OPTIMIZATION OF CRUDE PAPAYA (*Carica papaya*) PROTEASE IN CREAM CHEESE PREPARATION BY RESPONSE SURFACE METHODOLOGY

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Optimization of Crude Papaya (*Carica papaya*) Protease in Cream Cheese Preparation by Response Surface Methodology

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Approval Letter

This ***dissertation*** entitled ***Optimization of Crude Papaya (Carica papaya) Protease in Cream Cheese Preparation by Response Surface Methodology*** presented by **Nabindra Kumar Shrestha** has been accepted as the partial fulfilment of the requirement for the **B. Tech. degree** in **Food Technology.**

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_____

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Abstract

The effect of papaya latex as crude papaya protease on physicochemical, microbial and sensorial characteristics of cream cheese prepared were examined. Enzyme concentration, pH of milk and temperature of milk on milk clotting activity were optimized by surface response methodology. Also, the protease activity of the crude enzyme was also measured using optimized conditions. Finally, the cheese prepared by using crude papaya protease was compared with that prepared using rennet and commercial papain for physicochemical, microbial and sensorial properties.

Numerical optimization study revealed maximum milk clotting activity at pH 6.5, temperature 70°C and enzyme concentration 1% (1 ml/1000 ml milk) using papaya protease as coagulant. Protein, ash and calcium showed no significant ($p>0.05$) difference among the cheeses made using different coagulants. However, significantly ($p<0.05$) higher levels of moisture and ash, and lower levels of fat were observed in the cheese produced by papaya protease compared to that made using rennet. Papaya protease significantly enhanced the spreadability of cheese while the other sensory properties were similar to the control except aftertaste. Microbiologically, all samples were not significantly different and were free of coliforms. However, the presence of TPC and yeasts and molds was found. The results revealed that the papaya latex as crude papaya protease may have potential application for the manufacture of cream cheese and further could be utilized as a milk coagulant in cheese making.

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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
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
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 may be defined as a product made from the milk of cows and other mammals; the essential milk solid being reduced into a concentrated form. According to FAO/WHO, cheese is the fresh or matured solid or semi solid product obtained by coagulating milk, skimmed milk, partly skimmed milk, cream, whey cream or butter milk or any combination of these materials through the action of rennet or other suitable coagulating agents, and thereby partially draining the whey resulting from such coagulation. The above definition does not allow for cheese made by newer processes nor does it allow for whey cheese. Hence, cheese is a fresh or matured solid or semi-solid product obtained by processing techniques involving coagulation of milk and /or materials obtained from milk (provided that the whey protein/casein ratio does not exceed that of the milk) and which gives an end product which has similar physical, chemical and organoleptic characteristics as the product defined above. Whey cheese is the product obtained by concentration or coagulation of whey with or without addition of milk or milk fat (Upadhyay, 2003).

Historically, rennet used to coagulate milk in cheese making has generally been extracted from the fourth stomach of calves, although kids and lambs have also been used. World cheese production has doubled in less than 25 years from 1961-1994 (Farkye, 2004). Industrial cheese production is continuously growing. World cheese production in 2004, 2005, and 2006 were 28.4, 29 and 29.8 million MT respectively (IDF, 2007).

The coagulation of milk can be achieved by a number of proteolytic enzymes from various sources, such as different animal species (e.g., pig, cow, and chicken pepsins), microbial proteinases (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and proteinases extracted from plants. Certain plants that have been reported to yield promising rennet activity are Thistle (*Cirsium*) (Robinson and Wilbey, 1998); Silk tree (*Albizia julibrissin*) (Garg and Johri, 1994); Pineapple (*Ananas comosus*) (Cattaneo *et al.,* 1994); Sodom apple (*Calotropis procera*) (Aworth and Muller, 1987; Razzaq, 2003); Papaya (*Carica papaya*) (Veringa, 1961); Pumpkin (*Cucurbita pepo*) (Barbosa, 1983); Cardoon and artichoke (*Cynara cardunculus, C. humilis, C. scolymus*) (Vieira and Barbosa, 1970);

Lettuce (*Lactuca sativa*) (Piero *et al.,* 2002); Ginger (*Zingiber officinale*) (Hashim *et al.,* 2011; El-Aziz *et al.*, 2012) and Sunflower (*Helianthus annuus*) and Albizia (*Albizia lebbeck*) (Egito *et al.,* 2007), Sowthistle (*Sonchus oleraceous l.*) (Abu-Zeid, 1994), *Solanum dubium* (Kheir *et al.*, 2001; Mohamed *et al.*, 1997), *Withania coagulans* (Nawaz, 2007; Khan and Masud, 2013), etc.

Much research has been directed towards discovering a milk clotting enzyme of plant origin which would satisfactorily replace calf rennet in cheese manufacture (Duarte *et al.,* 2009). Plant extracts have the ability to hydrolyse the κ-casein, leading to curd formation, and they are also the main enzymes responsible for β-casein hydrolysis (Roseiro *et al.,* 2003). The use of these plant proteinases as milk coagulants is very interesting since they are natural enzymes and can also be used for producing cheeses aimed at lacto-vegetarian consumers and ecological markets (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

Calf rennet has been widely employed as a milk-coagulating agent from antiquity (Sousa and Malcata, 1997). The animal rennet contains two enzymes (chymosin and pepsin) that break the Met_{105}-Phe_{106} bond of the κ-casein present on the surface of the casein micelles. However, the increase in cheese production, coupled to a diminishing supply of natural animal rennet, is responsible for increases in the demand for alternative milk-coagulating sources (Chazarra *et al*., 2007). The increasingly higher prices of calf rennet as well as ethical concerns associated with the production of such enzymes for general cheese making have led to systematic investigations on the possibility and suitability of their substitution by other enzymes of plant origin (Sousa and Malcata, 1997). Due to this and a variety of factors (vegetarianism, religious beliefs, etc.), attention is being turned to the use of microbial coagulants and coagulants extracted from plants (Chazarra *et al*., 2007).

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. This problem raised a question in the cheese making industry about whether they could find a substitute to the rennet they have been traditionally using or

not. To solve this problem, a suitable cheap non-rennet coagulating agent has to be developed 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 study the optimization of papaya latex in cream cheese preparation by response surface methodology.

1.3.2 Specific objectives

- To prepare proteolytic enzyme from papaya latex to be used as a coagulating agent in cream cheese preparation.
- To determine milk coagulating activity (MCA) and protease activity of the enzyme from papaya latex.
- To prepare cream cheese from the enzyme in optimized conditions.
- To study physicochemical properties and evaluate the sensory quality of cream 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) and the most popular milk coagulating enzyme is calf rennet, which is found in the stomach of infant animals (Ishak *et al.,* 2006). There are many steps involved in the extraction and subsequent purification of calf rennet from the tissues of animal stomach, which makes this enzyme very expensive. Moreover, the reduced supply of calf rennet, calf diseases like bovine spongiform encephalopathy (BSE) has led to an increase in the demand for alternative sources of milk coagulants (Cavalcanti *et al.,* 2004). The consumer constraints on the use of animal rennet for religious reasons (e.g., Judaism and Islam) as well as diet (vegetarianism), or consumer concern regarding genetically engineered foods (e.g., Germany, Netherlands and France

forbid the use of recombinant calf rennet) have led to a growing interest in vegetable coagulants (Egito *et al.,* 2007).

The present study is conducted to investigate the possible presence of proteolytic enzymes with promising industrial application as regard milk-clotting activity of different plant proteases as coagulant. Therefore, the present work will be solely based on the optimization of the plant-based enzymes utilization in the preparation of cheese taking an account on the consumer acceptability of the product by varying proportion of the use of such enzymes.

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 done.
- Texture evaluation of the product was not done.

Part II

Literature review

2.1 History and development of cheese

Early records indicate that foods such as cheese and bread were staple foods in 'Fertile Crescent' situated between the rivers Euphrates and Tigris in present day Iraq. In this area, cheese is frequently referred to as the cradle of civilization (Scott, 1986; Fox, 1987 and Upadhyay, 2003).

It is very likely that first cheese is originated accidentally more than 800 years ago when man realized nutritive value of milk produced by his domesticated animals and contrived to share the mother milk with her offspring (Fox, 1987). However, the origin of cheese is lost in antiquity. But, most assuredly, milk was contaminated with lactic acid bacteria, which through acidification of the milk, created conditions unfavourable for growth of other bacteria. As the story goes, milk held in storage vessels (animal stomachs) clotted, making cream cheese, the ''mother of all cheeses''. The acid environment caused milk proteins to clot. It was a great leap forward when centuries later humans discovered the use of coagulating enzymes. This led to production of less sour cheeses. Natural contamination of milk or cheese by bacteria, yeasts, and molds led to development of a multitude of flavour sensations in cheese as it aged (Johnson, 2001).

Since animal skin bags were a convenient way of strong liquids for nomadic tribes, the surplus to daily needs would have been carried in such skin bags (Scott, 1986). Under such circumstances milk would extract coagulating enzymes (rennet) from the stomach tissue, leading to the coagulation during storage. Unfortunately, milk is also a rich source of nutrients for bacteria, some species of which utilize milk sugar, lactose, as a source of energy producing lactic acid as a byproduct. When sufficient acid has been produced, the milk protein coagulates i.e. at the isoelectric point of casein, to form a gel entrapping the fat (Fox, 1987). The acid curds would have been broken up by swaying animals during journeys, to produce curds and whey (Scott, 1986). It would have been realized quickly that the whey made a pleasant refreshing drink for immediate consumption while the curd could be consumed fresh or stored for future (Fox, 1987). The shelf life of the curd could be greatly extended by dehydration and salting. This activity gave rise to the evolution of cheese. The

rennet curd can be converted into more stable (due to low moisture curd) than acid curds. Thus, rennet coagulation has become predominant in cheese manufacture (Fox, 1987).

Cheese has been known to mankind for thousands of years. Cheese is an art that predates the biblical era. The origin of cheese has been dated to 6000 to 7000 BC. The worldwide annual production is more than 12 million tons and is growing at a rate of about 4 %. Throughout the ages numerous varieties of cheese have evolved. The development of cheese closely depended on local conditions, climate, type of milk, and other economical and geographical factors. Furthermore, it was a matter of technological experience and of finding, by trial and error, the manufacturing conditions that led to a product being tasty, easy to handle and durable. Along with this, the general level of technological and hygienic know-how was of great importance. Various process steps that now are very common are relatively new, say 100 years old: use of starter, use of industrially made rennet, washing of curd, pasteurization of cheese milk. Of more recent date are the uses of inocula for the surface flora, application of latex emulsions to the cheese rind, use of enzyme preparations to accelerate ripening, and so forth (Walstra *et al*., 2006).

Cheese manufacture obviously accompanied the spread of civilization throughout the Middle East, Egypt, Greece and Rome. Movements of Roman armies and administrators would have spread the use of cheese further. However, the most important agent contributing to the development of cheese 'technology' and to the evolution of cheese varieties were the monasteries and the feudal estates (Fox, 1987).

There are many references in literature to names of cheese varieties known today, a list of some of the important cheese varieties with the date first noted is shown in Table 2.1.

Table 2.1 Selected cheese varieties with the date first noted in literature

Cheese variety	Date first noted	Cheese variety	Date first noted
Gargonzola	AD 879	Parmesan	AD 1579
Roquefort	AD 1070	Gouda	AD 1697
Grana	AD 1200	Camembert	AD 1791
Cheddar	AD 1500	St. Paulin	AD 1816

Source: Scott (1986)

Cheesemaking remained an art rather than a science until relatively recently. With the gradual acquisition of knowledge on the chemistry and microbiology of milk and cheese, it became possible to direct the changes involved in cheesemaking in a more controlled fashion (Lawrence *et al*., 1984).

2.1.1 Scientific and technological developments

Key scientific and technological developments that have taken place during the last 100 years contributed tremendously to the growth of cheese industry are listed below (Fox, 1987).

- Isolation and characterization of the principal constituents of milk, especially those involved in cheese making.
- Microbiology of raw milk and the treatments (e.g. pasteurization, bactofugation, microfiltration, etc.) to reduce the micro flora.
- Elucidation of the mechanism of coagulation i.e. conversion of liquid milk to gel.
- Molecular basis of the conversion of gel to cheese curd.
- Acidification of curd through metabolic activity of cultures of lactic acid bacteria (e.g. mesophilic cultures, thermophilic cultures, etc.) and characterization of all aspects of these cultures
- Studies on the safety and public health aspects of cheese.

- Characterization of the maturation of cheese-biochemistry, microbiology, flavor, texture, functionality.

- Search and development of rennet substitute (a) Fungal rennet derived from *Rhizomucor miehei* (e.g. Hannilase, Marzyme, Rennilase, Fromase, etc.), *Rhizomucor pusillus* (e.g. Meito, Emporase etc) and Cryphonecteria *parastica* (e.g. Surecurd, Suparen) and (b) Fermentation derived chymosin from microorganisms by application of DNA recombination technology.

- Mechanism of cheese production line- semi automatic, automatic production lines.

- Development of technology for production of enzyme modified cheeses and cheese analogues.

2.2 Cheese production

In world as a whole, production has been continually growing for more than twenty years (about 4% per annum) - a little less than 6 million metric tonnes in 1961, 7.6 million in 1970-71, 12.3 million in 1984 and 14.65 million in 1994. Production doubled in less than twenty-five years (Farkye, 2004). World cheese production (29.8 million metric tonnes) has been increased by 1.02 in 2006 (IDF, 2007).

2.2.1 Cheese production- a Nepalese scenario

In Nepal, the production of cheese started from about 1953/54 when a public-sector cheese factory was established over the Langtang mountain range. The pioneers of cheese industry in Nepal are Warner Schulthess a Swiss nationale, senior specialist from FAO, and Gauri Pd. Sharma (Thapa, 2006).

Despite its history of about 47 years cheese production has not substantially improved qualitatively as well as quantitatively. In the recent years, DANIDA has been playing a major role in promoting cheese industry. Nepal produced some 350 MT cheese in 2000. In 1994, Nepalese cheese generated a foreign exchange of $ 525,000. The statistic may not be very impressive but is nevertheless encouraging. The demand for cheese is steadily increasing. At a very conservative estimate, the annual requirement of cheese in Nepal is around 800 MT. Nepal has been meeting the demand for cheese by importing them from foreign countries. Such cheeses are usually sold at prices about 5-10 times higher than the price for

Nepalese cheese. Cheese making can thus be a very important economic activity for Nepal (Acharya, 2010).

The important varieties of cheese produced in Nepal are Yak cheese, Kanchan cheese, mozzarella cheese and Processed cheese (Colavito, 1997) and Cheese spread (Panthi, 2007).

DDC runs six yak cheese factories 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). Till 1995, there were at least 10 private Yak cheese factories operating alongside DDC cheese plants (Thapa, 2006).

Till now different private and public industries has been established in the country especially in the higher alpine regions. The peasants who are unable to sell their milk in cheese factories and dairies still prepare *churpi*- a kind or very hard indigenous cheese from yak or cow milk, as a remedy of earning for their life and to preserve their surplus milk (Pradhan, 2000).

Cheese is not much of popular food item among the general Nepalese. Many are not accustomed to the flavor and taste of cheese. It is a relatively costly dairy product in Nepal food market. Growing awareness about dietary needs and hygienic products has enhanced the demand for the pasteurized milk and milk products, and among them mere popularly, cheese. This demand is bound to grow further in the future (Pradhan, 2000).

2.3 Varieties and classification

Cheese is used as a form of preserving essential nutrients in milk and is an excellent source of nutrients such as protein, fat, minerals and vitamins (Mahajan and Chaudhari, 2014). Thus, a considerable international trade exists in the principal varieties of cheese, many of which are produced in several countries but which may not be identical. To assist international trade, to provide nutritional information and perhaps for other reasons, e.g., research, number of attempts has been made to develop classification schemes for cheeses. There is no definitive list of cheese varieties (McSweeney *et al*., 2004). Sandine and Elliker (1970) suggest that there are more than 1000 varieties. Walter and Hargrove (1972) described more than 400 varieties and listed the names of a further 400 varieties, while Burkhalter (1981) classified 510 varieties (although some are listed more than once).

However, many of these varieties are very similar and should be regarded as variants rather than varieties. Walter and Hargrove (1972) suggested that there are probably only about 18 distinct types of natural cheese, no two of which are made by the same method, i.e., they differ with respect to: setting the milk, cutting the coagulum, stirring, heating, draining, pressing and salting of the curds or ripening of the cheese (McSweeney *et al*., 2004).

Attempts to classify cheese varieties exploit a number of characteristics of the cheese (McSweeney *et al*, 2004).

- Texture, which is dependent mainly on moisture content;
- Method of coagulation as the primary criterion, coupled with other criteria;
- Ripening indices.

However, International Dairy Federation report lists the characteristics of cheese under the following heads (Upadhyay, 2003).

