EXTRACTION, PARTIAL PURIFICATION AND UTILIZATION OF PROTEOLYTIC ENZYME FROM KIWI FRUIT (*Actinidia deliciosa*) FOR MAKING SOFT CHEESE

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2022

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

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March, 2022

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Approval Letter

This ***dissertation*** entitled ***Extraction, Partial Purification and Utilization of Proteolytic Enzyme from Kiwi Fruit (Actinidia deliciosa) for Making Soft Cheese*** presented by **Shiva Kumar Shrestha** has been accepted as the partial fulfilment of the requirement for the **B. Tech. degree** in **Food Technology.**

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March, 2022

Acknowledgements

It is my great privilege to work for the present investigation under the guidance and kind supervision of Asst. Prof. Mr. Bunty Maskey, Central Department of Technology, Tribhuvan University, Dharan. I express him my profound sense of gratitude for his continual supervision, encouragement and tireless effort throughout the work. His personal efforts, ideas and valuable time are the backbone of this dissertation work.

I am really thankful to Assoc. Prof. Dr. Dil Kumar Limbu (Campus Chief, Central Campus of Technology) and Asst. Prof. Nabin Gautam (Chairperson, Department of Food Technology) for providing adequate facilities to make every work convenient and fruitful.

Similarly, my special thanks goes to respected senior Mr. Nabindra Kumar Shrestha for his support and guidance throughout the work with great effort. I am also thankful to my seniors Mr. Kamal Ghimire and Mr. Ashish Koirala for their valuable advices. My heartly thanks goes to my classmates Niraj Adhikari, Chiranjivi Belbase, Bipin Aryal, Sanjog Kharel, Ashish Chhetri, Pragya Paudel, Sangita Shah, Archana Shrestha, Prashamsa Khanal, junior Barsha Baniya and all my batch mates from Central Campus of Technology for their help and support during the completion of this work.

I also express my sincere thanks to my teachers, staff of library and laboratory for their direct and indirect co-operation and suggestions without whom this work could not have been possible.

Finally, I am highly indebted to my parents and family members for their constant encouragement, love, inspiration and moral support without whom this work has not been completed successfully.

Date of submission:

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Abstract

The aim of the present study was to extract, partially purify and utilize the proteolytic enzyme from kiwi fruit (*Actinidia deliciosa*) for making soft cheese. The pulp of kiwi fruits was blended with sodium phosphate buffer (pH 7.0) in the ratio of 1:1 and the juice obtained was centrifuged at 4000 rpm for 10 min under cool condition (4°C). The partial purification of the suspended (crude extract) was obtained by ammonium sulphate precipitation at the saturation of 30, 40, 50, 60, 70 and 80%. The optimum saturation was selected on the basis of milk clotting activity (MCA). Optimum conditions of temperature and pH of milk for maximum MCA and minimum time of coagulation (TOC) were determined by response surface methodology. Milk clotting activity (MCA), proteolytic activity (PA) and protein content of the extracted protease were measured. The partially purified protease was used as milk coagulant in soft cheese preparation at optimized conditions. The physicochemical and microbiological properties of prepared cheeses were compared with rennet cheese.

