary effects on milk composition and cheddar cheese yield. Ph.D Thesis. The University of Queensland, Brisbane, Australia.

AOAC. (2005). "Association of Official Analytical Chemists".CRC, Washington.

Aquilanti, L., Babini, V., Santarelli, S., Osimani, A., Petruzzelli, A. and Clementi, F. (2011). Bacterial dynamics in raw cow's milk cheese manufactured with aqueous extracts of *Cynara cardunculus* dried flowers. *Lett. in Appl. Microbiol.* **52**, 651-659.

Asakura, T., Watanabe, H., Keiko, A. and Soichi, A. (1997). Oryzasin as an aspartic proteinase occurring in rice seeds: purification, characterization, and application to milk clotting. *J. Agric. Food Chem.* **45** (4), 1070-1075. [doi: 10.1021/jf960582x].

Bah, S., Paulsen, B. S., Diallo, D. and Johansen, H. T. (2006). Characterization of cysteine proteases in malian medicinal plants. *J. Ethnopharmacol.* **107** (2), 189-198. [doi: 10.10.16/j.jep.2006.03.008.].

Banks, J. M. (1992). Cheese. *In:* "The Technology of Dairy Products". (R. Early, Ed.). New York. VH Publishers Inc.

Banks, J. M. (2011). Cheese: Cheddar-type cheese. *In:* "Encyclopedia of Dairy Sciences". (J. W. Fuquay, Ed.). pp. 706-711. San Diego. Academic Press.

Barbano, D. M. (1999). Controlling functionality of mozzarella cheese through process control. Presented at Proc. Marshall Italian Cheese Sem. Rhone-Poulenc, Madison, WI.

Barros, R. M., Ferreira, C. A., Silva, S. V. and Malcata, F. X. (2001). Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. *Enzyme and Microbial Technol.* **29**, 541-547.

Beka, R. G., Carl, M. M., David, G. L., Didier, G., Dominique, V. M., François, K., Krasimir, D., Magali, B., Marie-Christine, S., Pauline, D. and Valentin, D. G. (2014). Characterisation of a milk-clotting extract from *Balanites aegyptiaca* fruit pulp. *Int. Dairy J.* **34** (1), 25-31. [doi: 10.1016/j.idairyj.2013.06.013].

Ben Amira, A., Makhlouf, I., Flaviu Petrut, R., Francis, F., Bauwens, J., Attis, H., Besbes, S. and Blecker, C. (2017). Effect of extraction pH on techno-functional properties of crude extracts from wils cardoon (*Cynara cardunculus* L.) flowers. *Food Chem.* **225**, 258-266.

Berridge, N. J. (1952). An improved method of observing the clotting of milk containing rennin. *J. Dairy Sci.* **19**, 328-329.

Bindhu, O. and Singh, M. (2014). Hemostatic, milk clotting and blood stain removal potential of cysteine proteases from *Calotropis gigantea* (L.) R. Br. latex. *Pharmacognosy Magazine*. **10** (38), 350-356. [doi: 10.4103/0973-1296.133294].

Borgono, C. A., Michael, I. P., Shaw, J. L. V., Lui, L. Y., Glosh, M. C. and Soosaipillai, A. (2007). Expression and functional characterization of the cancer-related serine protease, human tissue kallikrein. *J. Biol. Chem.* **161**, 2405-2522.

Broome, M. C., Powell, I. B. and Limsowtin, G. K. Y. (2011). Cheese starter cultures: specific properties. *In:* "Encyclopedia of Dairy Sciences" (2nd ed.). (J. W. Fuquay, Ed.). pp. 559-566. San Diego. Academic Press. [ISBN 978-0-12-374407-4].

Bruno, M. A., Lazza, C. M., Errasti, M. E., Lopez, L. M. I., Caffini, N. and Pardo, M. F. (2010). Milk clotting and proteolytic activity of an enzyme preparation from *Bromelia hieronymi* fruits. *LWT Food Sci Technol*. **43**, 695-701.

Bruno, M. A., Pardo, M. F., Caffini, N. O. and Lopez, L. M. I. (2002). Purification of a new endo peptidase isolated from the fruits of *Bromelia hieronymi* Mez (Bromeliaceae). *Acta Farm Bonaer*. **21**, 51-56.

Brutti, C. B., Pardo, M. F., Caffini, N. O. and Natalucci, C. L. (2012). *Onopordum acanthium* L. (Asteraceae) flowers as coagulating agent for cheesemaking. *LWT Food Sci. Technol*. **45**, 172-179.

Buranov, A. U. and Elmuradov, B. J. (2010). Extraction and characterization of latex and natural rubber from rubber-bearing plants. *J. Agric. Food Chem.* **58** (2), 734-743. [doi: 10.1021/jf903096z].

Bylund, G. (1995). "Dairy Processing Handbook". Tetrapak Processing System AB. S-22186 Lund, Sweden.

Cavalcanti, M. T. H., Teixeira, M. F. S., Filho, J. L. L. and Porto, A. L. F. (2004). Partial purification of new milk clotting enzyme produced by *Nocardiopsis* sp. *Bioresource Technol.* **93**, 29-35.

Cavalli, V. S., Claver, S., Priolo, N. and Natalucci, C. (2005). Etraction and partial charcterization of a coagulant preparation from *Silybum marianum* flowers. Its action on bovine caseinate. *J. Dairy Res.* **72**, 271-275.

Chazzara, S., Sidrach, L., Lopez, M. D. and Rodriguez, L. J. N. (2007). Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus*) flowers. *Int. J. Dairy*. **17**, 1393-1400.

Cimino, C., Cavalli, V. S., Spina, F., Natalucci, C. and Priolo, N. (2006). Callus culture for biomass production of milk thistle as a potential source of milk-clotting peptidases. *Electron J. Biotechnol.* **9**, 297-240.

Claverie-Martin, F. and Vega-Hernandez, F. C. M. (2007). Aspartic proteases used in cheese making. *In:* "Industrial Enzyme, Structure, Function and Appllication". (J. Polaina and A. P. MacCabe, Eds.). pp. 207-220. Dordrecht, Netherlands. Springer. [ISBN 978-1-4020-5377-1].

Cupp-Enyard, C. and Aldrich, S. (2008). Sigma's non-specific protease activity assay- casein as a substrate. *J. Visualized Experiments*. **19**. [doi:10.3791/899].

Davidson, P. M. and Zivanovic, S. (2003). The use of natural antimicrobials. *In:* "Food Preservation Techniques". (P. Zeuthen and L. Bøgh-Sørensen, Eds.). pp. 5-30. Woodhead Publishing. [ISBN 978-1-85573-530-9].

Davies, D. T. and Law, A. J. R. (1983). Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. *J. Dairy Res.* **50**, 67-75.

De, S. (2000). "Outlines of Dairy Technology". Oxford University Press, New Delhi.

Devaraj, K. B., Gowda, L. R. and Prakash, V. (2008). An unusual thermostable aspartic protease from the latex of *Ficus racemosa* (L.). *Phytochem.* **69**, 647-655.