- Country of origin.
- Raw milk: cow, buffalo, sheep, goat, etc.
- Type of cheese hard, semi-hard, soft, fresh, acid coagulated or whey cheese.
- Internal characters: close or open texture, large medium or small eyes/holes, slit openings in curd blue or white mould ripened, color of curds.
- External characters: rind hard, soft, smooth or rough, smear or mould ripened spices or herbal addition type of coating (plastic, ash, etc).
- Weight of cheese: shape and size.
- Fat in dry matter (FDM)/Fat-on dry basis (FDB): Percentage minimum/maximum.
- Water in fat free substances (WFFS)/ Moisture in fat free substance (MFFS).

Classification of cheese on the basis of texture as given by Codex Alimentarious, FAO/WHO, and Standard A6 has been shown in the Table 2.2.

Table 2.2 Classification of cheese according to Codex Alimentarious

MFBB[1] (%)	Types	FDB[2] (%)	Types
<41	Extra hard	>60	High Fat
49-56	Hard	45-60	Full Fat
54-63	Semi-hard	25-45	Medium Fat
61-69	Semi-soft	10—25	Low Fat
>67	Soft	<10	Skim

Source: Scott (1986)

[1] MFFB equals percentage moisture on fat free basis.

[2] FDB equals percentage of fat on dry basis.

Vedamutha and Washam (1983) classified cheese on the basis of (i) Composition, (ii) Firmness, and (iii) Maturation agents, which has been shown in the Table 2.3.

Table 2.3 Classification of cheese on the basis of composition, firmness and maturation agents.

Types of Cheese	Examples
1. Soft Cheese (50-80% moisture)	
a) Unripened low fat	Cottage, Quark, Baker's
b) Unripened high fat	Cream, Neufchatels
c) Unripened stretched curd or pasta filata	Mozzarella, Scamorze
d) Ripened by external mold growth	Camembert, Brie
e) Ripened by bacterial fermentation	Kochkgse, Handkgse, Caciotta
f) Salt cultured or pickled	Feta- Greek; Domiati- Egyptian
2. Semi-soft Cheese (39-50% moisture)	
a) Ripened by internal mold growth	Blue, Gorgonzola, Roquefort

b) Surface-ripened by bacteria and yeast (surface smear)	Limburger, Brick, Trappist, Port du Salut, St. Paulin Oka
c) Ripened primarily by internal bacterial fermentation but may also have some surface growth	Miinster, Bel Paese, Tilsiter
d) Ripened internally by bacterial fermentation	Pasta Filata, Provolone, Low-moisture mozzarella
3. Hard Cheese (39% moisture maximum)	
a) Internally ripened by bacterial fermentation	Cheddar, Colby, Caciocavallo
b) Internally ripened by bacterial fermentation (CO_2 production resulting in holes or "eyes")	Swiss (Emmental), Gruyere, Gouda, Edam, Samsoe
c) Internally ripened by mold growth	Stilton
4. Very Hard Cheese (34% moisture maximum)	Asiago Old, Parmesan, Parmigiano, Grana, Romano, Sardo
5. Whey Cheese	
a) Heat and acid denaturation of whey protein	Ricotta (60 % moisture)
b) Condensing of whey by heat and water evaporation	Gjetost (goat milk whey; 13% moisture), Myost, Primost (13-18 % moisture)
6. Spiced Cheese	Noekkelost - cumin, cloves,

Source: Vedamutha and Washam (1983)

Rennet-coagulated cheeses represent about 75% of total cheese production and almost all ripened cheeses. Acid-curd cheeses represent about 25% of total cheese production and are generally consumed fresh (McSweeney *et al.*, 2004). Fox (1993) suggested that the classification schemes of Davis (1965), Walter and Hargrove (1972) and Burkhalter (1981) can be applied to rennet-coagulated Cheeses. The classification of cheese on the basis of mode of coagulation given by Fox (1993) has been shown in the Table 2.4.

Table 2.4 Classification of cheese groups on the basis of mode of coagulation

Group	Example
Rennet cheese	Most major international varieties
Acid cheese	Cottage, Quarg, Queso-Blanco
Heat/acid	Ricotta, Ziger
Concentration/crystallization	Mysost

Source: McSweeney *et al.* (2004)

Yak cheese and Kanchan cheese produced in Nepal can be classified as hard cheese (MFFB, 55-56%, Full fat (FDB min. 45%), close textured, bacteria ripened, rennet cheese. Yak and Kanchan cheese are similar to Swiss cheeses (Emmenthal, and Gruyere) (Pradhan, 2000). *La Serena* cheese is a semi-hard Spanish variety manufactured from raw Merino ewes' milk, which has a high fat content, with vegetable rennet from *Cynara cardunculus* L. as coagulant (Nunez *et al.* 1991).

2.4 Cream cheese

Cream cheese according to the Food and Drug Directorate is the cheese made from cream or milk to which cream has been added. It may contain no more than 0.5% stabilizer and shall not contain more than 55% moisture and not less than 30% milk fat. It is a soft, spreadable white cheese with a high fat content and has a rich creamy taste that is mildly acidic, similar to that of Greek yogurt. Its soft and creamy characteristics allow it to be used as a spread and give particular foods the creamy like texture. It differs from other types of cheese because it is not naturally aged and is meant to be eaten fresh; making it similar to Boursin and Mascarpone cheese (Anon., 2019a). Cream cheese is relatively more difficult to manufacture than most other cheeses because small adjustments in the timing of the development process can result in distinctions of flavor and texture. Included in many

recipes, cream cheese is highly versatile and can be used as a spread to add flavor to the food or can be combined in cooking to provide a creamy and smooth mouth feel (Kalab, 2000).

2.4.1 History of cream cheese

Records indicate that soft cheeses have been consumed as far back as in ancient Rome and Greece; Europe however, is widely regarded as the birth place of the first soft, creamy cheese (Mahalo, 2007), with documents indicating its existence from as early as the 1650's (Ellis-Christensen, 2011) The first commercial preparation of cream cheese came from an American dairyman, William Lawrence, in New York, 1872. While he was struggling to produce a type of French cheese called Neufchatel (Anon., 2019d), Lawrence discovered a method for making a soft yet creamy cheese. Soon, this innovative cheese product was distributed in foil wrappers as the Philadelphia Brand Cream Cheese, under the Empire Company and became the product that we know today, Kraft (Anon., 2019e).

2.4.2 Major brands of cream cheese

Kraft's Philadelphia Brand cream cheese currently owns nearly 70% of the $800 million cream cheese market. After Kraft, Schreiber (Anon., 2019f) is the second largest producer of cream cheese, with an estimated/approximately 25% of the market.

2.4.3 Uses of cream cheese

Cream cheese is often spread on bread, bagels, crackers, etc., and used as a dip for potato chips and similar snack items, and in salads. It can be mixed with other ingredients, such as yogurt or pepper jelly, to make spreads.

Cream cheese can be used for many purposes in sweet and savory cookery, and is in the same family of ingredients as other milk products, such as cream, milk, butter, and yogurt. It can be used in cooking to make cheesecake and to thicken sauces and make them creamy. Cream cheese is sometimes used in place of or with butter (typically two parts cream cheese to one-part butter) when making cakes or cookies, and cream cheese frosting. It is the main ingredient in the filling of crab rangoon, an appetizer commonly served at U.S. Chinese restaurants. It can also be used instead of or with butter or olive oil in mashed potatoes, and in some westernized sushi rolls (Anon., 2019a).

2.5 Quality of milk in relation to cheese making

Milk as a starting material for cheesemaking exerts a decisive influence on the course of manufacture and effects yield and quality of resultant cheese. The cheesemaking properties of milk are sensitive to variations. The changes in the quality of raw milk could influence cheesemaking, entailing modification in the existing process of manufacture or may disrupt normal manufacturing operations or may give altered product yield, composition and quality. Hence there is need to use high quality milk for cheese manufacture (Upadhyay, 2003).

In general, quality of milk for cheesemaking may be defined as its ability to give a good cheese, under normal working conditions and with a satisfactory yield (Abd El-Gawad *et al.*, 2007). Cheese yield is very economically important. A cheese maker would like to pay for milk based on the yield of cheese obtained from that milk (Melilli *et al*., 2002). The theoretical yield of cheese is limited by the fat and casein content of the milk used, the ability of the cheesemaker to recover the fat and casein as cheese, and the target moisture and salt content (Emmons and Binns, 1990). The quality criteria of milk which have relevance to cheesemaking are discussed under the following groups.

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

2.5.1 The amount and composition of milk constituents

Milk is a complex biological system which varies in composition depending on the breed of animal, stage of lactation, age of lactating animals, intervals between milking, season of the year, feed, health, and nutritional level of the animal gestation (Banks, 1992). Cheese made of buffalo milk has higher fat, protein, ash and total solids than cheese made of cow milk (Mijan *et al.,* 2010). Cheese composition and ultimately the interaction between casein molecules and adjacent micelles determines the firmness, melt, and chewiness of a cheese (Johnson *et al.*, 2001). The composition of milk is closely linked to yield and properties of resultant cheese. The milk constituents of importance to cheesemaking are milk proteins (i.e.

casein), milk fat and minerals salts particularly calcium. The influence on cheesemaking of changes in their concentrations, composition and nature are delineated below (Scott, 1986).

2.5.1.1 Milk protein (casein) and its effect

Casein is the principal milk protein of interest to the cheese maker. It constitutes about 78-80 % if the total protein and essentially all the casein occurs in micellar form (> 90%). The casein micelles are composed of several fractions αs_1, αs_2, β and κ-casein and some minor components (γ casein) derived from degradation of β-casein. The concentration and composition of such a mixture of protein is not constant and subject to alteration due to influence of breed, nutrition, stage of lactation, seasonal condition, health of animal and environmental factors (Banks, 1992).

For manufacture of cheese milk that is high in casein and low in serum protein and lactose in desirable. During cheesemaking major portion of the latter two constituents lost in whey. Moreover, higher whey protein content in milk delays rennet coagulation time and produces a weak coagulum. A high casein content of milk, in general, means good cheesemaking properties. Higher concentration of casein in milk has been found to give curd of higher strength, rapid rate of curd firming, longer syneresis time, higher cheese yield and cheese with firm body and texture. Milk, very low in casein content (e.g. <0.7%) fails to develop coagulum with rennet, whereas concentration of 2.5% and above is desirable (Kosikowski, 1982).

2.5.1.2 Milk fat

The amount and composition of milk fat vary widely in milk depending on species, individuality of the animal, stage of lactation, management practices and such other factors. Variation in the quality and quantity of milk fat, have direct influence on cheesemaking. Increase in the fat content of the milk prolongs the time required for initial clot formation; whereas the time required between initial clot formation and cutting is reduce (Scott, 1986).

For cheese, fat contributes to the taste, texture, functionality, and appearance (Rudan *et al.*, 1999). Regarding the effects of fat content of milk on the curd strength, there are conflicting reports available in literature. Some workers have found no appreciable change in the curd strength with change in the fat content of milk, whereas others have observed weakening of the coagulum with elevated fat levels in the milk. Increase in the fat content

of milk gives reduced syneresis, thus longer draining time are required to achieve the desired moisture content in cheese. On the other hand, cheese made no fat or low-fat milk has problem of moisture retention and it dried out fat to give a hard, dry body (Pradhan, 2000).

The curd obtained from the milk containing low levels of fat is more leathery and resultant cheese mellow velvetiness. Too high fat content in milk on the other hand yields products which are too soft, buttery and too grease and may show fat leakage during ripening (Fife *et al*., 1996).

Fat content of milk is also directly associated with the yield of cheese as fat and casein constitute >90% of the total solid of cheese. The cheese yield/ kg of fat used, decreased with increase in the fat content of milk as high fat milk usually contains less casein in proportion of fat than does milk rich in fat (Upadhyay, 2003).

2.5.1.3 Milk salt

Milk salts normally classed as 'ash', contain a large proportion of the metallic and non-metallic components. Some of the components of ash are of utmost importance in cheesemaking for example, calcium (Ca^{++}). The calcium content (colloidal, soluble and ionic) of milk greatly influences the RCT, firmness of the curd and together with phosphate is important for the drainage of whey. The level of Ca^{++} retained in the curd also influences the body and texture characteristics of the cheese. Normal milk contains adequate amount of calcium which is needed for proper coagulation of milk by rennet. Variations in concentration of calcium as well as magnesium, phosphates, citrates sodium have a direct influence on rennet clotting of milk. High soluble phosphate citrates and sodium, and low soluble Ca^{++} and magnesium and low proportion of casein bound Ca^{++} have been found to give slow coagulation of milk by rennet (Kindstedt and Kosikowski, 1985). The characteristics of milk with slow and fast coagulation have been shown the Table 2.5.

Table 2.5 Characteristics of milk with slow and fast coagulation

Character	Slow coagulation	Fast coagulation
Coagulation time	>400	40-60
Calcium **:** Nitrogen	0.18	0.24
Colloidal inorganic phosphate	0.88	1.27
Soluble casein (% of total casein)	10-12	6-7

Source: Upadhyay (2003)

In the view of importance of Ca^{++} in coagulation of milk, addition of 0.01-0.02 % of $CaCl_2$ to 'slow renneting' overheated milk to assist rennet activity is a common practice. However, addition of excessive amount of $CaCl_2$ to milk imparts a bitter taste to cheese.

The amount and composition of important milk components in cheesemaking are different in cow and buffalo milk thus it behaves differently from cow's milk when used for manufacture of certain cheese varieties (Upadhyay, 2003).

2.5.2 Abnormal milk

Abnormal milk is the general term used to describe any type of milk that differs markedly from normal milk that usually includes mastitis milk, colostrums and late lactation milk. Abnormal milk is unsuitable for cheesemaking on account of factors or conditions resulting in 'slow starter', slow coagulation with rennet and formation of weak curd (De, 2000). Abnormal enzyme content which may affect both ripening and developing of flavor.

2.5.2.1 Mastitis milk

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

2.5.2.2 Colostrum

Colostrum or fore milk vastly differs from that of normal milk with respect to its composition and physicochemical properties. Milk containing colostrum may coagulate easily in pasteurization temperature, because of containing high lactoglobulin and lactalbumin. Use of such early milk for cheesemaking is undesirable on account of it gives curd, which lacks elasticity, and shrinkability, the curd retains more moisture and its drying is difficult. It favors growth of undesirable organism. The presence of somatic cell and natural inhibitors in such type of milk may interfere with the starter activity (Anon., 1995).

2.5.2.3 Late lactation milk

Late lactated milk is poor for cheese making, because of its large sodium and potassium content causing more protein hydrated than in normal milk (De, 2000).

2.5.3 Changes in the milk after production

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

2.5.3.1 Changes in casein micelles and salt equilibrium

The practice of holding cooled raw milk in bulk tanks for extended period not only increases the possibility of growth of psychrotrophs but also modifies 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 2.6.

Table 2.6 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)

2.5.4 Inhibitory substances

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

2.5.4.1 Natural inhibitory systems in milk

The natural inhibitory system in raw milk includes immunoglobulin, lactoferrin, lysozyme and lactoperoxidase-thiocyanate-hydrogen peroxide system. Presence of such inhibitory systems can influence cheesemaking, particularly when cheese is made from raw milk as these systems have been found active against lactic streptococci affecting their multiplication and acid production. Certain strains of lactic acid bacteria can produce sufficient hydrogen peroxide under aerobic conditions and self-inhibitory effect may be observed during cheesemaking (FAO, 1999).

2.5.4.2 Antibiotic residues

Antibiotics may gain entry into 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 both from public health point of view and because their presence can 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, various types of taints, cracks, open texture and sponginess.

2.5.4.3 Residues of detergents and sanitizers

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

2.5.5 Microbiological quality of milk

The groups of microorganism that may be found in raw milk and their relative predominance or otherwise in the total microbial population at any given time are influenced by a number of factors such as health of animal, hygienic practices employed at different stages of milk production, handling and storage, system of cleaning and sanitization and its efficacy, method of milking, handling and storage, design and condition of the utensils used, type and number of organisms initially contaminating the milk and their rate of multiplication (Robinson, 1985).

In general, for cheesemaking a low count milk, as free as possible 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, for public health reasons milk must be free from pathogens and also from organism which produces toxins involved in food poisoning (Upadhyay, 2003).

2.6 Pretreatments of milk for cheesemaking

Pretreatments have profound effect on cheese manufacturing schedule, cheesemaking efficiency, 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

2.6.1 Chilling and cold storage

Most raw milk in developed dairy countries 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. There is tendency for raw milk to be older and colder for much longer period than when milk is handled in cans. Such practice of hold in cooled raw and /or processed milk for extended period not only increases the possibility of growth of psychrotrophs, but also modifies the physico-chemical status of milk components, particularly casein and minerals. This in turn has been found to alter the behavior of milk during subsequent cheesemaking (Upadhyay, 2003).

The impaired technological properties as a result of chilling and cold storage of the raw milk, can be improved by adopting measures such as acidification with lactic acid to pH 6.5, addition of calcium chloride at 0.02%, addition of more rennet within permissible limit, use of higher renneting temperature and use of higher cooking temperature (Anon., 1995).