Out of the fractions obtained by precipitation, the highest milk clotting activity was observed in the 50% fraction. Numerical optimization study revealed that the optimum condition for milk clotting was found to be at 55°C at pH 6.5. The optimized TOC and MCA for partially purified kiwi protease was found to be 30 s and 841.65 unit. The cheese manufactured by kiwi protease showed similar textural properties and physico-chemical compositions to rennet cheese. The physico-chemical analysis showed non-significant ($P>0.05$) difference in protein content between soft cheeses prepared by kiwi protease and rennet. However, significantly ($P<0.05$) higher level of moisture, ash content, acidity and calcium content, and lower level of fat were observed in kiwi protease cheese as compared to rennet cheese. Microbiological analysis revealed significantly ($P<0.05$) higher level of total plate count in kiwi cheese. There was no significant difference ($P>0.05$) in yeast and mold count between kiwi protease and rennet cheeses.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
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
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 is a stabilised curd of milk solids produced through casein coagulation and entrapment of milk fat inside the coagulum. The water content is substantially reduced, in comparison with milk, via the separation and removal of whey from the curd. Excepts for some fresh cheeses, the curd is textured, salted, shaped, and pressed into moulds before storage and curing or ripening (Fernandes, 2009). According to the definition of FAO/WHO, cheese is a fresh or ripened solid or semi-solid product obtained from the coagulation of milk, skim milk, partially skim milk, cream, whey cream, or buttermilk, through the action of rennet or other suitable coagulating agents, and by draining the whey resulting from such coagulation. Cheese is rich in essential amino acids and binds large amounts of minerals and vitamins (Upadhyay, 2003).

Enzymatic coagulation is a necessary step in cheese manufacture where the casein component of the milk protein forms a gel network that entraps fat (Duarte *et al.*, 2009; Mazorra-Manzano *et al.*, 2018) . Most enzyme used for preparation of cheese are extracted from the fourth stomach of calves. The source of rennet enzyme is ruminant stomach especially that of the calf. It contains chymosin (EC 3.4.23.4) as the main enzyme component (Khan and Masud, 2013), which is responsible for specific cleavage between k-casein Phe_{105}–Met_{106} bond (Nawaz *et al.*, 2011), leading to the disruption of casein micelles and milk coagulation but coagulants from microbes and plants were also used at very early dates (Freitas *et al.*, 2016).

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 proteases (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and proteases extracted from plants. Some plants reported to have proteases that yields good milk clotting activity are *Helianthus annus* (Egito *et al.*, 2007); *Ficus racemose* (Devaraj *et al.*, 2008); *Solanum dubium* (Ahmed *et al.*, 2009); *Bromelia hieronymi* (Bruno *et al.*, 2010); *Ficus religiosa* (Kumari *et al.*, 2010); *Euphorbia neriifolia* (Yadav *et al.*, 2011); *Moringa oleifera* (Pontual *et al.*, 2012); *Zingiber officinale* (Gagaoua *et al.*, 2015; Hashim

et al., 2011); *Withania coagulans* (Pezeshki *et al.*, 2011; Salehi *et al.*, 2017); *Carica papaya* (Maskey and Shrestha, 2020) and *Actinidia deliciosa* (Sharma and Vaidya, 2018). Actinidin is the predominant enzyme in kiwi fruit (Boland, 2013).

The search for new plant proteases from different parts of the plant continues in order to industrialize applicable and cost-effective products. Unfortunately, most of the plant rennet obtained to date has been inappropriate for commercial cheese manufacture due to an excessive proteolytic activity, with few exceptions. Due to high level of proteolytic activity, it causes the production of short peptides that affects both the flavor and the texture of cheese, resulting in excessive acidic and bitter flavour (Beigomi *et al.*, 2014). Thus, it is important to study the degradation pattern of casein that has an impact on yield, consistency and flavour of the cheese when choosing a potential plant rennet substitute. It is very vital to minimize general non-specific proteolysis and ensure correct ratio between protein and peptides in commercial cheese production (Singh *et al.*, 2014).

1.2 Statement of the problem

The worldwide increase in cheese production, coupled to a diminishing supply of natural animal rennet, is responsible for increase in the demand for alternative milk-coagulating sources (Amira *et al.*, 2017a). The increasingly higher prices of calf rennet as well as ethical concerns associated with the production of such enzymes for general cheesemaking have led to systematic investigations on the possibility and suitability of their substitution by other enzymes of plant origin (Jacob *et al.*, 2011). Due to this reason and other factors such as high price of rennet, religious concerns, diet, or ban on recombinant calf rennet, attention is being turned to the use of microbial coagulants and coagulants extracted from plants (Nawaz *et al.*, 2011). The research has been directed towards discovering milk clotting enzymes which would satisfactory replace calf rennet in cheesemaking, including microbial, recombinant, and plant-based enzymes (Shah *et al.*, 2014).