Dhillon, A., Sharma, K., Rajulapati, V. and Goyal, A. (2016). Proteolytic Enzymes. *In:* "Current Developments in Biotechnology and Bioengineering Production, Isolation and Purification of Industrial Products" (1st ed.). (A. Pandey, N. Sangeeta and C. R. Soccol, Eds.). pp. 149-173. Amsterdam, Netherlands. Elsevier Radarweg. [ISBN 978-0-444-63662-1].

Duarte, A. R., Duarte, D. M. R., Moreira, K. A., Cavalcanti, M. T. H., De Lima-Filho, J. L. and Porto, A. L. F. (2009). A new vegetable source for milk-clotting enzymes. *Braz. Arch. Biol. Technol.* **52**, 1-9.

Düsterhöft, E. M., Engels, W. and Huppertz, T. (2017). Gouda and Related Cheeses. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 865-888. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

Egito, A. S., Girardet, J. M., Laguna, L. E., Poirson, C., Molle, D., Miclo, L., Humbert, G. and Gaillard, J. L. (2007). Milkclotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine κ-casein. *Int. Dairy J.* **17**, 816-825.

Emmons, D. B. and Binns, M. (1990). Cheese yield experiments and proteolysis by milk-clotting enzyme. *J. Dairy Sci.* **73** (8), 2028-2043.

Esteves, C. L. C., Lucey, J. A., Hyslop, D. B. and Pires, E. M. V. (2003). Effect of gelation temperature on the properties of skim milk gels made from plant coagulants and chymosin. *Int. Dairy J.* **13**, 877-885. [doi: 10.1016/S0958-6946(03)00114-6].

Fagan, C. C., O'Callaghan, D. J., Mateo, M. J. and Dejmek, P. (2017). The synersis of rennet-coagulated curd. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 145-177. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

FAO. (1999). "Manual on the Use of the LP System in Milk Handling and Preservation". Rome, Italy.

Farkye, N. Y. (2004). Cheese Technology. *J. Soc. Dairy Technol.* **57**, 91-98. [doi: 10.1111/j.1471-0307.2004.00146.x].

Fox, P. F. (2011). Cheese: Overview. *In:* "Encylopedia of Dairy Science". (J. W. Fuquay, Ed.). pp. 533-543. San Diego. Academic Press.

Fox, P. F. and Cogan, T. M. (2004). "Cheese: Chemistry, Physics and Microbiology" (3rd ed.). Chapman and Hall. London, UK. [ISBN 01-1226-3651-1].

Fox, P. F., Guinee, T. P., Cogan, T. M. and McSweeney, P. L. H. (2000). Overview of cheese manufacture. *In:* "Fundamentals of Cheese Science". (P. F. Fox, Ed.). pp. 1-20. Boston, MA. Springer Science and Buisness Media. [ISBN 978-1-4899-7681-9].

Fox, P. F. and McSweeney, P. L. H. (2017). Cheese: An Overview. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 9-19. San Diego Elsevier Applied Science. [ISBN 978-0-12-417012].

Freitas, C. D. T., Leite, H. B., Oliveira, J. P., Amaral, J. L., Egito, A. S., Vairo-Cavalli, S., Lobo, M. D., Monteiro-Moreira, A. C. and Ramos, M. V. (2016). Insights into milk-clotting activity of latex peptidases from *Calotropis procera* and *Cryptostegia grandiflora*. *Food Res. Int.* **87**, 50-59. [doi: 10.1016/j.foodres.2016.06.020].

Freitas, C. D. T., Oliveira, J. S., Miranda, M. R. A., Macedo, N. M. R., Sales, M. P., Villas-Boas, L. A. and Ramos, M. V. (2007). Enzymatic activities and protein profile of latex from Calotropis procera. *Plant Physiol. Biochem.* **45**, 781-789. [doi: 10.1016/j.plaphy.2007.07.020].

Gagaoua, M., Hoggas, N. and Hafid, K. (2015). Three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* roscoe rhizomes. *Int. J. Biol. Macromol.* **73**, 245-252. [doi: 10.1016/j.ijbiomac.2014.10.069].

Galan, E., Prados, F., Pino, A., Tejada, L. and Fernandes, F. J. (2008). Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on

the proteolysis and sensory characteristics of cheeses made with sheep milk. *Int. Dairy J.* **188**, 93-98.

Ghosh, B. C. and Singh, S. (1990). Effect of heat treatment on the quality of mozzarella cheese from buffalo milk. *Ind. J. Food Sci. Technol.* **27** (4), 218-220.

Giffel, M. C. and Wells-Bennik, M. H. J. (2010). Good hygienic practice in milk production and processing. *In:* "Improving the Safety and Quality of Milk". (M. W. Griffiths, Ed.). pp. 179-193. Woodhead Publishing. [ISBN 978-1-84569-438-8].

Godhia, M. L. and Patel, N. (2013). Colostrum-its composition, benefits as a nutraceutical : a review. *Curr. Res. in Nutr. Food Sci.* **1**, 37-47. [doi: 10.12944/CRNFSJ.1.1.04].

Gomez, R., Sanches, E., Vioque, M., Ferreira, J., Tejada, L. and Fernandes, S. J. (2001). Microbiological characteristics of ewe's milk cheese manufactured using aqueous extracts of flowers from various species of cardoon *Cynara I. Milchwissenschaft*. **56**, 16-19.

González-Rábade, N., Badillo-Corona, J. A., Aranda-Barradas, J. S. and Oliver-Salvador Mdel, C. (2011). Production of plant proteases in vivo and in vitro-a review. *Biotechnol. Adv*. **29** (6), 983-996. [doi: 10.1016/j.biotechadv.2011.08.017].

Grudkowska, M. and Zagdanska, B. (2004). Multifunctional role of plant cysteine proteinases. *Acta Biochem Pol*. **51**, 609-624.

Guinee, T. P. (2007). Salt in cheese. *In:* "Cheese Problems Solved". (P. L. H. McSweeney, Ed.). pp. 80-99. Woodhead Publishing. [ISBN 978-1-84569-060-1].

Guinee, T. P. and Fox, P. F. (2017). Salt in cheese: physical, chemical and biological aspects. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 317-375. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

Guinee, T. P. and McSweeney, P. L. H. (2006). Significance of milk fat in cheese. *In:* "Advanced Dairy Chemistry Volume 2 Lipids" (Vol. 2). (P. F. Fox and P. L. H. McSweeney, Eds.). pp. 337-440. Boston, MA. Springer Science+Business Media. [ISBN 978-0-387-28813-0].

Guinee, T. P., Muholland, E., O'Brien, B. and Murphy, J. (2001). Effect of diet quality on suitability of mid-lactation bovine milk for cheddar cheese manufacture. *Aust. J. Dairy Technol.* **56**, 3-8.