2.6.2 Lactoperoxidase treatment

Lactoperoxidase system can be employed as an alternative to chilling of raw milk for its preservation and to overcome the problem posed by psychrotrophs in such stored milks. The system comprises of three components viz., Lactoperoxidase, thiocynate and H_2O_2. A residual level of 61.4ppm has been reported in 70:30 (SCN : H_2O_2) lactoperoxidase treated milk. Most of the SCN added to milk is regenerated through interaction of oxidation products with SH-groups of protein. The ability of starter organisms to produce H_2O_2 results in reactivation of lactoperoxidase system unless the lactoperoxidase is inactivated by suitable heat treatment (e.g. 78°C/15 s). However, such drastic heat treatment is not commonly used for cheese milk and thus lactoperoxidase is expected to be always present in lactoperoxidase treated milk. Thus, potential exists for the reactivation of lactoperoxidase system leading to problems in manufacture of cheese due to suppression of starter activity (FAO, 1999).

2.6.3 Thermization

In countries, where cooling and storage of pooled raw milk is being practiced, proliferation of psychotropic bacteria poses certain problems in cheese manufacture such as reduced cheese yield and deleterious effects on its quality. These organisms produce unclean flavors, cause bitterness and even sour the milk when stored for extended period. They also secrete heat stable extra cellular lipolytic and proteolytic enzymes and thus can bring about reduced yield. Thermization (57-68°C for 10-20 s) of fresh raw milk prior to cooling and storage can be advocated to overcome such problem (Anon., 1995).

2.6.4 Bactofugation

Bactofugation process is the selective method of removing of bacteria; the size and density of bacteria being the criteria for their removal. The other factors which decide the efficiency of the bactofuge to remove bacteria are initial bacteria load, pretreatment of milk, throughput of machine, volume of through put, frequency of partial de-sludging and duration of the run. Bactofugation of milk is always applied in combination with heat treatment, a temperature of 55-65°C offering the optimum bactofugation effect. In the last two decades this technique has been applied on large scale particularly to decrease the danger of “late blowing” caused by butyric acid bacteria in cheese milk. Bactofugation facilitates substantial reduction in the quality of nitrates, usually added to prevent ‘late fermentation’ by clostridia in cheese, which minimizes the product loss. However, it leads to the slight weakening of coagulum during

cheese making. This can be overcome be the addition of calcium chloride in cheese milk (Kosikowski and Fox, 1968; Anon., 1995).

2.6.5 Microfiltration

Microfiltration efficiently removes the indigenous microorganisms from milk without the application of heat. Microfiltration appears to offer the ideal approach to assess the contribution of non-starter lactic acid bacteria (NSLAB) to cheese ripening without complicating heat induced changes to milk protein or enzymes, except those on fat globule membrane. Some years ago, microfiltration was introduced as an alternative to bactofugation in the so-called 'Bactocatch' process (Anon., 1995; Upadhyay, 2003).

2.6.6 Standardization

The necessity of standardization of cheese milk has arisen due to variation in the composition of milk and to ensure that the final product meets the legal requirements. Since fat and protein are the two components that constitute the main body of cheese 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 (Scott, 1986). According to Scott (1986), adjusting the casein/fat ratio (C/F) in cheese milk in the range of 0.69:1 to 0.70:1 yields a cheese with better body-texture characteristics.

2.6.7 Pasteurization

The purpose of pasteurization of cheese milk is to destroy microorganisms which are pathogenic and which may damage the cheese, and inactivate enzyme, which would affect the healthiness and palatability of the cheese (Kosikowski and Fox, 1968).

Pasteurization temperature-time combination varies considerably depending 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°C
2. HTST: 71-75°C /14-40s
3. LTLT: 61-65°C / 20-40 min.

Salient effects of heat treatment of cheese milk outlined are (De, 2000).

1. It enables the manufacturer of cheese with consistency higher quality and uniformity with ensured public health safety.

2. It permits better growth of starter bacteria as a consequence of elimination of antagonistic effect of contaminant organism.

3. Results in increased cheese yield owing to higher moisture retention and higher total solids recovery on account of denaturation of whey proteins with concomitant improved incorporation in curd matrix along with fat.

With increasing severity of heat treatment, the rennet coagulation time increases, yielding soft curd, lacking elasticity and coherence due to the heat induced complex of β-lactoglobulin and κ-casein, altered charges and surface properties of casein micelles and shift in the ionic and colloidal milk salt system. Acidification, slightly higher setting temperature and addition of calcium chloride can be used to overcome the adverse effect (Barbano, 1999).

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

2.6.8 Homogenization

Homogenization is not usually practiced in manufacture of most of the cheese verities such as Cream, Blue and Soft varieties. Homogenization of milk is done (Banks, 1992).

- To reduce the loss of fat (incorporation of small sized fat globules in the curd) in whey and therefore increase in the yield of cheese.

- Improve the texture of cheeses by making the curd smoother and finer yielding the higher moisture retention.

- Produce ‘white cheese’ from cows’ milk similar to that of goat milk cheese.

2.6.9 Lactose hydrolysis

Use of lactose hydrolyzed milk for cheesemaking has been reported to have the following effects on the manufacture (Upadhyay, 2003).

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

2.6.10 Concentration

Cheese manufacturing is essentially a controlled dewatering process, in which the fat and casein in milk concentrated around 6-12 fold, depending on the cheese variety being made. The concentration of milk for cheesemaking can be achieved by (Anon., 1995).

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

Ultrafiltration is most widely used concentration process generally in the manufacture of soft cheese varieties. Along with the reduction in manufacturing time, labor, transportation and storage three direct advantages of ultrafiltration in cheese milk are (Kosikowski, 1982).

- Increased cheese yield by incorporating the whey protein in the cheese
- Increased plant capacity due to reduction in volume of fluid to be processed
- Reduced used of rennet salt and color.

2.7 Additives in cheese milk

The essential additives the cheese making process are the starter culture and the rennet. Sometimes it may be necessary to supply other components such as calcium chloride ($CaCl_2$), and saltpeter and acidulants (Anon., 1995).

2.7.1 Calcium salt

Calcium plays an important role in the secondary phase of rennet action. Thus, calcium balance between the soluble, colloidal and complexed is very important for the successful coagulation. The lack of balance and disturbance of calcium is due to chilling and cold storage at 4-5°C for long periods causes dissociation of β-casein, sever heating of milk during pasteurization and dilution of milk with water (Lucey, 1993).

Calcium may be added to milk in different forms such as calcium chloride ($CaCl_2$) up to 0.02%, dibasic calcium phosphate is recommended for use with pepsin rennet (<0.01%), lime water and calcium lactate (Upadhyay, 2003).

The beneficial effects of addition of $CaCl_2$ on Rennet Coagulation Time (RCT) and gel strength is believed to be due to increase in Ca^{++}, increase in Colloidal Calcium Phosphate (CCP) and decrease in pH (De, 2000). Micellar calcium plays an important role in improving melt and other related functional properties of Mozzarella cheese (Joshi *et al.,* 2004).

2.7.2 Cheese colour

It is common practice to add extra color to pale colored milk to give cheese an attractive and appetizing appearance. Two important colors Riboflavin and Carotenoids are found in milk however they are lost in whey. The vegetable origin annatto cheese color is used at the rate of 88 g/1000 kg widely (Kosikowski, 1982).

2.7.3 Inhibitory salts

In the manufacture of less acid cheese like Edam, Gouda, Swiss inhibitory salts (Saltpeter) are added in milk to prevent the growth of gas producing organisms such as coliform /aerogenes groups of bacteria which are responsible for “early blowing” defect in cheese and butyric acid bacteria which are responsible for “late blowing” defect in cheese. A concentration of 10 to 100 ppm of nitrite or 2 to 5 ppm of nitrate is sufficient to inhibit the growth of spores. Color defects, possibly carcinogenic effects are the main limitation of using the nitrate in cheesemaking. Lysozyme has been introduced as a substitute for saltpeter for an inhibitor of clostridia organisms (Farkye, 2004).

2.7.4 Starters

Various starter types are used for cheesemaking. Starters play a role in the acidification of cheese milk to the desired pH during manufacture. In addition, starter bacteria play an important role in the maturation and flavor development of cheese. Current starter technologies include genetically modified starters, adjunct starters and fast-acid starters, which are available commercially as liquid, frozen or dried (Farkye, 2004).

Starters of lactic acid bacteria are added to cheese milk for manufacture of all cheese varieties. Production of cheese depends on fermentation of the lactose by those lactic acid bacteria to form mainly lactic acid, and to lesser extents aroma and flavor compounds and enzymes aiding in ripening of cheese (Cogan *et al*., 1997).

Lactic acid bacteria used as starters in cheesemaking include streptococci, lactococci, leuconostocs and lactobacilli. Selected species of these genera are used as combined cultures, or as single strain cultures, or as mixtures of single strain cultures. Thermophilic starters (optimum temperature 37-45°C) are used for the production of high temperature cooked cheese varieties (e.g., Swiss and Parmesan), 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).

2.7.5 Acidulants

- Lactic acid produced in situ by lactic acid bacteria.
- Chemically acidifying the milk.

The potential for substituting all or a portion of the acid-producing functions of lactic cultures has been tested for several varieties of cheese. The two successful methods involve adding an acidogen that hydrolyzes in milk to release an acid and adding acids to milk at low temperature (Olson, 1981). This method is used fairly widespread in manufacture of certain cheese varieties. Acids of food grade quality such as lactic acid, glacial acetic acid, lime juice/juice; D-glucono-delta-lactone, phosphoric acid, etc. are used to bring about coagulation of milk. Natural whey culture can be added as an Acidulants (FAO, 1995). However, acidulants do not show superior organoleptical potential in rennet cheese as compared to acid coagulated cheeses (Patel and Gupta, 1986).

2.7.6 Salt

Sodium chloride is added in some form, to all cheese at some stage in their manufacture. Salt has three major functions in cheese: it acts as a preservative, contributes directly to flavor, and is a source of dietary sodium. Together with the desired pH, water activity and redox potential, salt assists in cheese preservation by minimizing spoilage and preventing the growth of pathogens. In addition to these functions, salt level has a major effect on cheese composition, microbial growth, enzymatic activities and biochemical changes, such as glycolysis, proteolysis, lipolysis and Para -casein hydration, that occur during ripening (Guinee and Fox, 2004).

2.7.7 Rennet

The main purpose of adding rennet to cheese milk is to cause the milk to coagulate, which is necessary for whey exudation. However, action of rennet in cheese-making does not end here, but it also plays a part in breaking down the casein during cheese ripening.

Rennet is the commercial preparation of the milk clotting enzymes. For centuries, rennet has been made from calf stomach (abomasum, the fourth stomach or the veal). Chymosin (rennin) is a saline extract from the abomasum of the milk fed calves. Chymosin is the major protease in young calf (88-94%) but it is replaced by pepsin as the calf matures. Chymosin extracts contain some pepsin depending upon the age of the calf (Dalgleish, 1987).

Rennet activity is defined as the number of milliliters of milk, which can be clotted by one milliliter of liquid rennet within 40 minutes at a temperature of 35°C. Stability of calf rennet is maximum at pH from, 5.5-5.9 and photo-oxidation results in inactivation of chymosin (Upadhyay, 2003).

2.7.7.1 Calf rennet

For centuries, rennet has been made from calf stomachs (abomasums, the fourth stomach or the veal). The stomachs are sliced into strips which are extracted in a slightly acid salt solution. As rennet enzymes are destroyed by heating to 55-60°C, the rennet extracts cannot be preserved by heating. To obtain the best possible preservation, the pH value of the extract adjusted to approximately 5.5, and salt solution to 15-20%. The rennet must be protected from light and stored cold. Under these conditions, it will only lose about 1% of its activity (strength). The enzymes are destroyed by alkali and strong acid. Most of the coagulation

activity of calf rennet is caused by the enzyme chymosin (rennin). Part of the coagulation activity, however, is caused by another enzyme: bovine pepsin. As the calf matures the amount of pepsin increases and chymosin (rennin) decreases (Acharya, 2010).

2.7.7.2 Microbial rennet

The recent growth in the cheese industry and the scarcity on calf rennet have stimulated the research for milk clotting enzyme from alternative sources (Fox, 1987). In 1988, chymosin produced through recombinant DNA technology was first introduced to cheesesmakers for evaluation. Many microorganisms are known as producers of rennet such as proteinases, which can substitute the calf rennet. However, the microbial enzymes exhibited two major drawbacks, i.e., (i) the presence of high levels of nonspecific and heat-stable proteases, which led to the development of bitterness in cheese after storage; and (ii) a poor yield. Extensive research in this area has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases. Microorganisms like *Rhizomucor pusillus* and *Cryphonectria parasitica* (Egitoa *et al.,* 2007), *Thermomucor indicae-seudaticae* N31 (Merheb-Dini *et al.,* 2009), *Nocardiopsis* sp. (Cavalcanti *et al.,* 2004), *Rhizomucor miehei* (Park *et al.,* 2000), *Penicillium oxalicum* and *Aspergillus oryzae* (Hashem, 2000) are extensively used for rennet production in cheese manufacture.

2.7.7.3 Plant enzymes as rennet substitute

The use of plant coagulants in cheese making is associated with many advantages: they are natural, cheap, easy to prepare, allow straightforward process and used to produce cheese for ecological markets (Roseiro *et al.,* 2003). Plant proteases employed for cheese production in various areas of the world include papain, bromelain, ficin, oryzasin, cucumisin, sodom apple and *Jacaratia corumbensis* (Duarte *et al.,* 2009). The most promising plant protease that has potentiality in Nepal is discussed.

Papain (Papaya protease)

Papain is a cysteine hydrolase that is stable and active under a wide range of conditions. It is very stable even at elevated temperatures (Cohen *et al.*, 1986). The latex of Carica papaya is a rich source of four cysteine endopeptidases including papain, chymopapain, glycyl endopeptidase, and caricain. The proteins are synthesized as inactive precursors that become

active within two minutes of the plant being wounded and the latex expelled. Papain is a minor constituent, but has been more widely studied because it is more easily purified (Azarkan *et al.*, 2003).

- History

In 1873 G.C. Roy first investigated the action of papain in an article published in the Calcutta Medical Journal entitled "The Solvent Action of Papaya Juice on Nitrogenous Articles of Food". Papain was first named in the late nineteenth century by Wurtz and Bouchut who partially purified the product from the sap of papaya (Menard *et al.,* 1990). When named, it was simply recognized as a proteolytically active constituent in the latex of tropical papaya fruit (Wurtz and Bouchut, 1879). Throughout the mid-1950s and 1960s, purification and separation techniques improved greatly and pure papain was isolated. The study of papain allowed for great advances in understanding enzymes as proteins. In 1968, papain was the second enzyme to be crystallized and its structure determined by x-ray methods. Papain was the first cysteine protease to have its structure identified (Drenth *et al.*, 1968). In the 1980s, the geometry of the active site was reviewed and the three-dimensional structure was determined to a 1.65A° resolution (Kamphuis *et al.,* 1984). The precursors and inhibitors of papain were extensively studied into the 1990s (Vernet *et al.* 1995).

- Specificity

Papain has fairly broad specificity; it has endopeptidase, amidase, and esterase activities. The active site consists of seven subsites (S1-S4 and S1'-S3') that can each accommodate one amino acid residue of a substrate (P1-P4 and P1'-P3') (Schechter and Berger, 1967). Specificity is controlled by the S2 subsite, a hydrophobic pocket that accommodates the P2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite (Kimmel and Smith, 1954). Outside of the S2 subsite preferences, there is a lack of clearly defined residue selectivity within the active site. Activated papain attacks the peptide bonds between the carboxylic acid group of lysine or arginine and the adjacent amino acid residue. A small cleavage occurs at the carboxylic acid group of histidine and also of glycine, glutamic acid, glutamine, leucine tyrosine residues (Anon., 2019b).

The mechanism by which papain breaks peptide bonds involves the use of a catalytic triad with a deprotonated cysteine. Asn-175 helps to orient the imidazole ring of His-159 to allow it to deprotonate the catalytic Cys-25. This cysteine then performs a nucleophilic attack on the carbonyl carbon of a peptide backbone. This forms a covalent acyl-enzyme intermediate and frees the amino terminus of the peptide. The enzyme is deacylated by a water molecule and releases the carboxy terminal portion of the peptide. Papain prefers to cleave at: [(hydrophobic) - (Arg or Lys)- cleaves here - (not Val)]. Hydrophobic is Ala, Val, Leu, Ile, Phe, Trp, or Tyr (Anon., 2019c).

- Molecular Characteristics

The mature forms of all papaya proteinases are between 212 and 218 amino acids, and exhibit a strong degree of homology (Azarkan *et al.*, 2003). X-ray structure analysis has shown that they adopt identical three-dimensional folds (Pickersgill *et al.,* 1991, O'Hara *et al.,* 1995, and Maes *et al.,* 1996). Papain is synthesized as a zymogen with a 133 amino acid N-terminal region that is not part of the active enzyme (Cohen *et al.,* 1986). The papain precursor gene, prepropapain, has been cloned and expressed either in parts or as a whole (Cohen *et al.,* 1986 and Choudhury *et al.,* 2009).