In addition to these, plant proteases are superior to microbial derived enzymes mainly because of safety problems such as pathogenicity or other disadvantageous effects such as higher proteolysis and lower yield. Henceforth, comparing to microbial and animal proteases, plant sources are good and approachable alternative (Sharma and Vaidya, 2018). Thus, 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. To solve this problem, a suitable cheap non-rennet coagulating agent has to be identified which could benefit cheese makers as well as consumers.

1.3 Objectives

The objectives of the research was divided into two parts:

1.3.1 General objective

The general objective of the dissertation work is to extract, partially purify and utilize the proteolytic enzyme from kiwi fruit for making soft cheese.

1.3.2 Specific objectives

- To extract the crude proteolytic enzyme from kiwi fruit and partially purify it.
- To optimize the temperature and pH of kiwi protease for maximum MCA and minimum time of coagulation (TOC).
- To determine milk coagulating activity (MCA), proteolytic activity (PA), specific activity and protein of the kiwi protease.
- To prepare soft cheese in the optimized condition of pH and temperature.
- To study physicochemical, microbiological as well as sensory quality of soft cheeses.

1.4 Significance of the study

Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been widely used as a milk clotting enzyme (Ahmed *et al.*, 2009). Plant coagulants share many biochemical features with chymosin. Both chymosin and plant coagulants cleave the phenylalanine$_{105}$-methionine$_{106}$ peptide bond of k-casein, but plant coagulants are more proteolytic and have broader specificity than chymosin (Esteves *et al.*, 2003).

Kiwi fruit extract has the potential to be employed as an efficient and low cost milk clotting agent in the production of dairy products (Grozdanovic *et al.*, 2013). Piero *et al.* (2011) worked on actinidin and showed that actinidin does exhibited milk clotting activity, which was correlated with the enzyme concentrations, indicating that the activities of

actinidin enzymes depends upon both pH and temperature. Puglisi *et al.* (2014) reported that kiwi protease exhibited high levels of milk clotting activity probably due to the presence of clotting enzyme actinidin, might be a promising plant coagulant which would replace the calf rennet. Therefore, the kiwi protease can be used for cheese making at low cost.

1.5 Limitations of the study

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

- Purification of the enzyme was not carried out.
- Characterization of the enzyme was not performed.
- Texture evaluation of the product was not carried out.

Part II

Literature review

2.1 Introduction to cheese technology

Cheese is the generic name for a group of fermented dairy products produced around the world in a wide variety of flavours, textures and shapes (Singh *et al.*, 2003). Cheese has been defined as a product made from milk by coagulating the casein with rennetor similar enzymes in the presence of lactic acid produced by added or adventitious micro- organisms, from which part of the moisture has been removed by cutting, cooking and/or pressing, which has been shaped in a mould, and then ripened at suitable temperatures and humidity (Mijan *et al.*, 2010).

Cheese has been recognised by mankind for thousands of years. The cheese predates the biblical era. Cheese was originated from 6000 to 7000 BC. The worldwide annual production is more than 12 million tons and is growing at a rate of about 4%. Over the centuries, many varieties of cheese have evolved.The production of cheese closely depends on local conditions, climate, type of milk, and other economical and geographical factors (Walstra *et al.*, 2006). An important part of the cheesemaking process is the conversion of a milk (liquid) into a solid material (the curd), which contains the casein and fat of the milk, but it usually removes most of the water, the whey proteins and part of the lactose content (Law and Tamime, 2010). 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 *et al.*, 2001).