Guinee, T. P. and O'Kennedy, B. T. (2007). Reducing salt in cheese and dairy spreads. *In:* "Reducing Salt in Foods". (D. Kilcast and F. Angus, Eds.). pp. 316-357. Woodhead Publishing. [ISBN 978-1-84569-018-2].

Hashim, M. M., Iqbal, M. F., Xiahong, C. and Mingsheng, D. (2011). Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochem.* **72**, 458-464.

Heinemann, U., Pal, G. P., Hilgenfeld, R. and Saenger, W. (1982). Crystal and molecular structure of the sulfhydryl protease calotropin DI at 3.2 A resolution. *J. Mol. Biol.* **161** (4), 591-606. [doi: 10.1016/0022-2836(82)90410-7].

Hellmuth, K. and Van Den Brink, J. M. (2013). Microbial production of enzymes used in food applications. *In:* "Microbial Production of Food Ingredients, Enzymes and Nutraceuticals". (B. McNeil, D. Archer, L. Giavasis and L. Harvey, Eds.). pp. 262-287. Woodhead Publishing. [ISBN 978-0-85709-343-1].

Hill, A. R., Bullock, D. H. and Irvine, D. M. (1982). Manufacturing parameters of Queso Blanco made from milk and recombined milk. *J. Food Sci. Technol.* **15**, 47.

Hort, J. and Le Grys, G. (2001). Developments in the textural and rheological properties of UK cheddar cheese during ripening. *Int. Dairy J.* **11**, 475-481.

Islam, M. W. (2006). Shelf-life of cheese as affected by salt content with particular reference to mozzarella cheese. M.Sc. Thesis. Bangladesh Agricultural Univer., Bangladesh.

Jacob, M., Jaros, D. and Rohm, H. (2011). Recent advances in milk clotting enzymes. *Int. J. Dairy Technol.* **64**, 14-33.

Jain, S., Gupta, S., Kumar, A., Bangar, Y. and Ahlawat, S. S. (2019). Preparation and evaluation of cottage cheese using enzyme extracted from mustard oilseed cake. *Int. J. Curr. Microbiol. Appl. Sci.* **8** (12), 1202-1209.

Johnson, M. E., Chen, C. M. and Jaeggi, J. J. (2001). Effect of rennet coagulation time on composition, yield, and quality of reduced-fat cheddar cheese. *J. Dairy Sci.* **84** (5), 1027-1033. [doi: 10.3168/jds.S0022-0302(01)74562-6].

Joshi, N. S., Muthukumarappan, K. and Dave, R. I. (2004). Effect of calcium on microstructure and metability of part skim mozzarella cheese . *J. Dairy Sci.* **24**, 1027-1033.

Kazemipour, N., Salehi Inchebron, M., Valizadeh, J. and Sepehrimanesh, M. (2017). Clotting characteristics of milk by Withania coagulans: Proteomic and biochemical study. *Int. J. Food Prop.* **20** (6), 1290-1301. [doi: 10.1080/10942912.2016.1207664].

KC, J. B. and Rai, B. K. (2007). "Essentials of Food Chemistry." (1st ed.). Maya K.C. Kathmandu.

Keller, B., Olson, N. F. and Richardson, T. (1973). Mineral retention and rheological properties of mozzarella cheese made by direct acidifications. *J. Dairy Sci.* **57** (2), 174-180.

Khan, R. S. and Masud, T. (2013). Comparison of buffalo cottage cheese made from aqueous extract of *Withina coagulans* with commercial calf rennet. *Int. J. Dairy Technol.* **66** (3), 369-401. [doi: 10.1111/1471-0307.12048].

Kindstedt, P. S. and Kosikowski, F. V. (1985). Improved complexometric determination of calcium in cheese. *J. Dairy Sci.* **68**, 806.

Konkalmatt, P. and Virupaksha, T. (1990). Purification and characterization of a protease from jackfruit latex. **29**, 1763–1766. [doi: 10.1016/0031-9422(90)85011-4].

Kosikowski, F. V. (1982). "Cheese and Fermented Milk Foods". Edwards Bros. Inc. Michigan USA.

Kosikowski, F. V. and Fox, P. F. (1968). Low heat, hydrogen peroxide, and bactofugation treatments of milk to control coliforms in Cheddar cheese. *J. Dairy Sci.* **51** (7), 1018-1022. [doi: 10.3168/jds.0022-0302(68)87114-4].

Kumar, G., Loganathan, K. and Rao, B. (2011). A review on pharmacological and phytochemical profile of *Calotropis gigantea* Linn. **1**, 1-8.

Kumari, M., Sharma, A. and Jagannadham, M. V. (2010). Decolorization of crude latex by activated charcoal, purification and physico-chemical characterization of religiosin, a milk clotting serine protease from the latex of *Ficus religiosa*. *J Agric Food Chem* **58**, 8027-8034.

Lasztity, R. (2009). Milk and milk products. *Food Quality and Standards.* **2**, 156-168.

Law, B. A. and Tamine, A. Y. (2010). The quality of milk for cheese manufacture. *In:* "Technology of Cheesemaking." (2nd ed., Vol. 1). (T. P. Guinee and B. O'Brien, Eds.). pp. 1-67. UK. Wiley. [ISBN 9781444323740].

Lawrence, R. C., Gilles, J., Creamer, J. K., Crow, V. L., Heap, H. A., Honore, C. G., Johnston, K. A. and Samal, P. K. (2004). Cheddar cheese and related dry-salted cheese varieties. *In:* "Cheese: Chemistry, Physics, and Microbiology." (Vol. 2). (P. F. Fox, P. L. H. McSweeney, T. M. Cogan and T. P. Guinee, Eds.). pp. 72-102. Amsterdam; London:Elsevier.

Lee, M. R., Johnson, M. E. and Lucey, J. A. (2005). Impact of modifications in acid development on the insoluble Ca content and rheological properties of Cheddar cheese. *J. Dairy Sci.* **88**, 3798-3809.

Legg, A. K., Carr, A. J., Bennett, R. J. and Johnston, K. A. (2017). General aspects of cheese technology. *In:* "Cheese". (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 643-675. San Diego. Academic Press.

Li, Z., Scott, K., Hemar, Y., Zhang, H. and Otter, D. (2018). Purification and characterisation of a protease (tamarillin) from tamarillo fruit. *Food Chem.* **256**, 228-234. [doi: 10.1016/j.foodchem.2018.02.091].

Lo Piero, A. R., Puglisi, I. and Petrone, G. (2002). Characterization of "lettucine", a serine like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting. *J Agric Food Chem*. **50**, 695-701.

Lopes, A., Teixeira, G., Liberato, M. C., Pais, M. S. and Clemente, A. (1998). New vegetal source for milk clotting enzymes. *J. Mol. Cat. B: Enzymatic.* **83**, 181.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1), 265-275.

Lucey, J. A. (1993). Importance of calcium and phosphate in cheese manufacture: a review. *J. Dairy Sci.* **76**, 1714-1724.