- Composition

Papain is a single-chained polypeptide with three disulfide bridges and a sulfhydryl group necessary for the activity of the enzyme. Papain is expressed as an inactive precursor, prepropapain. The formation of active papain requires several cleavage steps including an initial cleavage of the 18 amino acid preregion (the signal sequence), followed by further cleavage of the glycosylated 114 amino acid proregion (Vernet *et al.,* 1995). This proregion serves as an intrinsic inhibitor and folding template.

The papain precursor protein contains 345 amino acid residues (Anon., 2018), and consists of a signal sequence (1-18), a propeptide (19-133) and the mature peptide (134-345). The amino acid numbers are based on the mature peptide. The protein is stabilized by three disulfide bridges. Its three-dimensional structure consists of two distinct structural domains with a cleft between them. This cleft contains the active site, which contains a catalytic diad that has been likened to the catalytic triad of chymotrypsin. The catalytic triad is made up of the amino acids - cysteine-25 and histidine-159. Aspartate-158 was thought to

play a role analogous to the role of aspartate in the serine protease catalytic triad, but that has since been disproved (Menard *et al.,* 1990).

- Protein Accession Number: P00784
- Molecular Weight: 23.4 kDa (Theoretical)
- Optimal temperature: 60-70°C
- Optimal pH: 6.0-7.0
- Isoelectric Point: 8.88 (White and White, 1997)
- Production

The papain is a natural proteolytic enzyme that is extracted from the latex in the leaf, the stem and the papaya's unripe fruits (Baeza *et al.*, 1990). The papaya is a small, sparsely branched tree, usually with a single stem growing from 5 to 10 m (16 to 33 ft) tall, with spirally arranged leaves confined to the top of the trunk. The lower trunk is conspicuously scarred where leaves and fruit were borne. The leaves are large, 50–70 cm (20–28 in) in diameter, deeply palmately lobed, with seven lobes. All parts of the plant contain latex in articulated laticifers. Papayas are dioecious. The fruit is a large berry about 15–45 cm (5.9–17.7 in) long and 10–30 cm (3.9–11.8 in) in diameter. It is ripe when it feels soft (as soft as a ripe avocado or a bit softer) and its skin has attained an amber to orange hue (Menard *et al.,* 1990).

In 2016, global production of papayas was 13.05 million tonnes, led by India with 44% of the world total. Global papaya production grew significantly over the early 21st century, mainly as a result of increased production in India and demand by the United States. Both green papaya fruit and the plant's latex are rich in papain, a protease used for tenderizing meat and other proteins, as practiced currently by indigenous Americans, people of the Caribbean region, and the Philippines. It is now included as a component in some powdered meat tenderizers (Shuren, 2008).

Papain is usually produced as a crude, dried material by collecting the latex from the fruit of the papaya tree. The latex is collected after scoring the neck of the fruit, where it may either dry on the fruit or drip into a container. This latex is then further dried. It is now classified as a dried, crude material. A purification step is necessary to remove contaminating substances. This purification consists of the solubilization and extraction of the active papain enzyme system through a government-registered process (CBS, 2019). This purified papain may be supplied as powder or as liquid (Anon., 2019b).

- Papain family

Papain belongs to a family of related proteins with a wide variety of activities, including endopeptidases, aminopeptidases, dipeptidyl peptidases and enzymes with both exo- and endo-peptidase activity (Rawlings and Barrett, 1994). Members of the papain family are widespread, found in baculoviruses, eubacteria, yeast, and practically all protozoa, plants and mammals (Rawlings and Barrett, 1993). The proteins are typically lysosomal or secreted, and proteolytic cleavage of the propeptide is required for enzyme activation, although bleomycin hydrolase is cytosolic in fungi and mammals (Sebti *et al.,* 1987). Papain-like cysteine proteinases are essentially synthesized as inactive proenzymes (zymogens) with N-terminal propeptide regions. The activation process of these enzymes includes the removal of propeptide regions, which serve a variety of functions in vivo and in vitro. The pro-region is required for the proper folding of the newly synthesized enzyme, the inactivation of the peptidase domain and stabilization of the enzyme against denaturing at neutral to alkaline pH conditions. Amino acid residues within the pro-region mediate their membrane association, and play a role in the transport of the proenzyme to lysosomes. Among the most notable features of propeptides is their ability to inhibit the activity of their cognate enzymes and that certain propeptides exhibit high selectivity for inhibition of the peptidases from which they originate (Yamamoto *et al.,* 2002).

- Utilization in cheese production

The papaya latex can successfully be used as a coagulating agent in cottage cheese (Rana *et al.*, 2017). The cheese which is produced by using papain enzyme as coagulant is *Dangke*, it originates from Enrekang, a regency of the South Sulawesi province of Indonesia (Prasetyo *et al.*, 2015).

2.8 Cheese technology

Cheese manufacturing is a dehydration process in which the casein, fat and colloidal salts of milk are concentrated 6-12 fold in the manufacturing phase with the removal of 90% of the milk and essentially all of the lactose, whey protein and soluble milk salts (Anon., 1995).

The object of the various cheese manufacturing procedures is to establish conditions suitable for the biochemical and physical changes which are responsible for the development of characteristic body and flavor (Harper *et al*., 1966).

Though no two cheeses, even from the same batch of production are identical (Pradhan 2000), basic cheese making principles are concentration, preservation and ripening (Scott, 1986).

2.8.1 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).

2.8.2 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).

2.8.2.1 Protein

As the casein is the principle constituents in the coagulum, the retention figure for rennet casein will be close to 100. As small loss of casein may occur through very small cheese particles being lost with the whey. Rennet casein makes up some 74% of the milk protein,

and as some of the whey proteins also remain in the cheese, the retention figure for protein is about 75%. If the cheese milk is high temperature pasteurized, the retention figure for protein will be higher because whey protein is also denatured will precipitate together with the casein. However, the retention figure cannot be higher approximate 88% because low molecular weight nitrogenous compounds and peptides, as well as peptides, which are cleaved from the casein by the rennet, will always follow the whey (Acharya, 2010). Demott (1983) found 85.37% protein retention with 18.1% fat in the final product. Omueti and Jaiyeola (2006) reported that retention of whey in final cheese might increase the protein contents as well. The microorganisms and enzymes may also contribute to the increase in total protein content retention in cheese but in small amount (Fox and McSweeney, 2004).

2.8.2.2 Fat

The fat globules are retained by the fine-meshed casein network. The usual retention for fat is around 92 (88-95) %. The rest of the fat is released during cutting of the curd and enters the whey from the surface of the curd grains (Acharya, 2010).

2.8.2.3 Lactose

Lactose is found in true solution in milk and therefore freely follows the water out of the curd grains. For this reason, the retention figure for lactose is low, usually in the range of 3-5%, equivalent to the amount of whey remaining in the cheese (Acharya, 2010).

2.8.2.4 Ash

When rennet cheese is manufactured, approximate 30-40% of ash goes into cheese. When acid curd cheese in manufactured, the retention figures is lower because calcium and phosphate are released from the casein during acidification (Acharya, 2010).

2.8.2.5 Citric acid

Most of the citric acid is found in true solution just like lactose. Approximate 10% of the citric acid is linked to the casein together with the calcium in the same way as phosphate. The retention figure will depend partly on the whey content of the cheese, partly on the acid development during whey exudation or prior to ultrafiltration (Acharya, 2010).

2.8.2.6 Bacteria

The diameter of the bacteria is of the same order of the fat globules and therefore, the bacteria behave in similar fashion during whey exudation from the curd grains. Normally, approximately 90% of the bacteria of milk are concentrated in the curd grains (Acharya, 2010).

2.8.3 Basic steps in cheese-making

The basic steps in cheese-making (Acharya, 2010) are:

1. Acidification
2. Coagulation
3. Curd Treatment
    (i) Cutting
    (ii) Stirring
    (iii) Scalding/cooking
    (iv) Whey draining
4. Shaping (molding and pressing)
5. Salting

2.8.3.1 Acidification

Acidification of milk *in situ* decreases the pH of milk affecting the several aspects of cheese manufacture.

- Decrement of the rennet coagulation time of milk

Chymosin activity is reported to be optimum at pH in the range of 5.0-5.4. Additionally, increase in acidity results in solubilization of Colloidal calcium, which in turn make the calcium readily available for secondary phase of rennet action (Adhikari *et al*., 2000).

- Increased rate of syneresis

Casein has been reported to have least water holding property at isoelectric point (i.e. 4.6). Thus, the gradual shifting of pH towards the isoelectric point influences the final moisture content of cheese (Upadhyay, 2003). This controls the growth of bacteria and the activity of enzymes in the cheese, it thus strongly influences the rate and pattern of ripening and the quality of the finished cheese (Pradhan, 2000).

- Curd strength of gel which influences cheese yield

Decrease in pH and increase in calcium content of milk increases the firmness of curd, resulting in improved retention and thereby the yield (Upadhyay, 2003).

- Retention of the coagulant in the curd

Decrease in draining pH increases the amount of rennet retained in the curd during manufacture this influences the rate of proteolysis and thereby cheese texture and flavor during ripening (Quarne *et al*., 1968).

- Solubilization of Colloidal Calcium Phosphate (CCP)

Approximately 65% calcium and 55% phosphorus are insoluble and are associated with the casein micelles as colloidal calcium phosphate (CCP) (Keller *et al*., 1973). Acidity controls the growth of many species of non-starter bacteria, especially harmful (pathogenic and spoilage) bacteria, in cheese. In addition to acid production, many starter bacteria produce antibiotics that also check the growth of non-starter microorganisms (Upadhyay, 2003).

- Acidification in situ by starter culture

Pure cultures are widely used for acid production in situ for consistently good quality cheese manufacture. For the uniform quality the amount of starter culture added should be kept constant (Pradhan, 2000). Nielsen and Ullum (1989) have stated that when the starter used is grown in milk, the normal amount of inoculums is 0.5-2% and the pre-ripening time is usually 10-30 minute.

2.8.3.2 Coagulation of cheese-milk

The property of the protein, casein, which permits its coagulation by acid or rennet, is a key essential to cheese-making. Coagulation of the milk or curd formation is usually

accomplished by addition of the enzyme, rennin, to the milk. Formation of milk coagulum is due to the destabilization of the casein micelles (Harper and Hall, 1976).

- Stability of casein micelle

In bovine milk, casein which represents the 80% of the total nitrogen comprises of 4 principle fraction, α s-1, α s-2, β and κ, existing in the approximate ratio of 40:10:35:12. More than 90% of casein exists as micelles in milk. It is widely supported view that calcium sensitive α_s and β-caseins form the core of the sub-micelles with κ-casein predominantly located on the surface. The micelles are stabilized by a zeta potential of about -20 mV and by stearic hindrance caused by protrusion of C-terminal segment of κ-casein on the surrounding medium (Walstra *et al*., 2006).

- Mechanism of coagulation of milk by rennet

Rennet coagulation of milk is a 2-stage process, the first involving conversion of casein to paracasein by the rennin, the second involving the precipitation of paracasein by calcium ions at temperature above 20°C (Anon., 1995). The protein chain of κ-casein (1-169 residues) consists of two quite different segments. On the C-terminal, one third of the protein (106-169) is highly negatively charged and is very hydrophilic. The remaining two third of the molecule (1-105 residue) on the N terminal carries a net positive charge and is hydrophobic in nature (Upadhyay, 2003). Successful milk clotting agents split the κ-casein at the bond between the phenylalanine residue (105) and the methionine residue (106) diffusing casino-peptide in the serum. As a consequence, the net charge on the micelles is reduced and the positively charged sites are exposed which can react with negatively charged groups on the other micelles. Removal of protruding peptides from the surfaces also contributes to decrease in inter-micelles repulsive force. As a result, the altered casein micelles in presence of calcium interact to form a three-dimensional network which becomes the milk coagulum. The micelles begin to gel only when about 85% of the total κ-casein has been hydrolyzed (Upadhyay, 2003).

- Setting the vat

When correct acidity has been developed, rennet is added to the cheese milk, usually at the rate of 20 ml liquid extract or 1g powder per 100 kg milk (Irvine and Hill, 1985). The correct amount of rennet mixture is diluted with 10-20 times its volume of sterile cold water in order

to obtain a uniform distribution. The rennet is mixed into the cheese milk, the milk having been stirred just prior to its addition. It is usually safe to stir the milk 3-5 min after adding rennet which will be a sufficient time for good mixing. Inadequate stirring allows fat to raise the surface of the milk. This leads to fat losses during cutting. Too vigorous or prolonged stirring of milk causes the newly forming coagulum to breakup These curds whey-off quickly and do not reunite but loose fat in the whey (Scott, 1981). Vat is left undisturbed until the coagulation occurs (Scott, 1986). At first the curd formed is very soft and weak, but gradually it becomes firmer and more cohesive. Curd tension determines the moisture content and cheese yield (Fox, 1987).

- Factors affecting the rennet activity

The factors affecting rennet activity have been discussed by Scott (1986); Dalgleish, (1987); De (2000) and (Upadhyay, 2003).

1. pH

In connection with the splitting of the κ-casein in milk (the primary phase), the chymosin optimum pH is at 5.4 (5.0-5.5) (Dalgleish, 1987). Even small changes in the acidity of the milk greatly influence the activity of rennet enzymes. In the case of chymosin, the enzymes act twice as fast when the pH is lowered from 6.7 to 6.4. The effect of pepsins-both bovine and porcine- increases even when the pH value is lowered from 6.7 to 6.4 (De, 2000).

2. Temperature

The optimum temperature of rennet is 42°C and the activity is lower at both higher and lower temperatures. At 55-60°C the enzyme is destroyed. Changes in milk temperature influence the coagulation time as coagulation takes two to thrice times longer at 30°C than 42°C (Adhikari *et al.,* 2000).

3. Calcium ion concentration

Removal of colloidal calcium phosphate by more than 20% will not allow coagulation of rennet altered micelles and normal bovine milk does not coagulate below 18°C unless calcium is increased. Variation in the content of free calcium ion in the milk will cause

changes in the coagulation time, firmness of curd, and whey exudation. It has also been demonstrated that these ions increase the rate of the enzyme reaction (Upadhyay, 2003).

2.8.3.3 Curd treatment

The purpose of curd treatment in the cheese vat after coagulation is to promote the contraction of the casein network and resulting whey exudation (syneresis) without losing too much fat and curd in the whey (Harper and Hall, 1976).

- Cutting the coagulum

After the coagulum has reached the desired firmness it is cut. The object of cutting is to divide the large mass of curd into smaller, uniform particles in order that moisture may be more easily expelled (Harper and Hall, 1976).

Curds which need to be scaled to higher temperature, are cut into smaller pieces, while those curd, which are scalded to lower temperatures, can be left in larger pieces unless the curds are very acid (Scott, 1986).

Curd is normally cut with cheese harp wired curd knives, horizontal and vertical, which are spaced from 3 mm for Swiss to 1.2 cm apart for cottage (Irvine and Hill, 1985). It is important that all cubes be identical so that the expulsion of whey on heating will be uniform (Fox, 1987).

- Stirring

During stirring the curd is agitated gently just enough to keep it from matting (Harper and Hall, 1976) otherwise curd particles may fuse together preventing free release of whey (Irvine and Hill, 1985).

Stirring maintains the curd in discrete particles allowing the uniform cooking and uniform release of whey. The curd after cutting is quite soft and fragile and must be handled gently to prevent undue crushing and loss of fat and curd dusts. Stirring (agitation) can become more rapid when enough whey is released so that the curd may be stirred without breakage (Irvine and Hill, 1985). The stirring phase usually lasts for about 15-25 minutes (Nielsen and Ullum, 1989).

- Heating/cooking/scalding

When the curd is firm enough for handling, the curd whey mixture may be heated (cooked) to increase their concentration and whey exudation. Heat causes contraction of the curd particles with increased expulsion of whey (Rowney *et al.*, 1999). More importantly, heat is used to regulate the growth of the lactic acid bacteria and control acid development in the curd. The time and temperature program for heating is determined by the type of cheese and the method of heating (direct heating by adding hot water; indirect heating in a jacketed vat) (Fox, 1987). Heating to temperatures above 40°C is sometimes called cooking and beyond 44°C is called scalding. At 37-38°C the activity of the mesophilic lactic acid bacteria is retarded and they are totally deactivated at above 44°C. Further, they are killed if held at 52°C for periods of 10-20 min (Upadhyay, 2003). Too rapid application of heat may result in lumping and formation of a film on the surface of the curd particles, preventing proper expulsion of whey. After heating, the curd is cooked, usually under constant agitation, until desired firmness has been reached. The heating may favor further degradation of α-casein and provide for increased cross-linking in the gel which results in a firmer, more rubbery and compact curd (Scott, 1981; Dalgleish, 1982). Soft cheese with higher moisture content necessitates a lower scalding temperature resulting in more whey in the curd (FAO, 1995).