Cheese manufacture is aimed at making an attractive and durable product, in which essential nutrients of the milk are concentrated but it is a complicated process, involving many process steps and several biochemical transformations (Walstra *et al.*, 2005). Cheese manufacture is a process of dehydration and acidification where the fat and protein (casein) of milk are concentrated between 6 to12 folds, and the pH is reduced from about 6.6 in milk to between 4.6 and 5.4 in freshly made curd. Although the production process differs greatly from variety to variety, the basic production steps common to all varieties are coagulation, acidification, dehydration, forming (shaping) and salting (Kheir *et al.*, 2011). In turn, the moisture and salt levels, pH and micro flora of the cheese regulate and control the

biochemical changes that occur during aging and thus determine the flavour, aroma and texture of the finished product. The quality of the finished cheese is determined largely by the manufacturing procedures. However the characteristic flavor and texture of the individual cheese varieties develop during the ripening phase (Guinee *et al.*, 2004).

2.2 Cheese production

The production of cheese has been growing continuously in the world as a whole for more than twenty years (about 4% per annum) – just under 6 million metric tons in 1961, 7.6 million in 1970-1971, 12.3 million in 1984 and 14.65 million in 1994 (Farkye, 2004). World cheese production in 2015, 2016, 2017, 2018, 2019, 2020 and 2021 were 19.52, 19.81, 20.44, 20.72, 20.98, 21.22 and 21.69 million metric tons respectively. In 2020, the European Union was the top producer of cheese worldwide, with a production volume of around 10.35 million metric tons of cheese (Shahbandeh, 2021).

2.2.1 Cheese production- a Nepalese scenario

The first cheese industry in Nepal dates back to in 1953, when the government- started producing Yak cheese with the support of the FAO in Langtang and Rasuwa district. A Dairy Development Section was established under the Department of Agriculture (DOA) and also a small-scale milk processing plant was started in Tusal village in Kavre district. In 1955, a Dairy Development Corporation was formed (Khanal *et al.*, 2019). Alongside government support, there has been a recent increase in private dairy plants with medium-sized facilities (that can process 10,000-30,000 L/day) (NDDB, 2021).

In Nepal, there are 53 small and medium scale cheese industries so far registered in the Department of Food Technology and Quality Control (DFTQC) and other offices under this department (DFTQC, 2020). Until now, exact data on the export of cheese is not available in Nepal. In recent years, the Himalayan Dog Chew company has started exporting dry cheese (called *Chhurpi* in Nepali) which is commonly referred to as “dog chew” to the United States, Britain, Canada and Japan (Khanal *et al.*, 2019).

Cheese is not much of popular food item among the general Nepalese. Many are not familiar to the flavor and taste of cheese. It is a relatively costly dairy product in Nepalese food market. Increased understanding of health requirements and hygienic products has

increased demand for pasteurized milk and milk products, including cheese in general. This demand is bound to grow further in the future (Pradhan, 2000).

2.3 Varieties and classification

There are more than 2,000 different types of cheese. They can be classified on the basis of their origin, composition, firmness, the maturation agents employed in their production, and the processes employed in their manufacture and maturation (Bamforth and Ward, 2018). McSweeney *et al.* (2017) suggested that there are probably about 18 distinct types of natural cheese i.e., they differ in many steps setting the milk, cutting the curd, stirring, heating, draining, pressing, and salting of the curds or ripening of the cheese. Some hard cheeses are ripened by moulds: Roquefort and Gorgonzola are representatives of this group. In addition, several semi-hard cheeses, such as brick, are ripened by bacteria. Soft cheeses consist of un-ripened, acid curd, such as cottage cheese; Camembert, ripened by mould; and Limberger, ripened by bacteria (Mijan *et al.*, 2010). Brined cheeses are some of the oldest varieties of cheese, originating from the Middle East and the Mediterranean (Dimitreli *et al.*, 2017).

According to Khanal *et al.* (2019), there are different schemes of cheese classification and they are:

- Based on texture,
- Based on method of coagulation and
- Based on 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 mold ripened, color of curds.
- External characters: rind hard, soft, smooth or rough, smear or mold 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 Alimentarius, FAO/WHO, and Standard A6 has been shown in the Table 2.1.