Lucey, J. A. (2017). Formation, structural properties and rheology of acid-coagulated milk gels. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 179-193. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

Lufrano, D., Faro, R., Castanheira, P., Parisi, G., Verissimo, P., Vairo-Cavalli, S., Simoes, I. and Faro, C. (2012). Molecular cloning and characterization of procirsin, an active aspartic protease precursor from *Cirsium vulgare* (Asteraceae),. *Phytochem.* **69**, 647-655.

MacGibbon, A. K. H. and Taylor, M. W. (2006). Composition and structure of bovine milk lipids. *In:* "Advance Dairy Chemistry: Lipids" (3rd ed., Vol. 2). (P. F. Fox and P. L. H. McSweeny, Eds.). pp. 1-35. New York. Springer Science+Business Media Inc.

Mahajan, R. T. and Chaudhari, G. M. (2014). Plant latex as vegetable source for milk clotting enzymes and their use in cheese preparation. *Int. J. Adv. Res.* **2** (5), 1173-1181.

Maskey, B. and Shrestha, N. K. (2020). Optimization of crude papaya (*Carica papaya*) protease in soft-unripened cheese preparation. *J. Food Sci. Technol. Nepal*. **12**, 1-8.

Mazorra-Manzano, M. A., Moreno-Hernández, J. M., Ramírez-Suarez, J. C., De Jesús Torres-Llanez, M., González-Córdova, A. F. and Vallejo-Córdoba, B. (2013). Sour orange *Citrus aurantium* L. flowers: a new vegetable source of milk-clotting proteases. *LWT-Food Sci. Technol.* **54** (2), 325-330.

McSweeney, P. L. H., Fox, P. F., Cotter, P. D. and Everett, D. W. (2017). Legislation in relation to cheese. *In:* "Cheese" (4th ed.). (M. Hickey, Ed.). pp. 757-778. San Diego. Academic Press. [ISBN 9780124170124].

Melili, C., Lynch, J. M., Carpino, S., Barbano, D. M., Lichitra, G. and Cappa, A. (2002). An empirical method for prediction of cheese yield. *J. Dairy Sci.* **85**, 2699-2704.

Mijan, M. A., Haque, M. A., Habib, M. A. and Wadud, M. A. (2010). Evaluation of quality of mozzarella cheese. *The Bangladesh Veteriarian*. **21** (1), 36-42.

Mullan, W. M. A. (2008). Determination of the theoretical yield of cheddar cheese using milk composition only and a modified version of the Van Slyke yield equation. Retrieved from https://www.dairyscience.info/newCalculators/yield-01.asp. [Accessed 23 January, 2021].

Murugan, T. (2012). Antimicrobial activity of leaves and latex extracts of the herbal plant *Calotropis gigantea* (Erukku in Tamil). *Int. J. Biol., Pharmacy Appl. Sci.* **1**, 261-270.

Nassar, K. and Emirie, A. T. (2015). Milk quality for cheese production. B.Sc. Thesis. Swedish Univ. of Agricultural Sciences, Sweden.

Nawaz, M., Masud, T. and Sammi, S. (2011). Quality evaluation of mozzarella cheese made from buffalo milk by using paneer booti (*Withania coagulans*) and calf rennet. *Int. J. Dairy Technol.* **64**, 218-226. [doi: 10.1111/j.1471-0307.2010.00653.x].

Nunez, N., Fernandez, P. B., Rodr, M. M., Gaya, P. and Medina, M. (1991). Effect of vegetable and animal rennet on chemical, microbiological,rheological and sensorial characteristics of La Serena cheeses. *J. Dairy Res.* **58**, 511-519.

Oliveira, A., Perira, C., Soares da Costa, D., Teixeira, J., Fidalgo, F., Pereira, S. and Pissarra, J. (2010). Characterization of aspartic proteinases in *C. carfunculus* L. callus tissue for its prospective transformation. *Plant Sci.* **178**, 146-146.

Ong, L., Lawrence, R. C., Gilles, J., Creamer, L. K., Crow, V. L., Heap, H. A., Honore, C. G., Johnston, K. A., Samal, P. K., Powell, I. B. and Gras, S. L. (2017). Cheddar cheese and related dry-salted cheese varieties. *In:* "Cheese: Chemistry, Physics and Microbiology" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 829-863. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

Oseni, O. A. and Ekperigin, M. M. (2013). Distribution of proteolytic and milk clotting enzymes in the plant of Sodom apple *Calotropis procera* (Ait.) R. Br. (Asclepiadaceae). *Int. J. Biotechnol. Res.* **1** (2), 24-27.

Palma, J. M., Sandalio, L. M., Corpas, F. J., Romero-Puertas, M. C., McCarthy, I. and Del Rio, L. A. (2002). Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. *Plant Physiol. Biochem.* **40**, 521-530.

Panthi, R. R., Jordan, K. N., Kelly, A. L. and Sheehan, J. J. (2017). Selection and treatment of milk for cheesemaking. *In:* "Cheese" (4th ed., Vol. 1). (P. L. H. McSweeney, P. F. Fox, P. D. Cotter and D. W. Everett, Eds.). pp. 23-44. San Diego. Academic Press. [ISBN 978-0-12-417012-4].

Patel, G. R. and Gupta, S. K. (1986). Some aspects of sensory evaluation of paneer. *Ind. Dairyman*. **38**, 135-140.

Patel, M. T., Kilara, A., Huffman, L. M., Hewitt, S. A. and Houlihan, A. V. (1990). Studies on whey protein concentrates: 1. Composition and thermal properties. *J. Dairy Sci.* **73**, 1439-1449.

Pérez, A., Laudat, T., Mora, M., Carvajal, C., Aragón, C., González, J., Escalona, M., Daquinta, M., Trujillo, R., Hernández, M. and Lorenzo, J. C. (2013). Micropropagation of *Hohenbergia penduliflora* (A. Rich.) Mez. for sustainable production of plant proteases. *Acta Physiologiae Plantarum*. **35** (8), 2525-2537. [doi: 10.1007/s11738-013-1288-8].

Peri, C. (2006). The universe of food quality. *Food Quality and Preference*. **17**, 3-8.

Pezeshki, A., Hesari, J., Zonoz, A. A. and Ghambarzadeh, B. (2011). Influence of *Withina coagulans* protease as a vegetable rennet on proteolysis of Iranian UF white cheese. *J Agric Sci Technol*. **13**, 567-576.

Pontual, E. V., Carvalho, B. E., Bezerra, R. S., Coelho, L. C., Napoleo, T. H. and Paiva, P. M. (2012). Caseinolytic and milk-clotting activities from *Moringa oleifera* flowers. *Food Chem.* **135**, 1848-1854.

Pradhan, S. K. (2000). Effect of renneting temperature and pH on the fat, protein and total solid retention in cow milk cheese. B.Tech Thesis. Tribhuvan Univ., Nepal.

Rajagopalan, A., Singh, M. and O S, B. (2014). Characterisation of potential milk coagulants from Calotropis gigantea plant parts and their hydrolytic pattern of bovine casein. *Eur.Food Res. Technol.* **238**. [doi: 10.1007/s00217-014-2177-0].