Cheeses prepared from pasteurized milk will be superior to those prepared from milk heated to high temperature. Melting and stretching characteristics of cheese made from milk heated to high temperature will also be inferior to the cheeses made from pasteurized milks (Ghosh and Singh, 1996).

2.8.3.4 Molding and pressing

When the curd grains have released a suitable amount of whey and have attained the proper degree of firmness and/or when pH (acidity) has reached a suitable point, the curd grains are collected into a collective mass and the shape is given (Scott, 1986). Pressing assist to mold the cheese into a desired shape to make the grains fuse and to assist in expelling any free whey (Anon., 1995). The difficulty in whey drainage may arise from clogging of the drainage screen by fine particles (Hill *et al*., 1982).

2.8.3.5 Salting

Salting of curd is a traditional and integral part of the manufacture of most cheese varieties. The techniques of salting are very varied such as (Guinee and Fox, 2004):

- Mixing dry salt with broken or milled curd at the end of manufacture (e.g. Cheddar). Submerging cheese in brine (e.g., Swiss cheese).
- Rubbing dry salt on the surface of the cheese.

Salt content directly influences cheese flavour, provides sodium, essential for control of blood pressure and healthy cell function within the body, and crucially acts as a preservative. Salt lowers the water activity within the cheese matrix and subsequently controls microbial growth, enzyme activity, extent of protein hydration and aggregation along with rheological and cooking properties of cheese (Islam, 2006).

2.8.4 Curing and ripening

Curing/ ripening refer to the storage of cheese under controlled conditions of time, temperature and humidity during which the desired body, flavor and texture of cheese develops. Ripening embodies the physical and chemical changes that occur in the curd, which results in the desirable characteristic flavor and body (Kosikowski, 1982).

Extensive knowledge of the primary degradation pathways of milk constituents in cheese curd, glycolysis (lactose and citrate), lipolysis (milk lipids), and proteolysis (caseins), has been accumulated. During ripening, primary degradation of milk constituents leads to the formation of a whole range of precursors of flavor compounds. Only some of the compounds formed by glycolysis, lipolysis, and proteolysis directly contribute to cheese flavor; for example, shortchain fatty acids, acetaldehyde diacetyl, peptide, and amino acids. Primary degradation of major caseins; for example, α_{s1}-caseins, has major consequences for cheese texture. These changes are followed and/or overlapped by a concerted series of secondary catabolic reactions which are responsible for the unique aroma profile of a particular variety or type of cheese (Singh *et al.,* 2003).

2.9 Cream cheese manufacturing method

The methodology for cream cheese manufacturing given by Phadungath (2005) has been shown in Fig. 2.1.

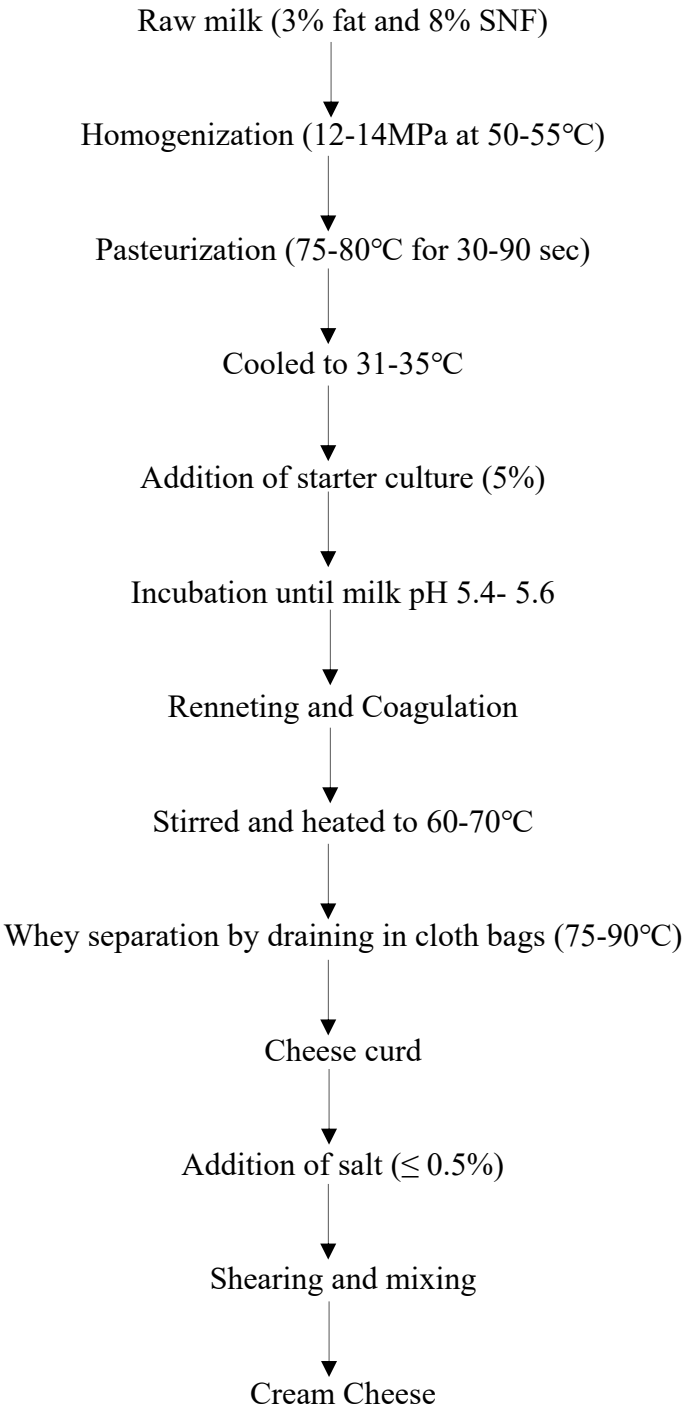

Fig. 2.1 Processing steps for cream cheese making

2.10 Manufacturing steps involved in cream cheese

2.10.1 Milk

Good quality fresh milk must be used for cheese production. Either cow milk or buffalo milk or both can be used for cream cheese preparation (Scott, 1986).

2.10.2 Pre-treatments of milk

Various pre-treatments such as standardization, heat treatment, homogenization are performed to improve the characteristics of cream cheese.

- Standardization

The starting milk for making cream cheese is standardized to 8-14% fat for double cream cheese, and to 3-5% fat for single cream cheese (Phadungath, 2005).

- Heat treatment

Ghosh and Singh (1990) concluded that buffalo milk cheese prepared from raw milk and pasteurized (63ºC/30 min) were superior to those prepared from heated to higher temperature. According to Ghosh and Singh (1990), the flavour characteristics of pasteurized milk cheese was superior to that of the raw milk cheese, whereas the body and texture was similar. The milk is pasteurized at 66-68ºC for 30 min or 72-75ºC for 30-90 s and cooled to the desired setting temperature (31-35ºC) (Phadungath, 2005).

- Homogenization of milk

The homogenization of milk in cream cheese leads to white appearance and improved body and mouthfeel, enhance whiteness, reduced fat loss in whey, increased cheese yield. Furthermore, homogenization of the cream instead of the milk improves cheese-making performance by reducing the amount of curd shattering and fines, and by reducing the amount of fat lost during the manufacturing process (Breene *et al.*, 1964; Quarne *et al.*, 1968). Milk must be homogenized at 12-14 MPa (50-55ºC) for cream cheese (Phadungath, 2005).

- Starter culture

Starter culture is added during the cheese making process for acid production and proteolysis of the cheese. The proteolytic activity of the starters affects the properties of the cheese. Different types of starter culture have different effects on the cheese properties (Oberg *et al.*, 1991). The milk is inoculated with D-type starter culture (i.e. *Lactococcus* starter). The level of starter culture and the set temperature depend on the incubation period; two of the common incubation conditions are the short-set incubation with 5% starter culture, an incubation temperature of 31ºC, and the long-set incubation with 0.8-1.2% starter culture, a temperature of 22-23ºC. The mix is held at the specific temperature until reaching the desired pH of 5.4-5.6 (Phadungath, 2005).

2.10.3 Renneting and cooking

The coagulant used in cheese making has a dual role. Its primary function is to coagulate milk, thereby producing curd that is subsequently transformed into cheese. In addition, a small proportion of the coagulant is carried into the cheese. This residual coagulant remains proteolytically active during aging, playing an important role in the development of texture and flavour (Nunez *et al.*, 1991). Rennet is added to the acidified milk when it reaches pH 5.4-5.6 at 31-35ºC. After the coagulum is completely formed, the curds are stirred and cooked with whey to a temperature of about 60-70ºC (Phadungath, 2005).

2.10.4 Whey draining, salting and mixing

Once cooking is completed, whey is then separated from the curd by several methods; the traditional method involves letting the hot curd (75-90ºC) drain in cloth bags overnight, while the modern methods use a cream cheese centrifugal separator operating at 70-85ºC or ultrafiltration at 50-55ºC. After whey separation, the hot curd is cooled down to 10-20ºC, then mixed with salt (0.5-1%) (Phadungath, 2005).

Part III

Materials and methods

3.1 Materials

3.1.1 Milk

Raw cow milk of local breed was obtained from Phushre, Dharan-13.

3.1.2 Rennet

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

3.1.3 Papaya latex

The latex of papaya (*Carica papaya*) was collected from Mangalbare, Dharan-11.

3.2 Methods

3.2.1 Preparation and optimization of enzyme

3.2.1.1 Extraction of crude papaya latex

The extracted latex was obtained by several longitudinal incisions with a rustless-steel blade on the unripe fruits (1-month-old) using protocol given by Nitsawang *et al.* (2006). This latex was allowed to run down the fruit and drip in plastic containers. Before being stored at -20ºC, 0.3 M NaOH was added to avoid oxidation (Ortiz *et al.*, 1980).

3.2.1.2 Drying of papaya latex

The papaya latex was dried at 40°C in tray drier for 2 h and then stored in freezer at -20°C (Puig *et al.,* 2008).

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).

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

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

3.2.1.4 Protease activity

Protease enzymatic activity was determined using protocol given by Sigma (1999). This uses casein as protease substrate. 0.05 g of dried samples were 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 minutes 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 minute at pH 7.5 and 37°C (color by Folin's reagent) and was calculated by the following reaction (Sigma, 1999).

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

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 Experimental design

The experimental design, data analysis and model building were performed using "Design Expert" software (Version 11.1.1, Stat-Ease Inc., USA). The non-rennet cream cheese was

prepared with variations in: (a) pH of milk and (b) temperature of milk during enzyme addition, and (c) enzyme concentrations used shown in Table 3.1. The independent variables and their levels were selected on the basis of literature and preliminary experiments. According to White and White (1997), the optimal temperature and pH range for papain is 60-70℃ and 6-7 respectively. A three-level, three-factor central composite rotatable 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

Name	Goal	Range
Enzyme concentration	To be minimized	1-5%
Temperature of milk	To be in range	60-70℃
pH of milk	Target = 6.5	6-7
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, 2 and 3) by a second-degree polynomial equation

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 . X_2 + \beta_{13} X_1 . X_3 + \beta_{14} X_2 . X_3 + \varepsilon$$

The coefficients of the polynomial were represented by β_0 (constant), $\beta_1, \beta_2, \beta_3$ (linear effects); $\beta_{12}, \beta_{13}, \beta_{14}$ (quadratic effects); $\beta_{11}, \beta_{22}, \beta_{33}$ (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.

3.2.1.6 Analysis of data

A complete second order quadratic model employed to fit the data and adequacy of the model was tested considering R^2 (the coefficient of multiple determination, a measure of 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 how good the model predicts a response value) and Fischer's F-test. Coefficient of determination R^2 is defined as the ratio of the explained variation to the total variation and is measure of the degree of fit (Haber and Runyon, 1980). It is also the proportion of the variability in the response variables, which is accounted for by the regression analysis. When R^2 approaches unity, the better the empirical model fits the actual data. The smaller the value of R^2, the less relevance the dependent variables in the model. The models were then used to interpret the effect of various predictors (terms) on the response. The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at probability (p) of 0.05. The regression coefficients were then used to make statistical calculation to generate response plot from the regression models. Optimization of process parameters was done by partially differentiating the model with respect to each parameter, equating to zero and simultaneously solving the resulting functions.

3.2.2 Preparation of cream cheese

Cream cheese were prepared by using three variations which is shown in Fig 3.1. The cream cheese prepared using calf rennet by direct acidification was labelled as cheese A, prepared using dried papaya latex was labelled as cheese B and prepared using commercial papain was labelled as cheese C. Milk was heated until it reached the temperature of 75-80°C followed by stirring for 1 min. The addition of enzyme for cheese A was done after the milk attained pH (5.4-5.6) by citric acid solution (5%) and temperature (37°C) while for cheeses B and C pH (6.5) and temperature (70°C) of the milk were adjusted, and then the milk was stirred gently for about 15 min maintaining constant respective temperature for all the cheeses. The whey and the curd were separated and the curds were drained using filter cloth. The draining process was done for 15 min and the drained curds were mixed with 2% common salt. The cream cheese curds were then drained again for 15 min. The drained curds were worked gently to form cream texture. The cream cheese was stored in refrigerator at below 5°C (Barry and Tamime, 2010; Kingsley, 2008).

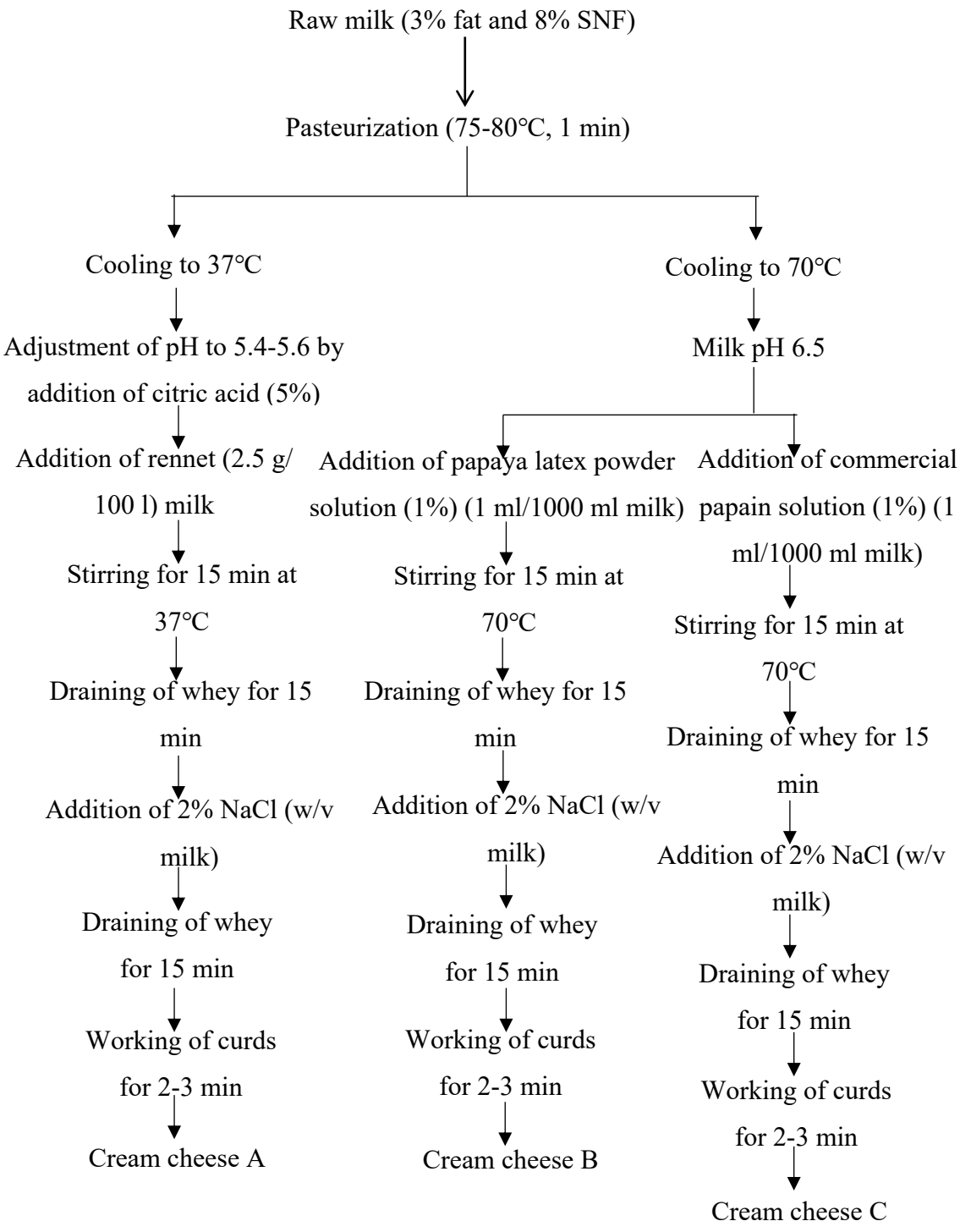

Fig. 3.1 Preparation steps of cream cheese with slight modification (Barry and Tamime, 2010; Kingsley, 2008)

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 as per NDDB (2001).