Table 2.1 Classification of cheese according to Codex Alimentarius

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.

Bamforth and Ward (2018) classified cheese on the basis of (i) Composition, (ii) Firmness, and (iii) Maturation agents, which has been shown in the Table 2.2.

Table 2.2 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: Bamforth and Ward (2018)

Rennet-coagulated cheeses account for about 75% of total cheese production and almost all ripened cheeses. Acid-curd cheeses account for about 25% of total cheese production and are generally consumed fresh. Coagulation using a combination of heat and acid is used for a limited number of varieties, including Ricotta and Manouri. Rennet-coagulated varieties were subdivided into relatively homogeneous groups based on the characteristic ripening agents or manufacturing technology (McSweeney *et al.*, 2017).

The classification of cheese on the basis of mode of coagulation given by (Fox *et al.*, 2004) has been shown in the Table 2.3

Table 2.3 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: Fox *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).

2.4 Soft cheese

Robinson (2002) define soft cheeses as containing water in the fat free cheese matter greater than 61% and fat in the dry matter, (FDM) of 10-50 %. The FAO/WHO classifies soft cheese as cheese containing over 67% water in the fat free cheese matter. Data on the composition of various soft cheeses in the literature reveal moisture contents in the range of 50-80%. Because most soft cheeses are consumed fresh and also, to eliminate the risk of food poisoning, it is important that the milk or other dairy ingredients used for soft cheese manufacture be adequately pasteurized.

Soft cheeses vary widely in nutritional composition, depending on whether they belong to the ripened or unripened varieties. Fresh, unripened cheeses, such as cottage cheese, are low in fat, relatively low in calcium, high in moisture and contain unfermented lactose (Buttriss, 2003). According to Bamforth and Ward (2018), different types of soft cheese has been shown in the Table 2.4.

Table 2.4 Types of soft cheese

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

Source: Bamforth and Ward (2018)

2.5 Raw milk quality

Cheese can be made from the milk of many mammals including goats, sheep, buffalo, reindeer, camel, zebra and yak. The milk of ruminants is the best milk for cheesemaking because it contains high levels of the milk protein casein which is required to provide an adequate coagulum (Goff, 2003). Fox *et al.* (2000) stated that traditionally, all cheese was made from raw milk, a practice that remained widespread until the 1940s. Even today, significant amounts of cheese are made from raw milk in Europe. The use of raw milk is undesirable for two reasons:

- Public health safety and
- The presence of undesirable microorganisms, which may cause defects in flavor and/or texture

According to Acharya (2010), only high grade milk can yield high grade cheese. The quality of finished depends upon the initial quality of milk from which it is made. Milk for cheesemaking requires the use of good quality milk, both at the level of its composition, and microbial flora, and in relation to its capacity to ferment and coagulate properly. If these requirements are not met, this may lead to processing problems, and the development of various defects in milk, including flavour. The standard requirements are as follows:

- Few bacteria.
- The milk must come from healthy cows.
- Good ability to rennet.
- Milk must be of a natural composition.
- Good smell and taste.
- Few butyric acid bacteria.

The yield of cheese is directly related to the content of casein in milk. Fat influences texture, flavour, mouth feel and consistency of cheese whereas the casein fraction of cheese is responsible for curd firmness, syneresis rate and moisture retention rate of cheese. Milk with high composition of casein and fat, low levels of enzymes and low somatic cell count is generally good quality milk for cheese manufacturing (Nassar and Emirie, 2015).

2.5.1 Chemical composition of milk

The principal constituents of milk of any species are water, fat, protein (caseins and whey proteins), sugar (lactose) and minerals (salts such as calcium and phosphate), with trace quantities of vitamins and enzymes (McSweeney, 2007). It may contain as many as 10^5 different kinds of molecules. The composition of the milk, especially the content of fat, protein, calcium and pH value effects the composition of cheese. The constituents and composition of milk are influenced by several factors, including species, breed, individual animal variations, nutritional and health status, and stage of lactation of the milk-producing animals (Haenlein and Park, 2013).