Rana, M. S., Hoque, M. R., Nahar, T. N., Habib, R. and Siddiki, M. (2017). Preparation of cottage cheese from buffalo milk with different levels of papaya latex. *Bangladesh J. Animal Sci.* **46**, 128-133. [doi: 10.3329/bjas.v46i2.34443].

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **62** (3), 597-635.

Raposo, S. and Domingos, A. (2008). Purification and characterization milk-clotting aspartic proteinases from Centaurea calcitrapa cell suspension cultures. *Process Biochem.* **43**, 139-144. [doi: 10.1016/j.procbio.2007.11.003].

Rawlings, N. D. and Barrett, A. J. (2004). Families of serine peptidases. *Methods Enxymol*. **244**, 19-61.

Rawlings, N. D., Barrett, A. J. and Bateman, A. (2010). MEROPS: the peptidase database. *Nucleic Acids Res.* **38**, D227-233. [doi: 10.1093/nar/gkp971].

Razzaq, S. (2003). Preparation of cottage cheese using Sodom Apple (*Calotropis procera*) latex as a plant rennet. M.Sc. (Hons) Thesis. Arid Agric. Univ., Pakistan.

Roseiro, L. B., Barbosa, M., Ames, J. M. and Wilbey, R. A. (2003). Cheese making with vegetable coagulants the use of *Cynara L*. for the production of ovine milk cheeses. *Int. J. Dairy Technol.* **56**, 76-85.

Rowney, M. K., Roupas, P., Hickley, M. W. and Everett, D. W. (1999). Effect of fat reduction on checimal composition,poroteolysis, functionality, and yield of mozerella cheese. *J. Dairy Sci.* **82**, 661-672.

Rudan, M. A., Barbano, D. M., Yun, J. J. and Kindstedt, P. S. (1999). Effect of fat reduction on chemical composition, proteolysis, functionality, and yield of mozzarella cheese. *J. Dairy Sci.* **82**, 661-672.

Salehi, M., Aghamaali, M. R., Saajedi, R. H., Asghari, S. M. and Jorjani, E. (2017). Purification and characterization of a milk clotting aspartic protease from *Withinia coagulans* fruit. *Int. J. Biol. Macromol.* **98**, 847-854.

Sayed, A. and Tanboly, A. (2003). Production of plant proteinase from jackfruit (*Artocarpus integrifolis*) as a source of dairy enzyme I. Isolation, partial purification and some properties. *Pakistan J. Bio. Sci.* **6** (16), 1435-1441. [doi: 10.3923/pjbs.2003.1435.1441].

Schaller, A. (2005). A cut above the rest: The regulatory function of plant proteases. *Planta*. **220**, 183-197. [doi: 10.1007/s00425-004-1407-2].

Scott, R. (1986). Cheese making practice. *In:* "Cheese: Chemistry, Physics and Microbiology" (2nd ed., Vol. 1). (P. F. Fox, Ed.). London. Elsevier Applied Science.

Scott, R., Robinson, R. K. and Wibley, R. A. (1998). Introduction to cheese making. *In:* "Cheesemaking Practice" (2nd ed.). (R. Scott, Ed.). pp. 37-43. Gaithersburg. Md: Aspen Publication.

Sengupta, A., Bhattacharya, D., Pal, G. and Sinha, N. K. (1984). Comparative studies on calotropins DI and DII from the latex of *Calotropis gigantea*. *Arch. Biochem. Biophys.* **232** (1), 17-25. [doi: 10.1016/0003-9861(84)90517-4].

Shah, M. A. and Mir, S. A. (2019). Plant protease in food processing. *In:* "Bioactive Molecules in Food. Reference Series in Phytochemistry.". (J. M. Merillon and K. Ramawat, Eds.). pp. 4443-4464. Springer, Cham. [ISBN 978-3-319-78030-6].

Shah, M. A., Mir, S. A. and Paray, M. A. (2014). Plant proteases as milk-clotting enzymes in cheesemaking: a review. *Dairy Sci. Technol.* **94** (1). [doi: 10.1007/s13594-013-0144-3].

Shahbandeh, M. (2021). Annual cheese production worldwide from 2015 to 2020. Retrieved from https://www.statista.com/statistics/1120911/cheese-production-worldwide/#:~:text=In%202020%2C%20global%20cheese%20production,tons%20of%20cheese%20that%20year. (Last update 19 January, 2021).

Sharma Khanal, B., Pradhan, M. and Bansal, N. (2019). Cheese: Importance and introduction to basic technologies. *J. Food Sci. Technol.* **11**, 14-24. [doi: 10.3126/jfstn.v11i0.29666].

Silvestre, M. P. C., Carreira, R. L., Silva, M. R., Corgosinho, F. C., Monteiro, M. R. P. and Morais, H. A. (2012). Effect of pH and temperature on the activity of enzymetic extracts from pineapple peel. *Food Bioprocess. Technol.* **2**, 139-162.

Singh, S. J., Singh, L. R., Devi, S. K., Singh, S. S., Devi, C. B. and Rully, H. (2015). Purification and Characterization of a Thermostable Caseinolytic Serine Protease from the Latex of Euphorbia heterophylla L. *Protein Pept Lett.* **22** (9), 828-835. [doi: 10.2174/0929866522666150707114548].

Siritapetawee, J., Thammasirirak, S. and Samosornsuk, W. (2012). Antimicrobial activity of a 48-kDa protease (AMP48) from *Artocarpus heterophyllus* latex. *Eur. Rev. Med. Pharmacol. Sci.* **16** (1), 132-137.

Solorza, F. J. and Bell, A. E. (1998). Effect of calcium on the minerals retention and cheesemaking parameters of milk. *Int. J.Dairy Technol.* **51**, 37-43.

Somavarapu, S., Vemula, S. and Reddy, I. (2017). Extraction, purification and characterization of a novel cysteine protease from the latex of plant *Vallaris solanacea*. *J. Plant Biochem. Biotechnol.* **27**, 1-13. [doi: 10.1007/s13562-017-0429-3].

Sousa, M. J., Ardö, Y. and McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.* **11** (4), 327-345. [doi: 10.1016/S0958-6946(01)00062-0].

Swami, S. and Kalse, S. (2018). Jackfruit (*Artocarpus heterophyllus*): Biodiversity, Nutritional Contents, and Health. *Phytochem.*, 1-23. [doi: 10.1007/978-3-319-54528-8_87-1].

Swart, G. J., Blignaut, C. M. and Jooste, P. J. (2003). Pasteurization-other pasteurization processes. *In:* "Encyclopedia of Food Sciences and Nutrition " (2nd ed.). (B. Caballero, Ed.). pp. 4401-4406. Oxford. Academic Press. [ISBN 978-0-12-227055-0].

Tamer, M. I. and Mavituna, F. (1996). Protease from callus and cell suspension cultures of *Onopordum turcium* (Compositae). *Biotechnol Lett*. **18**, 361-366.