3.2.3.2 Determination of SNF in milk

SNF in milk was determined by Lactometer test as per NDDB (2001).

3.2.3.3 Determination of pH in milk and cheese

The pH in milk and cheese was determined as per NDDB (2001).

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 as per NDDB (2001).

3.2.3.5 Determination of acidity in milk and cheese

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

3.2.3.6 Determination of calcium in cheese

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

3.2.3.7 Determination of moisture in cheese

Moisture in cheese was determined by the oven-drying method as per NDDB (2001).

3.2.3.8 Determination of ash in cheese

The ash content in cheese was determined by dry ashing method as per NDDB (2001).

3.2.3.9 Theoretical yield and actual yield

Theoretical yield was calculated using Van Slyke yield equation (VanSlyke and Publow, 1910).

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

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 was calculated by weighing the curd after pressing as described by Razzaq (2003). 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 Sensory evaluation of cheese

The cheeses were evaluated organoleptically by 10 semi-trained panelists, following the recommendations of IDF (1992). The members evaluated cheese for texture, spreadability, flavour (taste and odour), aftertaste and overall acceptance using a five-point hedonic scale, with 1 being poor and 5 being the excellent quality. The specimen of sensory evaluation card is shown in appendix E.

3.2.5 Microbiological analysis of cheese

3.2.5.1 Coliform count

Total Coliform of cheese was determined by pour plate technique on Violet Red Bile Agar (VRBA) medium (NDDB, 2001).

3.2.5.2 TPC of cheese

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

3.2.5.3 Yeast and molds count

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

3.2.6 Statistical analysis

Data was statistically processed by GenStat (12th 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, plant protease was extracted from papaya latex. The impact of enzyme concentration, pH of milk and temperature of milk on time of coagulation (TOC) and milk coagulating activity (MCA) were analyzed by response surface methodology. The cream cheeses thus prepared from rennet (A), dried papaya latex (B) and commercial papain (C) were analyzed for physico-chemical properties such as moisture, fat, protein, ash, acidity, pH and calcium. Various sensory attributes (texture, spreadability, flavor, aftertaste and overall acceptability) and microbiological qualities of prepared cheeses were analyzed.

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

The measured expansion of the time of coagulation and milk clotting activity varied from 58-159 s and 150.9-413.8 units respectively (Appendix A). Table B.1 and B.2 show the coefficients of the model and other statistical attributes of time of coagulation (TOC) whereas Table B.3 and B.4 show that of MCA.

$$TOC = 250.284 - 8.59447A - 6.45358B + 0.938571C + 1.25AB - 0.75AC + 1.5BC - 60.4374A^2 - 60.1323B^2 - 57.1271C^2 \quad \text{.....4.1}$$

$$MCA = -23.5966 + 33.393A + 27.0257B - 2.80561C - 2.10075AB + 4.15162AC - 5.88182BC + 129.043A^2 + 120.137B^2 + 105.089C^2 \quad \text{.....4.2}$$

Where A, B and C are the coded values of enzyme concentration, temperature of milk and pH of the milk. A, B, C, A^2, B^2, C^2, AB, AC and BC are model terms.

In the quadratic equation 4.1, TOC had significant ($P<0.05$) negative effect of enzyme concentration (A) and temperature of milk (B) at 95% confidence level. But the term pH of milk (C) had non-significant ($P>0.05$) positive effect on TOC. The quadratic terms of enzyme concentration, temperature of milk and pH of milk had highly significant ($P<0.05$) negative effect on TOC as given in Table B.2. The interaction terms of enzyme concentration and temperature of milk (AB), and temperature of milk and pH of milk (BC) had non-

significant (P>0.05) positive effect on TOC. Other interaction terms enzyme concentration and pH of milk (AC) had non-significant negative effect (P>0.05).

Similarly, the quadratic equation 4.2 of MCA shows that enzyme concentration (A) and temperature of milk (B) had significant (P<0.05) positive effect at 95% confidence level whereas the term pH of milk (C) had non-significant (P>0.05) negative effect on MCA. The quadratic terms of enzyme concentration, temperature of milk and pH of milk had highly significant (P<0.05) positive effect on MCA as given in Table B.4. The interaction terms of enzyme concentration and temperature of milk (AB), and temperature of milk and pH of milk (BC) had non-significant (P>0.05) negative effect on MCA. Other interaction terms enzyme concentration and pH of milk (AC) had non-significant positive effect (P>0.05).

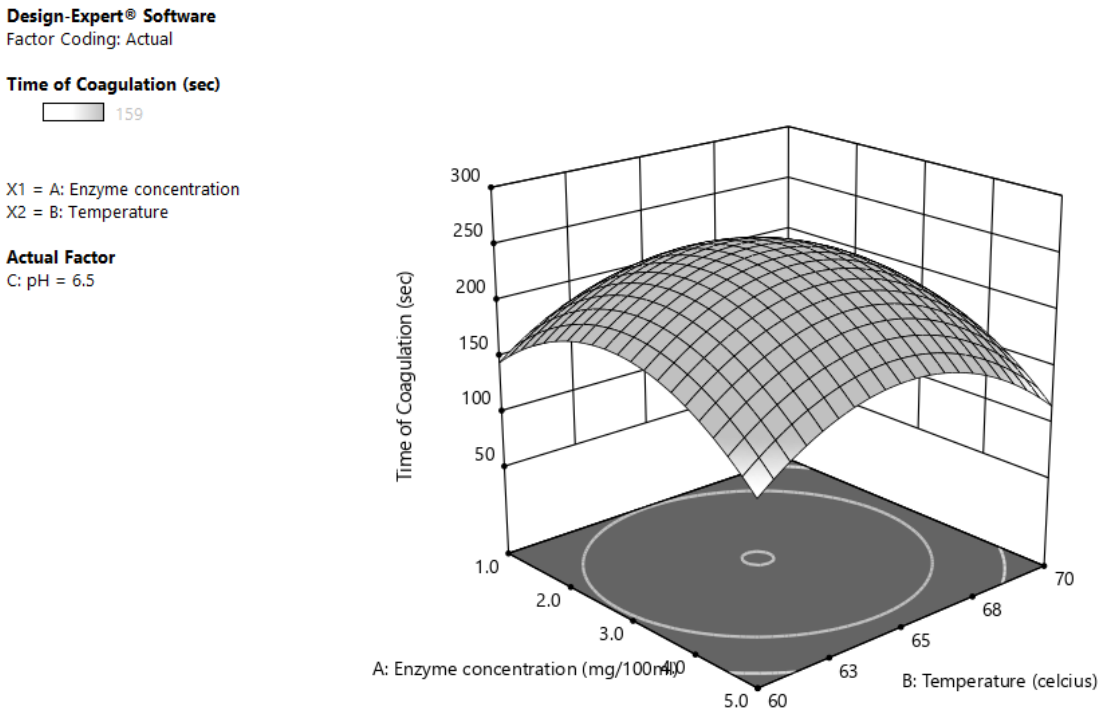

Fig. 4.1 Response surface plot for TOC as a function of temperature of milk and enzyme concentration at pH 6.5.

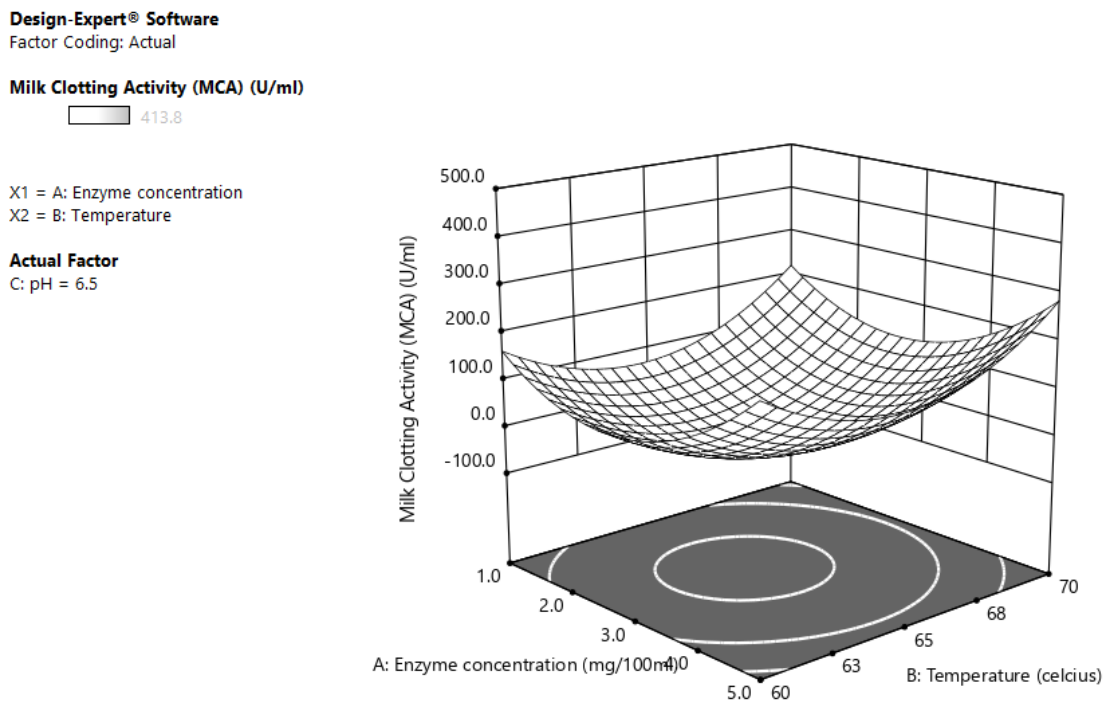

Fig. 4.2 Response surface plot for MCA as a function of temperature of milk and enzyme concentration at pH 6.5.

An increase in temperature of milk resulted in a lower TOC (Fig. 4.1 and Fig. 4.3) and higher MCA (Fig. 4.2 and Fig. 4.4). However, the effect was not linear as the TOC increased and reached maximum until further temperature was increased which led to decline in the TOC as shown in Fig. 4.1. Similarly, in Fig. 4.2, MCA was first decreased before increasing as the temperature kept on increasing. Similar findings were observed by Abd EL-Gawad Mona *et al.* (2007).

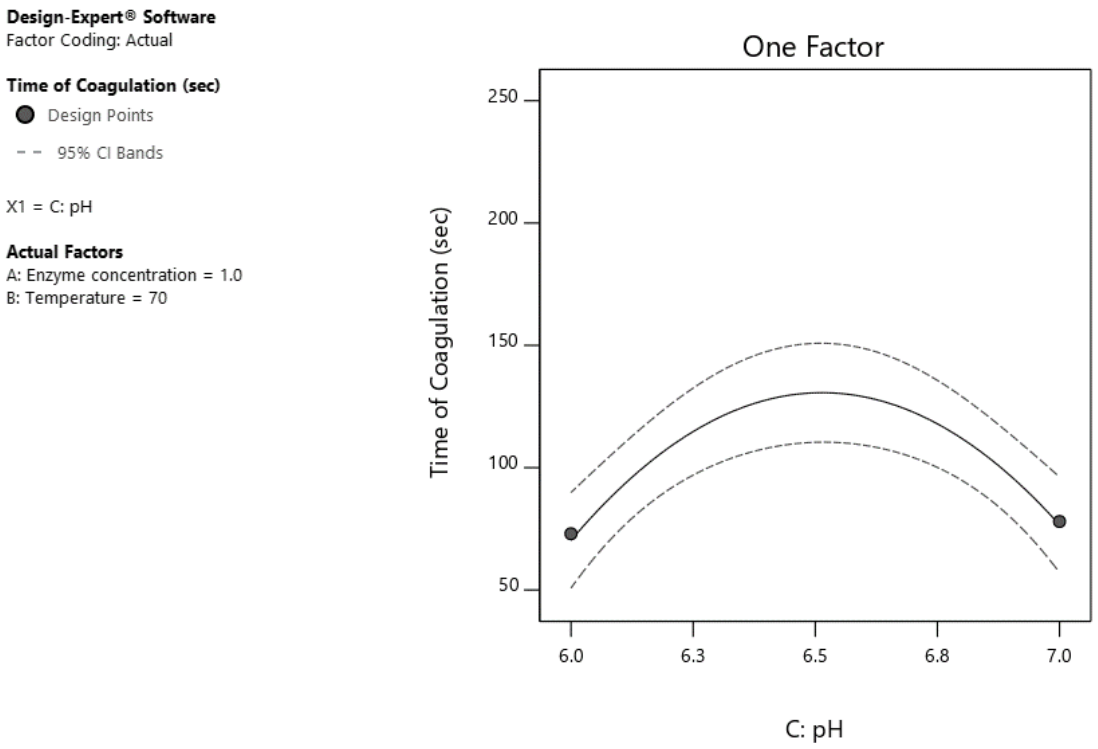

Fig. 4.3 Single factor interaction graph of TOC for individual factor C: pH

Also, an increase in enzyme concentration resulted in a lower TOC (Fig. 4.1) and higher MCA (Fig. 4.2). However, the effect was not linear as the TOC increased and reached maximum until further enzyme concentration was increased which led to decline in the TOC as shown in Fig. 4.1. Similarly, in Fig. 4.2, MCA was first decreased before increasing as the enzyme concentration kept on increasing. These findings correlate to the findings of Abd EL-Gawad Mona *et al.* (2007).

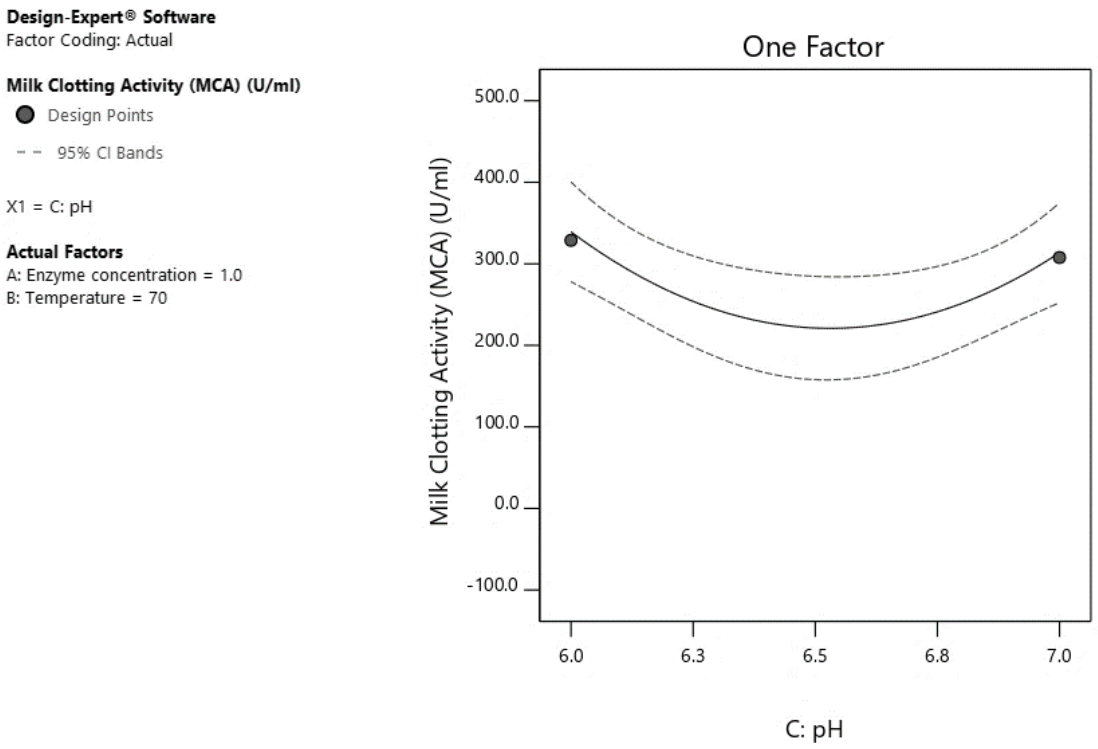

Fig. 4.4 Single factor interaction graph of MCA for individual factor C: pH

The pH of the milk has the important linear effect on the final specific activity of the crude enzyme. The maximum TOC and minimum MCA was observed at pH 6.5 (Fig. 4.3 and 4.4). However, the pH had non-significant effect on both TOC and MCA.

4.1.1 Protease activity of crude enzyme at different temperature

Crude enzyme was subjected to protease activity determination at different temperatures keeping enzyme concentration (1%), pH 6.5 and incubation time (10 min) constant. The results are calculated using equation deduced from standard curve (Fig. D.1) and are presented in Table 4.1.

Table 4.1 Protease activity at different temperature

Temperature (℃)	OD (at 660 nm)	Protease activity (Umoles Tyrosine)
60	0.451±0.043	0.218±0.021
65	0.519±0.09	0.250±0.004
70	0.514±0.031	0.248±0.015

From the Table 4.1, protease activity of crude papain enzyme increased with the increase in temperature upto 65℃ and it slightly decreased when the temperature reached 70℃ which is in agreement with the data given by CBS (2019). It may be due to the fact that papain starts to denature in temperatures above 70℃.

4.1.2 Optimization

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

Table 4.2 Different constraints for optimization

Name	Goal	Lower Limit	Upper Limit
Enzyme concentration	Minimize	0.4	6.4
Temperature of milk	is in range	60	70
pH of milk	is target = 6.5	6	7
Time of coagulation	Minimize	58	159
Milk clotting activity	Maximize	150.9	413.8

Under the assumptions by Design Expert (version 11.1.1), the optimum operating conditions for minimum time of coagulation and maximum milk clotting activity of enzyme were found to be 1% enzyme concentration (1 ml/100 ml milk), 6.5 pH and 70℃ of milk temperature. The responses predicted by the software for these optimum conditions reported MCA of 286.396 units at 83.841 s of coagulation time.

4.1.3 Verification of the model

Within the scope of the variables investigated in Central Composite Rotatable 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 4.3.

Table 4.3 Predicted and actual values of the responses at the optimized condition

Response	Conditions			Predicted value	Mean Observed value	Deviation
	Enzyme concentration	Temperature of milk	pH of milk			
Time of Coagulation (TOC)	1	70	6.5	83.841	79.33	3.18976
Milk Clotting Activity (MCA)	1	70	6.5	286.396	302.534	11.4113

4.2 Physicochemical properties

4.2.1 Chemical composition of raw milk

The proximate composition of raw cow milk is given in Table 4.4.

Table 4.4 Proximate composition of raw cow milk

Parameters	Cow milk
Moisture (%)	87.1(1.89)
Fat (%)	3.8(0.15)
Protein (%)	3.3(0.2)
Ash (%)	0.7(0.3)
pH	6.5(0.015)

Note: Values are the means of three determinations. Figures in the parentheses are the standard deviation.

The resulted presented in Table 4.3 revealed that the moisture, fat, protein, ash and pH in cow milk were 87.1%, 3.8%, 3.3%, 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, milking time and so on.

4.2.2 Chemical composition of cream cheese

The chemical composition of the cream cheeses made from rennet (A), dried papaya latex (B) and commercial papain (C) has been shown in Table 4.5.

The moisture percentage is in the line with the findings of Johnson *et al.,* (2001) but lower than the findings of Nawaz (2007) and higher than that of Rana *et al* (2017). The results were similar to 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 B. 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). Analysis of variance (Table G.1) regarding moisture content revealed that significant differences ($P<0.05$) was observed between all three samples. Moisture content in cream cheeses made using commercial papain and papaya latex were higher than that of rennet. The variation could be attributed to the molecular forces involved in the coagulation of casein by crude enzymes, which resulted into a greater water binding capacity of protein matrix of cheese. The significant difference might be due to the longer

coagulation time for papain that results in more moisture retention in the final product (Johnson *et al.,* 2001).

Table 4.5 Chemical composition of cream cheeses A, B and C.

Parameters	A	B	C	LSD
Moisture (%)	47.43^{a}(0.27)	54.22^{c}(1.15)	50.42^{b}(0.08)	2.167
Fat (%)	28.2^{b}(0.36)	27.4^{a}(0.44)	27.2^{a}(0.36)	0.774
Protein (%)	19.47^{a}(0.58)	19.2^{a}(0.45)	19.09^{a}(0.25)	0.894
Ash (%)	2.57^{a}(0.35)	3.3^{a}(0.13)	3.27^{a}(0.21)	0.786
pH	5.63^{a}(0.11)	6.61^{b}(0.09)	6.55^{b}(0.04)	0.269
Acidity	0.23^{b}(0.008)	0.16^{a}(0.011)	0.18^{a}(0.009)	0.029
Calcium (mg/100g)	628^{a}(7.94)	614.7^{a}(6.50)	618.3^{a}(5.50)	13.11

Note: Values are the means of three determinations. Figures in the parentheses are the standard deviation. Values in the row bearing similar superscript are not significantly different at 5% level of significance.

The fat level correlated with findings of Nawaz (2007). The results were slightly higher than the findings of Ghosh and Singh (1996) and Islam (2006) who found fat level to be 24.8% and 23.5%, respectively. 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 Mona *et al.,* 2007). The lower value fat contents recorded in cheese made using papain 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 G.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 (2007). Proteolytic enzymes such as papain 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, 2004). Cheese serves as a store house of essential amino acids, having similar proportion of essential amino acids that is present in milk except the methionine and cysteine. However, the slight variation might be due to the cheese making techniques and the quality of milk used, as the milk quality changes with the lactating stage of animal, nutrition, breed and age of milking animal (Razzaq, 2003). Among three samples, the average protein contents were more in A. Analysis of variance (Table G.3) regarding protein contents revealed that non-significant difference ($P>0.05$) was recorded among all treatments. Disparity could be due to crude enzyme as it might contain proteinaceous material in it. Omueti and Jaiyeola (2006) reported that retention of whey in final cheese might increase the protein contents as well.

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 B. The higher value was recorded in case of plant coagulant as compared to rennet, which is probably due to the remnants of plant materials in the crude enzyme. Analysis of variance (Table G.4) regarding ash contents revealed that non-significant difference ($P>0.05$) was recorded among all treatments.

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 cream cheese are greatly influenced by the pH (Rowney *et al.,* 1999). Analysis of variance (Table G.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 B 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. As Mohamed *et al.* (1997) experimented that higher the strength of the coagulant, higher will be the pH.

The titratable acidity of cream cheeses is similar to the findings of Nawaz (2007). Table G.5 also revealed a non-significant ($P>0.05$) effect on acidity of cheeses prepared by both

coagulants. However, slight increase in acidity was observed in cream 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. Conversely, these results are not similar with the findings of Abu-Zeid (1994) and Kheir *et al.* (2011). Analysis of variance (Table G.5) regarding titratable acidity revealed that non-significant difference ($P>0.05$) was seen between B and C while significant difference ($P<0.05$) was found between A and B, and A and C.

The calcium content of cream cheeses A, B and C range from 610-628 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 G.7) regarding calcium content revealed that non-significant difference ($P>0.05$) among all treatment.

4.2.3 Theoretical and actual yield

The theoretical and actual yield of cream cheeses A, B and C have been presented in Table F.1 and shown in Fig. 4.5.

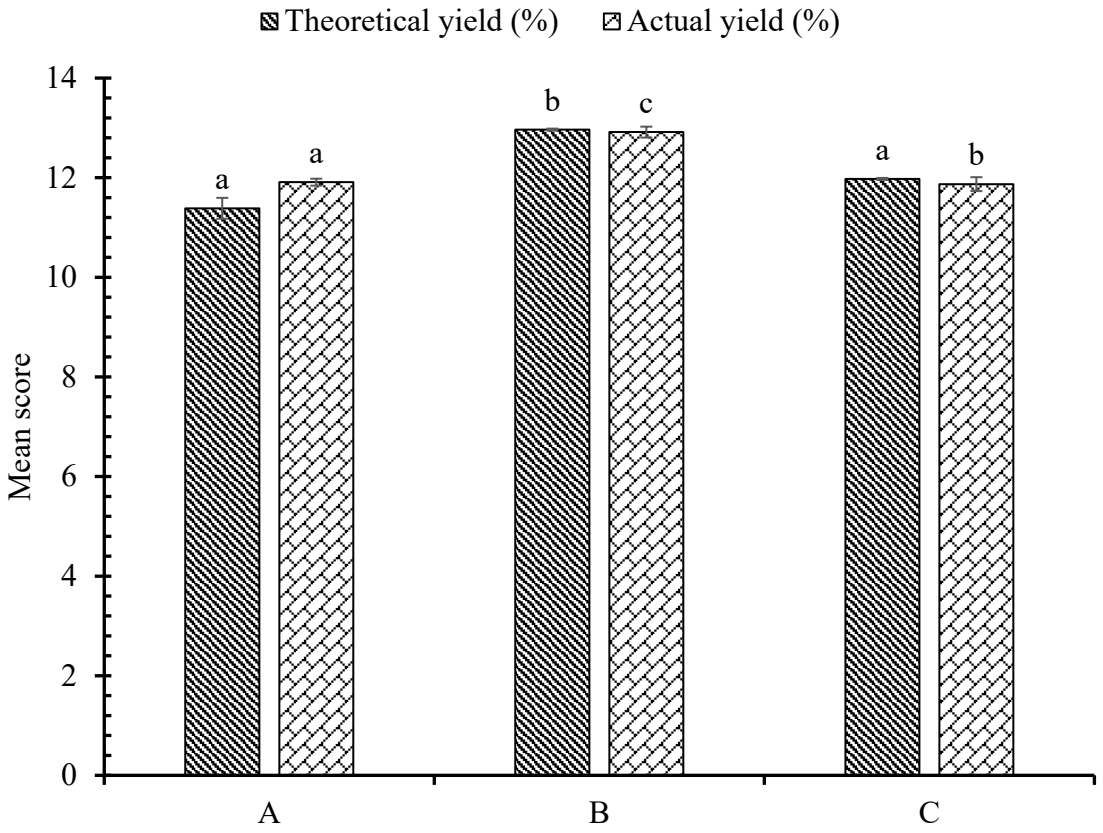

Fig. 4.5 Theoretical and Actual yield of cream cheeses

*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 cream cheeses is shown in Table F.1. Among them the average theoretical yield was more in B. The slight variation in theoretical yield might be due to the moisture contents in final cheese (Melilli *et al.,* 2002). Analysis of variance (Table G.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 cream cheeses is shown in Table F.1. Actual yield of all samples is lower than the findings of Mahajan and Chaudhary (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 B. The higher actual 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 G.9) regarding actual yield revealed that there was significant difference ($P<0.05$) among all treatments.

4.3 Sensory evaluation of cheese

The sensory scores of (5-point hedonic scales) the cream cheeses are shown in Table F.2 (Appendix F). Graphical representation is given in Fig. 4.6.

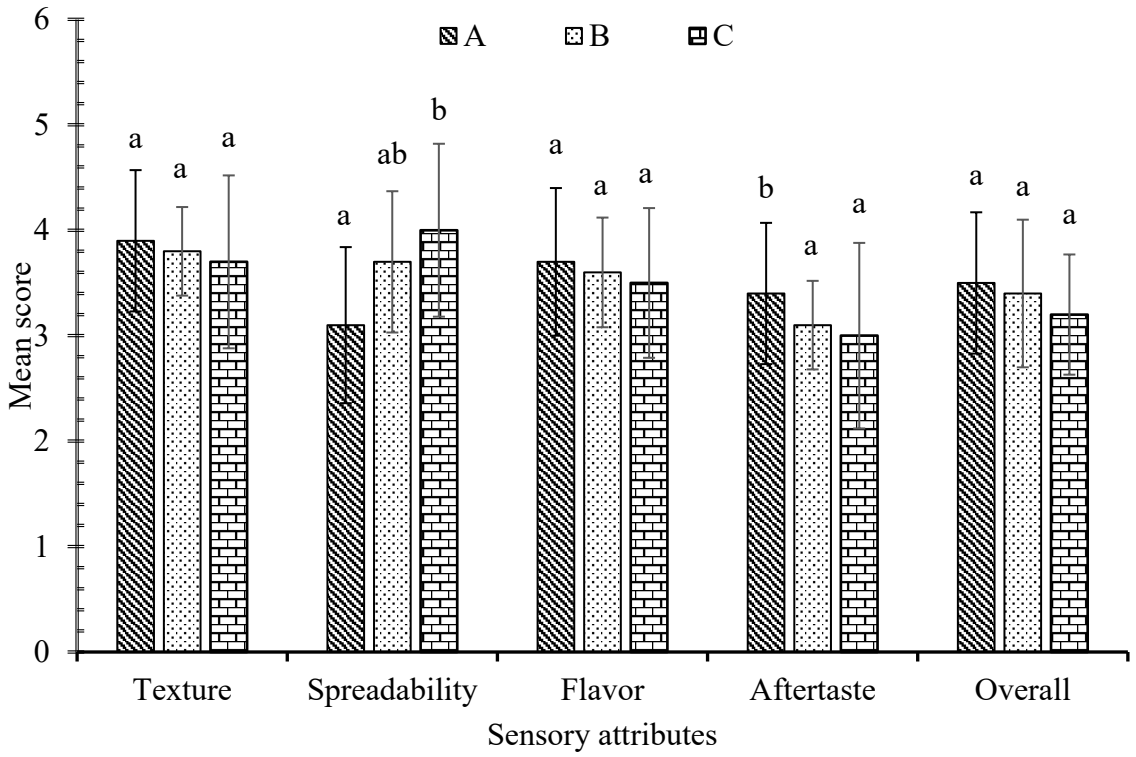

Fig.4.6 Graphical view of mean sensory scores of cream cheeses.

Note: Values in Fig. 4.6 are the means of 10 panelists. Values on top bars bearing similar superscript are not significantly different at 5% level of significance.

The average texture points were highest for cheese A. As cream cheeses are meant for use as a spread, so its texture must naturally be smooth without any grainy feel. This parameter defines whether the product has been justified or not. If the texture is not smooth or creamy then it is not cream cheese (Anon., 2019a). Analysis of variance (Table G.12) regarding texture revealed non-significant difference ($P>0.05$) among all samples.

Cheese made using commercial papain and papaya latex had significantly higher score ($P<0.05$) for spreadability as compared to cheese made using rennet (Table F.2). The average spreadability points were highest for sample C. This may be due to the variation in moisture content and fat content as well as proteolytic effect exhibited by the different enzymes used. Analysis of variance (Table G.13) regarding spreadability showed that there is non-significant difference between all the samples at 5% level of significance.

In terms of flavor the cheeses had similar scores where sample A was found to be slightly better. Although the cheese might be attributed to the inherent property of the crude papain enzyme the flavor of the cheese (El-Aziz *et al.,* 2012), it showed no difference in flavor to that compared with rennet cheese. Analysis of variance (Table G.14) regarding flavor revealed that non-significant difference (P>0.05) was observed between all the samples.

The cheese made using rennet had higher scores for aftertaste than cheese made using papain. (Table F.2). The average aftertaste score was higher for cheese A. However, non-significant difference was observed among the samples B and C as per Analysis of variance (Table G.15). A slight bitter taste was observed in cheese made from plant coagulant which may be the reason for low taste score in B and C. Aqueous extracts of *Withinia coagulans* has also showed a bitter taste in the final product. This bitterness is associated with accumulation of the bitter peptides that contain more hydrophobic amino acid residues when coagulants from plant sources are used (Singh *et al.,* 2003).

Finally, the overall acceptance of the sample A was slightly higher than that of B and C. Since the overall acceptance accounts for all the parameters of the cheeses including texture, flavor, aftertaste and spreadability and does not overlook other parameters for one. Hence it can be said that sample A was better than B and C but not by far margin which concludes they are nearly same. Also, Analysis of variance (Table G.16) regarding overall acceptance respectively revealed that non-significant difference (P>0.05) was recorded among all treatments.

4.4 Microbiological analysis

The result of microbiological analysis of cream cheese samples are presented in Fig.4.7 and in Table F.3 (Appendix F). It showed the average value for TPC and yeasts and molds of sample.

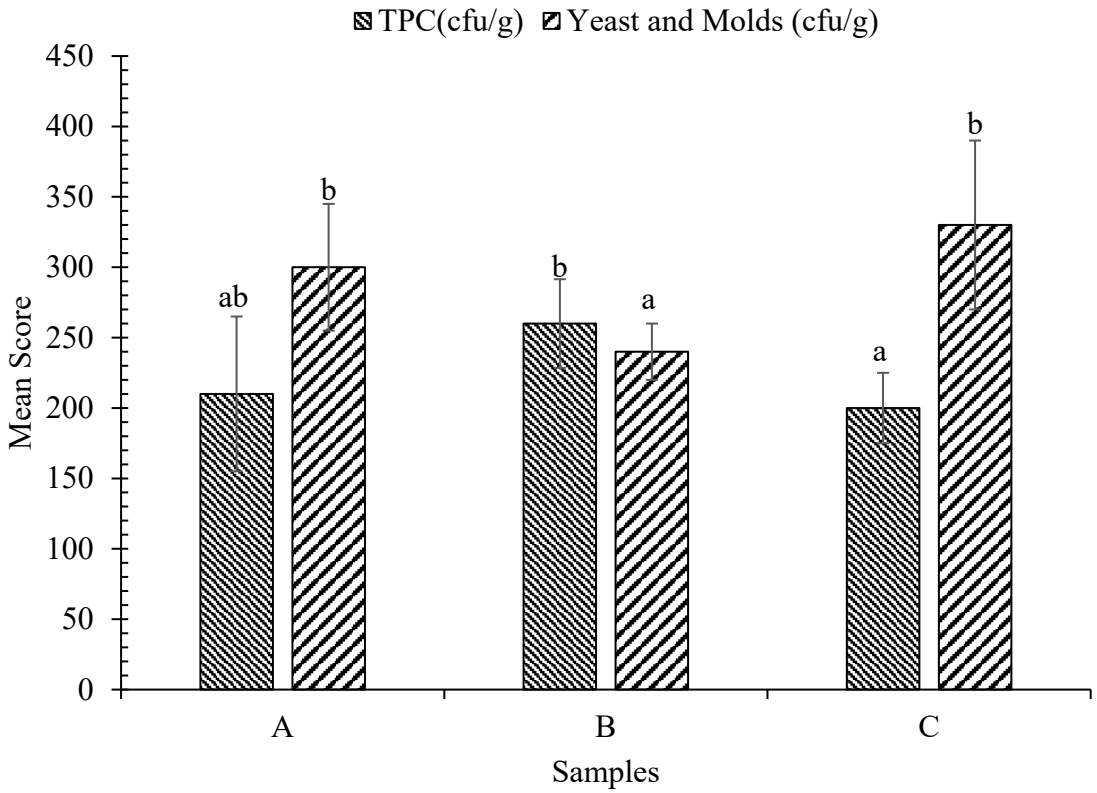

Fig.4.7 Microbiology of Cream cheese

*Values are the means of two determinations. Values of yeast and molds count multiplied by a factor of 10. Values on top bars bearing similar superscript are not significantly different at 5% level of significance.