Tamer, M. I. and Mavituna, F. (1997). Protease from freely suspended and immobilized *Mirabilis jalapa*. *Process Biochem*. **32**, 195-200.

Thapa, T. B. (2006). Yak cheese production in Nepal [Report]. ICIMOD (International Centre for Integrated Mountain Development). Kathmandu, Nepal.

Thomas, P. C. (1980). Influence of nutrition on the yield and content of protein in milk: dietary protein and energy supply. *Int. Dairy Federation Bulletin*. **125**, 142-151.

Tripathi, P., Tomar, R. and Jagannadham, M. V. (2011). Purification and biochemical characterisation of a novel protease streblin. *Food Chem*. **125**, 1005-1012.

Tunick, M. (2014). "The Science of Cheese". Oxford University Press. New York.

Turk, B., Turk, V. and Turk, D. (1997). Structural and functional aspects of papain-like cysteine proteases and their protein inhibitors. *Biol. Chem.* **378**, 141-150.

Uchikoba, T. and Kaneda, M. (1996). Milk clotting activity of cucumisin, a plant serine protease from melon fruit. *Appl. Biochem. Biotechnol.* **56**, 325-330.

Upadhyay, K. G. (2003). "Essential of Cheesemaking" (1st ed.). Ananad Press.

Walstra, P., Wouters, J. T. M. and Geurts, T. J. (2006). "Dairy Science and Technology" (2nd ed.). Taylor & Francis Group, LLC. London.

Wolfschoon-Pombo, A. F. (1997). Influence of calcium chloride addition to milk on the cheese yield. *Int. Dairy J.* **7**, 249-254.

Yadav, R. P., Patel, A. K. and Jagannadham, M. V. (2011). Purification and biochemical characterization of a chymotrypsin like serine protease from *Euphorbia nerifolia* Linn. *Process Biochem*. **46**, 1654-1662.

Yegin, S., Fernandez-Lahore, M., Salgado, J. G., Guvenc, U., Goksungur, Y. and Tari, C. (2011). Aspartic proteinases from *Mucor* spp. in cheese manufacturing. *Appl. Microb. Biotechnol.* **89**, 949-960.

Appendices

Appendix A

Table A.1 Product responses by independent variables of crude enzymes from *C. gigantea*

Std. no.	Factor 1 A: pH	Factor 2 B: Temperature ℃	Response 1 Time of Coagulation S	Response 2 Milk Clotting Activity (MCA) U/ml
1	5	35	190	63.1579
2	5.5	40	86	139.535
3	6	50	288	41.6667
4	5	30	190	63.1579
5	6	45	225	53.3333
6	5	40	40	300
7	5.5	50	114	105.263
8	5.5	30	250	48
9	6	35	313	38.3387
10	5	45	100	120
11	6.5	30	180	66.6667
12	6	40	105	114.286
13	5.5	35	195	61.5385
14	6.5	35	155	77.4194
15	6.5	40	50	240
16	6.5	45	40	300
17	6	30	315	38.0952
18	6.5	45	55	218.182

Table A.2 Product responses by independent variables for crude jackfruit enzyme

Std. no.	Factor 1 A: pH	Factor 2 B: Temperature ℃	Response 1 Time of Coagulation S	Response 2 Milk Clotting Activity (MCA) U/ml
1	7	75	137	87.59
2	7	35	1464	8.20
3	7.5	75	325	36.92
4	6.5	35	1160	10.34
5	7	55	188	63.83
6	7.5	55	579	20.73
7	6.5	55	180	66.67
8	7.5	45	1381	8.69
9	8	75	517	23.21
10	8	35	10800	1.11
11	7	45	580	20.69
12	6.5	45	330	36.36
13	6.5	65	110	109.09
14	7.5	65	188	63.83
15	8	45	10800	1.11
16	8	55	1055	11.37
17	6.5	75	126	95.24
18	6.5	65	108	111.11

Appendix B

Table B.1 Model summary statistics for Time of Coagulation for *C. gigantea* enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	83.82	0.2219	0.1384	-0.0534	1.617E+05	
2FI	86.40	0.2219	0.0761	-0.1352	1.743E+05	
Quadratic	**63.22**	**0.6355**	**0.5053**	**0.3126**	**1.055E+05**	**Suggested**
Cubic	56.57	0.7916	0.6040	0.1529	1.300E+05	
Quartic	33.49	0.9562	0.8612	0.4651	82109.59	Aliased

*Case(s) with leverage of 1.0000: PRESS statistic not defined.

Table B.2 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Time of Coagulation for *C. gigantea* enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	97547.84	5	19509.57	4.88	0.0086	significant
A-pH	136.89	1	136.89	0.0342	0.8558	
B-Temperature	33930.62	1	33930.62	8.49	0.0113	
AB	2.20	1	2.20	0.0006	0.9816	
A^2	47531.25	1	47531.25	11.89	0.0039	
B^2	15946.87	1	15946.87	3.99	0.0656	
Residual	55961.91	14	3997.28			
Cor Total	1.535E+05	19				

Table B.3 Model summary statistics for Milk Clotting Activity for crude *C. gigantea* enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	80.07	0.2066	0.1132	-0.1066	1.520E+05	
2FI	81.01	0.2356	0.0923	-0.1718	1.610E+05	
Quadratic	**63.95**	**0.5832**	**0.4343**	**0.0884**	**1.252E+05**	**Suggested**
Cubic	61.60	0.7238	0.4752	0.0751	1.271E+05	Aliased
Quartic	37.24	0.9394	0.8082	0.3269	92463.17	

* Case(s) with leverage of 1.0000: PRESS statistic not defined.

Table B.4 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Milk Clotting Activity for crude *C. gigantea* enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	80.116.67	5	16023.33	3.92	0.0197	significant
A-pH	1656.75	1	1656.75	0.4051	0.5347	
B-Temperature	26721.67	1	26721.67	6.53	0.0228	
AB	3990.50	1	3990.50	0.9757	0.3400	
A^2	40683.27	1	40683.27	9.95	0.0070	
B^2	7064.49	1	7064.49	1.73	0.2099	
Residual	57256.51	14	4089.75			
Cor Total	1374E+05	19				

Table B.5 Model summary statistics for Time of Coagulation for crude *A. heterophyllus* enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	2421.02	0.5053	0.4471	0.2654	1.480E+08	
2FI	1879.70	0.7193	0.6667	0.5183	9.702E+07	
Quadratic	**1614.40**	**0.8188**	**0.7541**	**0.6249**	**7.554E+07**	**Suggested**
Cubic	1459.12	0.8943	0.7992	0.4901	1.027E+08	
Quartic	1507.12	0.9323	0.7857	-0.6997	3.423E+08	Aliased

*Case(s) with leverage of 1.0000: PRESS statistic not defined.

Table B.6 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Time of Coagulation for crude *A. heterophyllus* enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.649E+08	5	3.299E+07	12.66	<0.0001	significant
A-pH	4.831E+07	1	4.831E+07	18.54	0.0007	
B-Temperature	5.346E+07	1	5.346E+07	20.51	0.0005	
AB	4.311E+07	1	4.311E+07	16.54	0.0012	
A^2	1.256E+07	1	1.256E+07	4.82	0.0455	
B^2	7.488E+06	1	7.488E+06	2.87	0.1122	
Residual	3.649E+07	14	2.606E+06			
Cor Total	2.014E+08	19				

Table B.7 Model summary statistics for Milk Clotting Activity for crude *A. heterophyllus* enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	18.94	0.7671	0.7397	0.6727	8573.52	
2FI	16.12	0.8412	0.8114	0.7482	6594.72	
Quadratic	15.86	0.8656	0.8176	0.7326	7003.42	
Cubic	**8.94**	**0.9695**	**0.9420**	**0.8661**	**3506.56**	**Suggested**
Quartic	6.05	0.9916	0.9734	0.7579	6340.51	Aliased

* Case(s) with leverage of 1.0000: PRESS statistic not defined.

Table B.8 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Milk Clotting Activity for crude *A. heterophyllus* enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	22671.02	5	4534.20	18.03	<0.0001	significant
A-pH	8114.33	1	8114.33	32.26	<0.0001	
B-Temperature	11976.63	1	11976.63	47.62	<0.0001	
AB	1941.36	1	1941.36	7.72	0.0148	
A^2	65.31	1	65.31	0.2597	0.6183	
B^2	573.39	1	573.39	2.28	0.1533	
Residual	3520.95	14	251.50			
Cor Total	26191.96	19				

Appendix C

Table C.1 Solutions of optimization result for crude *C. gigantea* enzyme

Number	pH	Temperature	Time of coagulation	MCA	Desirability	
1	**6.500**	**35**	**122.445**	**146.197**	**1**	**Selected**
2	6.500	30	201.880	73.255	1	
3	6.500	40	76.760	196.676	1	
4	6.500	50	86.640	230.243	1	
5	6.500	45	64.825	224.691	1	
6	6.500	39.819	77.825	195.240	1	

Table C.2 Solutions of optimization result for crude *A. heterophyllus* enzyme

Number	pH	Temperature	Time of coagulation	MCA	Desirability	
1	**6.500**	**71.628**	**1131.781**	**107.694**	**0.936**	**Selected**
2	6.500	71.500	1113.217	107.488	0.936	