Significant difference ($P<0.05$) was found for both TPC (Table G.10) and yeast and molds count (Table G.11) of samples. For TPC, all the samples were significantly different from each other whereas for yeast and molds count, sample A and C had not significant difference but there was significant different between A and B, and B and C 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°C 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. Optimum time of coagulation (TOC) and milk clotting activity (MCA) was obtained at pH 6.5, temperature 70℃ and enzyme concentration 1% using papaya protease as coagulant.
2. The protease activity of the prepared papaya enzyme was found to be 0.248 Umoles Tyrosine at 70℃.
3. The physico-chemical analysis, showed that protein, ash and calcium were non-significant ($P>0.05$) among cream cheeses made using rennet and papaya protease while disparity was observed in moisture, fat, acidity, pH and yield of the cheeses.
4. Sensory attributes of cream cheese made using papaya protease were found non-significant ($P>0.05$) to that of rennet cheese.
5. Coliforms were not detected in all samples and the average TPC and Yeasts and Molds counts were 223.33 cfu/g and 29 cfu/g respectively for the cheese samples.

5.2 Recommendations

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

1. Extraction and purification of crude enzyme extract of papaya protease to enhance milk clotting activity (MCA) of milk.
2. Use of other plant extracts such as bromelain, ficin etc as rennet substitute to prepare cream cheese.

Part VI

Summary

The main emphasis of this study was to utilize papaya latex as a source of papaya protease for the preparation of cream cheese using this enzyme as milk coagulant. The cream cheese thus obtained was compared with control for physico-chemical, sensory and microbiological quality.

The impact of three processing parameters namely enzyme concentration, pH of milk, and temperature of milk was investigated on time of coagulation and milk clotting activity 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. The optimized time of coagulation (83.841 s) and milk clotting activity (286.396 U/ml) were obtained at pH of milk 6.5, temperature of milk 70°C and 1% papaya protease solution (1ml/1000ml milk). No significant difference ($p>0.05$) was observed between the experimental and predicted values. 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 cream cheese.

The physicochemical parameters like protein, ash and calcium showed no significant ($p>0.05$) difference but significantly ($p<0.05$) higher levels of moisture and ash, and lower levels of fat were observed in the cheese produced by papaya protease compared to that made using rennet. Sensory evaluation revealed that there was non-significant difference ($p>0.05$) among all samples in overall acceptance. Yet rennet cream cheese was found high scoring than cream cheeses made using papaya protease. This variation may be due to crude enzyme extract and can be overcome by purifying the enzyme. Microbiological analysis showed that coliforms were not detected in all cheese samples. However, TPC of about 223.33 cfu/g and yeasts and molds count of 29 cfu/g was observed which may be due to handling contamination.

Therefore, it was observed that crude papaya protease from papaya latex showed good results in the production of cream cheese. The quality of the cheese made from this easily available source can further be improved by initiating the process of purification of papaya protease.

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Appendices

Appendix A

Table A.1 Product responses by independent variables

Std. no.	Factor 1 A: Enzyme concentration mg/100ml	Factor 2 B: Temperature °C	Factor 3 C: pH	Response 1 Time of Coagulation s	Response 2 Milk Clotting Activity (MCA) U/ml
1	3.0	65	7.3	91	263.7
2	5.0	60	7.0	69	347.8
3	1.0	60	6.0	89	269.7
4	1.0	60	7.0	85	282.4
5	3.0	73	6.5	62	387.1
6	6.4	65	6.5	58	413.8
7	5.0	70	6.0	65	369.2
8	3.0	57	6.5	93	258.1
9	1.0	70	6.0	73	328.8
10	3.0	65	5.7	81	296.3
11	5.0	60	6.0	73	328.8
12	5.0	70	7.0	64	375.0
13	0.4	65	6.5	159	150.9
14	1.0	70	7.0	78	307.7

Appendix B

Table B.1 Model summary statistics for Time of Coagulation

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	21.19	0.4469	0.2810	0.0369	7822.00	
2FI	25.23	0.4512	-0.0192	-0.9762	16049.46	
Quadratic	**8.02**	**0.9683**	**0.8971**	**0.2867**	**5793.01**	**Suggested**
Cubic					*	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

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7864.29	9	873.81	13.59	0.0115	significant
A-Enzyme concentration	734.84	1	734.84	11.43	0.0278	
B-Temperature	568.79	1	568.79	8.85	0.0410	
C-pH	12.03	1	12.03	0.1871	0.6876	
AB	12.50	1	12.50	0.1944	0.6820	
AC	4.50	1	4.50	0.0700	0.8044	
BC	18.00	1	18.00	0.2800	0.6247	
A^2	2816.65	1	2816.65	43.81	0.0027	
B^2	3898.25	1	3898.25	60.64	0.0015	
C^2	3518.34	1	3518.34	54.73	0.0018	
Residual	257.14	4	64.29			
Cor Total	8121.43	13				

Table B.3 Model summary statistics for Milk Clotting Activity

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	43.52	0.6801	0.5841	0.4185	34422.54	Suggested
2FI	51.39	0.6877	0.4200	-0.2816	75868.60	
Quadratic	**25.11**	**0.9574**	**0.8615**	**0.2555**	**44072.60**	**Suggested**
Cubic					*	Aliased

* 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

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	56674.29	9	6297.14	9.99	0.0203	significant
A-Enzyme concentration	11093.45	1	11093.45	17.59	0.0138	
B-Temperature	9974.84	1	9974.84	15.82	0.0164	
C-pH	107.50	1	107.50	0.1705	0.7009	
AB	35.31	1	35.31	0.0560	0.8246	
AC	137.89	1	137.89	0.2187	0.6644	
BC	276.77	1	276.77	0.4389	0.5439	
A^2	12840.68	1	12840.68	20.36	0.0107	
B^2	15559.99	1	15559.99	24.67	0.0077	
C^2	11905.96	1	11905.96	18.88	0.0122	
Residual	2522.40	4	630.60			
Cor Total	59196.69	13				

Appendix C

Table C.1 Solutions of optimization result

S. No.	Enzyme concentration	Temperature	pH	Time of Coagulation	Milk Clotting Activity (MCA)	Desirability	
1	**1.001**	**70.000**	**6.500**	**83.841**	**286.396**	**0.554**	**Selected**
2	1.000	70.000	6.241	113.642	256.134	0.552	
3	1.000	70.000	6.328	122.761	238.145	0.548	
4	1.000	70.000	6.335	123.360	236.941	0.547	
5	1.000	69.991	6.163	102.808	277.081	0.542	
6	1.000	69.894	6.209	112.107	258.647	0.538	
7	1.000	70.000	6.612	128.435	223.743	0.529	
8	1.000	70.000	6.122	95.491	291.188	0.527	
9	1.000	70.000	6.676	124.656	229.808	0.526	
10	1.000	70.000	6.753	117.635	241.653	0.525	
11	1.000	69.795	6.177	109.847	262.551	0.523	
12	1.035	70.000	6.765	118.186	240.183	0.513	
13	1.000	69.734	6.156	108.083	265.647	0.513	
14	1.000	69.868	6.794	115.985	243.267	0.505	
15	1.115	70.000	6.795	118.942	237.786	0.486	

Appendix D

Calibration curve for protease activity

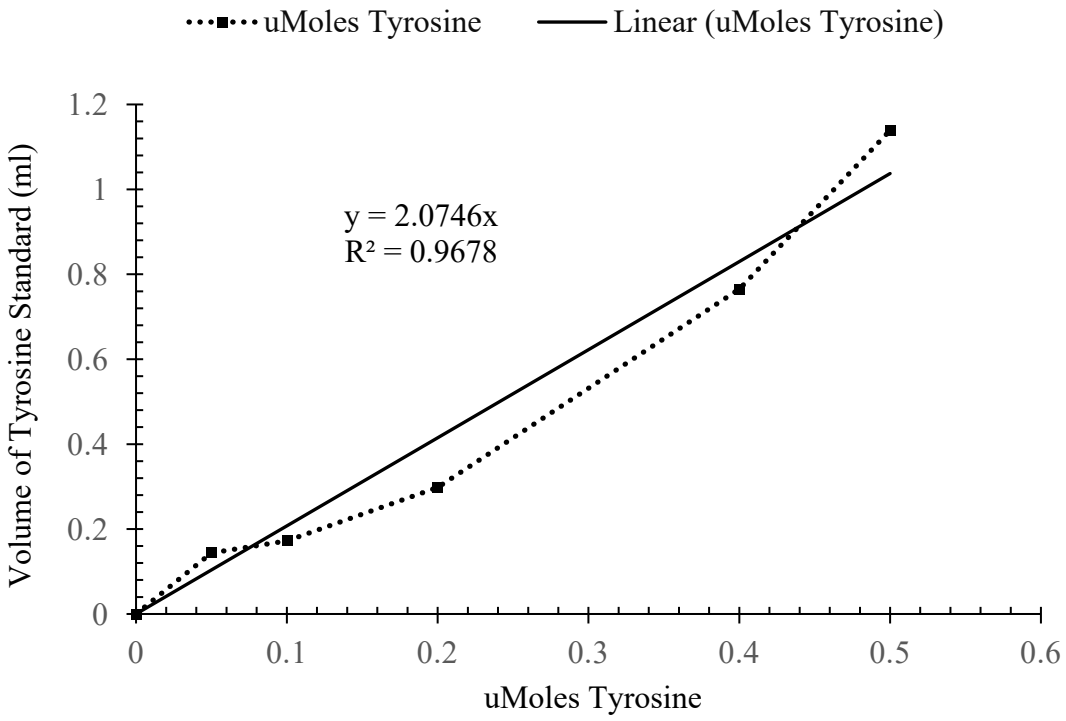

Fig. D.1 Standard curve of L-Tyrosine for protease activity

Appendix E

Sensory Evaluation Card

Date: _____

Name: _____

Product: Cream Cheese

Please conduct the sensory analysis based on the following parameter using the scale given. Panelists are requested to give ranks on their individual choice.

Perception	Points
Excellent	5
Good	4
Satisfactory	3
Fair	2
Poor	1

Samples	Parameters				
	Texture	Spreadability	Flavor	Aftertaste	Overall
A					
B					
C					

Comments (if any) ……...

……

_____

Signature

Appendix F

Table F.1 Theoretical and Actual yields of cream cheese

Source of Variation	Actual Yield	Theoretical Yield
A	11.910^{a}(0.07)	11.385^{a}(0.21)
B	12.915^{c}(0.11)	12.965^{b}(0.02)
C	11.870^{b}(0.14)	11.975^{a}(0.02)
LSD	0.3498	0.3808

Table F.2 Mean scores of sensory attributes of cream cheese

Source of variation	Texture	Spreadability	Flavor	Aftertaste	Overall
A	3.90^{a}(0.67)	3.10^{a}(0.74)	3.70^{a}(0.7)	3.30^{b}(0.67)	3.50^{a}(0.67)
B	3.80^{a}(0.42)	3.70^{ab}(0.67)	3.60^{a}(0.52)	3.20^{a}(0.42)	3.40^{a}(0.7)
C	3.70^{a}(0.82)	4.00^{b}(0.82)	3.50^{a}(0.71)	3.10^{a}(0.88)	3.30^{a}(0.57)
LSD	0.580	0.739	0.591	0.574	0.521

Values in Table F.2 are the means of 10 panelists. Figures in the parenthesis are the standard deviation. Values in the column bearing similar superscript are not significantly different at 5 % level of significance.

Table F.3 Microbiological analysis of cream cheese

Sample	Coliform (cfu/g)	TPC (cfu/g)	Yeast & Mold (cfu/g)
A	ND	210^{ab}(55)	30^{b}(6.0)
B	ND	260^{b}(31.5)	24^{a}(2.0)
C	ND	200^{a}(25)	33^{b}(4.5)
LSD		58.82	4.159

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

Appendix G

Statistical analysis (ANOVA Tables)

Table G.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	46.3832	23.1641	49.98	0.005
Residual	3	1.3905	0.4635		
Total	5	47.7187			

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

Table G.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	1.6800	0.8400	5.60	0.042
Residual	3	0.9000	0.1500		
Total	5	2.5800			

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

Table G.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	0.2219	0.1109	0.55	0.601
Residual	3	1.2011	0.2002		
Total	5	1.4230			

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

Table G.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	0.68703	0.32352	5.63	0.097
Residual	3	0.18310	0.06103		
Total	5	0.87013			

Since there is no significant difference between the samples for the ash at 5% level of significance, LSD testing is not necessary.

Table G.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	0.00469733	0.00234867	27.36	0.012
Residual	3	0.00025750	0.00008583		
Total	5	0.00495483			

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

Table G.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	1.212700	0.606350	84.61	0.002
Residual	3	0.021500	0.007167		
Total	5	1.234200			

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

Table G.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	544.67	181.56	3.74	0.060
Residual	3	388.00	48.50		
Total	5	932.67			

Since there is no significant difference between the samples for the calcium at 5% level of significance, LSD testing is not necessary.

Table G.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	2.54973	1.27487	89.05	0.002
Residual	3	0.04295	0.01432		
Total	5	2.59268			

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

Table G.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	1.40243	0.70122	58.03	0.004
Residual	3	0.03625	0.01208		
Total	5	1.43868			

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

Table G.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	6200.0	3100.0	3.58	0.045
Residual	6	5200.0	866.7		
Total	8	11400.0			

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

Table G.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	126.000	63.000	14.54	0.005
Residual	6	26.000	4.333		
Total	8	152.000			

Since there is significant difference between the samples for the Yeast and Molds count at 5% level of significance, LSD testing is necessary.

Table G.12 Two-way ANOVA (no blocking) for texture taking the samples

Variate: Texture

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Panelist	9	4.8333	0.5370	1.41	0.256
Sample	2	3.8000	1.9000	4.98	0.069
Residual	18	6.8667	0.3815		
Total	29	15.5000			

Since there is no significant difference between the samples for the texture at 5% level of significance, LSD testing is necessary.

Table G.13 Two-way ANOVA (no blocking) for spreadability taking the samples

Variate: Spreadability

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Panelist	9	3.8667	0.4296	0.69	0.706
Sample	2	4.2000	2.1000	3.40	0.046
Residual	18	11.1333	0.6185		
Total	29	19.2000			

Since there is significant difference between the samples for the spreadability at 5% level of significance, LSD testing is not necessary.

Table G.14 Two-way ANOVA (no blocking) for flavor taking the samples

Variate: Flavor

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Panelist	9	4.1667	0.4630	1.17	0.370
Sample	2	0.2000	0.1000	0.25	0.780
Residual	18	7.1333	0.3963		
Total	29	11.5000			

Since there is no significant difference between the samples for the flavor at 5% level of significance, LSD testing is not necessary.

Table G.15 Two-way ANOVA (no blocking) for aftertaste taking the samples

Variate: Aftertaste

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Panelist	9	3.3333	0.3704	0.72	0.686
Sample	2	2.0667	1.0333	2.01	0.043
Residual	18	9.2667	0.5148		
Total	29	14.6667			

Since there is significant difference between the samples for the aftertaste at 5% level of significance, LSD testing is not necessary.

Table G.16 Two-way ANOVA (no blocking) for overall taking the samples

Variate: Overall

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Panelist	9	5.8667	0.6519	2.12	0.084
Sample	2	0.4667	0.2333	0.76	0.483
Residual	18	5.5333	0.3074		
Total	29	11.8667			

Since there is no significant difference between the samples for the overall at 5% level of significance, LSD testing is not necessary.