Appendix D

Calibration curve for protease activity

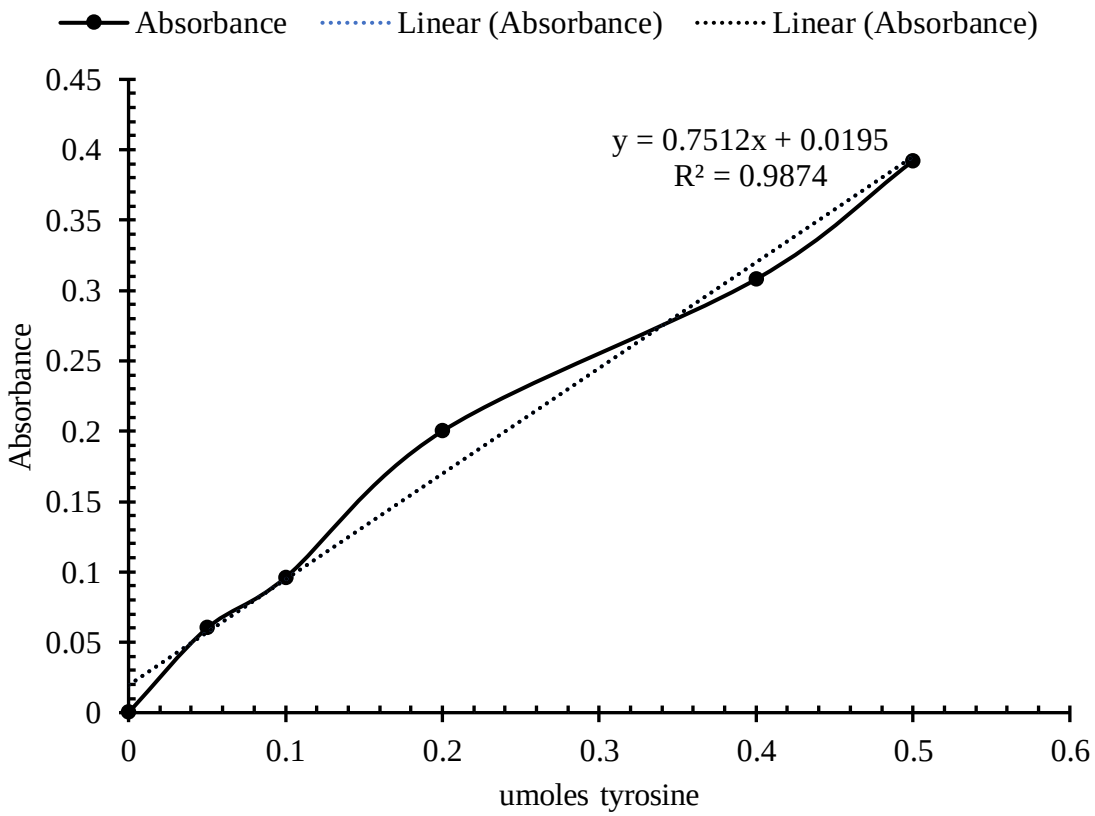

Fig. D.1 Standard curve of L-Tyrosine for protease activity

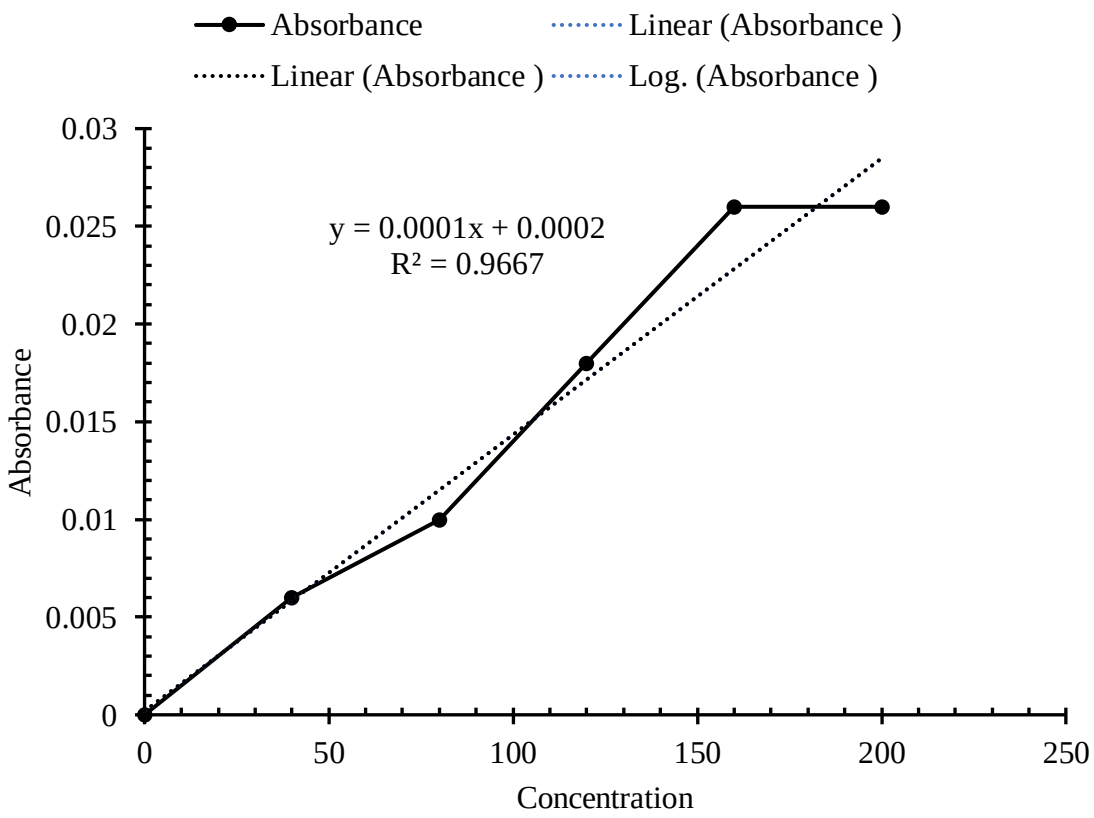

Fig. D.2 Standard curve of protein concentration for protein determination

Table D.1 Paired t test for two sample of means of protease activity of crude aank protease and crude jackfruit protease

	Variable 1	*Variable 2*
Mean	0.544	0.286
Variance	9E-06	0.000001
Observations	3	3
Pearson Correlation	-0.5	
Hypothesized Mean Difference	0	
Df	2	
t Stat	123.939191	
P(T<=t) one-tail	3.25469E-05	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	6.50939E-05	
t Critical two-tail	4.30265273	

Table D.2 Paired t test for two sample of means of protein concentration of crude aank protease and crude jackfruit protease

	Variable 1	*Variable 2*
Mean	0.139	0.106
Variance	1.9E-05	0.000025
Observations	3	3
Pearson Correlation	0.802955	
Hypothesized Mean Difference	0	
Df	2	
t Stat	19.05256	
P(T<=t) one-tail	0.001372	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.002743	
t Critical two-tail	4.302653	

Appendix E

Table E.1 Theoretical and Actual yields of cream cheese

Source of Variation	Actual Yield	Theoretical Yield
A	$11.84^{a} \pm 0.005$	$14.02^{a} \pm 0.015$
B	$13.9^{b} \pm 0.01$	$14.48^{b} \pm 0.066$
C	$14.83^{c} \pm 0.015$	$15.56^{c} \pm 0.026$

Table E.2 Microbiological analysis of cottage cheese

Sample	Coliform (cfu/g)	TPC (cfu/g)	Yeast & Mold (cfu/g)
A	ND	$218.67^{a} \pm 6.51$	$26.33^{a} \pm 3.05$
B	ND	$270^{b} \pm 6.08$	$19^{a} \pm 3.61$
C	ND	$197^{c} \pm 11.79$	$26^{a} \pm 5.57$

Note: ND = not detected, Values in the tables are the mean of two determinations.

Appendix F

Statistical analysis (ANOVA Tables)

Table F.1 One-way ANOVA (no blocking) for moisture content taking the samples

Variate: Moisture Content

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	51.81540	25.90770	1235.66	<0.001
Residual	6	0.12580	0.02097		
Total	8	51.94120			

Since there is significant difference between the samples for the moisture content at 5% level of significance, Tukey HSD testing is necessary.

Table F.2 One-way ANOVA (no blocking) for fat taking the samples

Variate: Fat

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	35.4772	17.7386	17.95	0.003
Residual	6	5.9286	0.9881		
Total	8	41.4058			

Since there is significant difference between the samples for the fat at 5% level of significance, Tukey HSD testing is necessary.

Table F.3 One-way ANOVA (no blocking) for protein taking the samples

Variate: Protein

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	0.1921	0.0960	0.11	0.894
Residual	6	5.0305	0.8384		
Total	8	5.2226			

Since there is no-significant difference between the samples for the protein at 5% level of significance, Tukey HSD testing is not necessary.

Table F.4 One-way ANOVA (no blocking) for ash taking the samples

Variate: Ash

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	0.6073	0.303633	32.45	<0.001
Residual	6	0.0561	0.009356		
Total	8	0.6634			

Since there is significant difference between the samples for the ash at 5% level of significance, Tukey HSD testing is necessary.

Table F.5 One-way ANOVA (no blocking) for acidity taking the samples

Variate: Acidity

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	0.0038	0.0019	8.14	0.020
Residual	6	0.0014	0.0002333		
Total	8	0.0052			

Since there is significant difference between the samples for the acidity at 5% level of significance, Tukey HSD testing is necessary.

Table F.6 One-way ANOVA (no blocking) for pH taking the samples

Variate: pH

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	0.4418667	0.22093333	3976.8	<0.001
Residual	6	0.00033333	0.00005556		
Total	8	0.4422			

Since there is significant difference between the samples for the pH at 5% level of significance, Tukey HSD testing is necessary.

Table F.7 One-way ANOVA (no blocking) for calcium taking the samples

Variate: Calcium

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	393.643	196.821	67.55	<0.001
Residual	6	17.483	2.914		
Total	8	411.125			

Since there is significant difference between the samples for the calcium at 5% level of significance, Tukey HSD testing is necessary.

Table F.8 One-way ANOVA (no blocking) for theoretical yield taking the samples

Variate: Theoretical Yield

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	4.946156	2.473078	1382.47	<0.001
Residual	6	0.010733	0.001789		
Total	8	4.956889			

Since there is significant difference between the samples for the theoretical yield at 5% level of significance, Tukey HSD testing is necessary.

Table F.9 One-way ANOVA (no blocking) for actual yield taking the samples

Variate: Actual Yield

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	14.0410889	7.0205444	57440.82	<0.001
Residual	6	0.0007333	0.0001222		
Total	8	14.041822			

Since there is significant difference between the samples for the actual yield at 5% level of significance, Tukey HSD testing is necessary.

Table F.10 One-way ANOVA (no blocking) for TPC taking the samples

Variate: TPC

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	8433.56	4216.78	57.94	<0.001
Residual	6	436.67	72.78		
Total	8	8870.22			

Since there is significant difference between the samples for the TPC at 5% level of significance, Tukey HSD testing is necessary

Table F.11 One-way ANOVA (no blocking) for Yeast and Molds taking the samples

Variate: Yeast and Molds

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	102.89	51.44	2.89	0.132
Residual	6	106.67	17.78		
Total	8	209.56			

Since there is no-significant difference between the samples for the Yeast and Molds count at 5% level of significance, Tukey HSD testing is not necessary.