The chemical composition of milk is of paramount importance. Fat has an influence on texture, flavor, yield, and has some effect on colour. Lactose acts as a substrate for the formation of acid, and thus coagulation of milk, drainage, and texture of the curd, as well as the growth of the microorganism. The degradation of casein, which coagulates and forms the basic material for the production of cheese, contributes to the development of whole range of flavours. 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).

2.5.1.1 Milk protein

Milk contains hundreds of types of protein, most of them in very small amounts (Haenlein and Park, 2013). Milk proteins are of two distinct types, whey protein (serum proteins) and caseins. Casein constitutes over 80% of the total protein in milk, although the relative proportion of whey protein to casein varies according to the stage of lactation (Chandan, 2008). Caseins can be fractionated into four distinct proteins: α_{s1}-, α_{s2}-, β- and κ-casein and some minor components (γ casein) derived from degradation of β-casein (Robinson, 2002). Whey protein also known as serum protein accounts for 20% milk protein and contains β-lactoglobulin (8.5%), lactalbumin (5.1%), immunoglobulins (1.7%), and serum albumins. From a cheesemaking standpoint, the proteins of milk are its most important constituents (Fox *et al.*, 2004).

For manufacture of cheese, milk that is high in casein and low in serum protein and lactose is 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 (Kosikowski, 1982).

2.5.1.2 Milk fat

Milk fat is the most variable of all the milk constituents. It is mainly a mixture of triglycerides (TG). The other 1-2% of milk fat is composed of phospholipids, steroids, carotenoids, and fat soluble vitamins A, D, E, and K (Chandan, 2008). The average fat content of cow, goat, sheep, and buffalo milk is 3.5, 3.5, 6.5, and 7 g/L, respectively. Within any particular species, there are considerable variations due to breed, individuality, stage of lactation, age, animal health, nutritional status, interval between milking, and so on (Fox *et al.*, 2000).

In most cheese varieties, fat is a major compositional variable and major changes in its level result in changes in moisture and protein levels and in the yield of cheese. It is one of the key variables in the determination of the characteristic body, composition, microstructure, yield, rheological and textural properties, cooking properties, cheese texture and taste (Abd El-Gawad *et al.*, 2007).

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

After milk has been heated in a muffle furnace at around 600°C for 5 h, a residue (ash), representing roughly 0.7 g/100 ml of the mass of the milk sample, remains (Fox *et al.*, 2000). Milk salts are mainly made up of chlorides, phosphates, citrates, sulfates, and bicarbonates of sodium, potassium, calcium, and magnesium. Some of the milk salts (i.e., the chlorides, sulfates, and compounds of sodium and potassium) are soluble and are present almost entirely as ions dissolved in milk whey. Others calcium and phosphate in particular are much less soluble and at the normal pH of milk exist partly in dissolved and partly in insoluble (i.e., colloidal) form, in close association with the casein micelles (Robinson, 2002).

Milk calcium concentration is described to influence cheese texture, which is an important rheological property of cheese quality. The total calcium content of several cheese varieties varies mostly due to differences in the manufacturing pH values (Lee *et al.*, 2005). Salt in cheese serves two major roles, namely it acts as a preservative and contributes directly to flavor and quality. The preservative action of NaCl is due to its depressing effect on the water activity (a_w) of the cheese (McSweeney, 2007).

2.5.1.4 Lactose

Lactose is the main carbohydrate in milk and its concentration varies with milk production between 4.2 and 5%, the lactose content is generally lower in lactation milk and in the milk of animals with mastitis (Chandan, 2008). Milk contains only trace amounts of other sugars, including glucose, fructose, glucosamine, galactosamine, neuraminic acid, and neutral and acidic oligosaccharides (Fox *et al.*, 2000).

Lactose is a disaccharide and comprises α-D-glucose and β-D molecules. Three solid forms of lactose exist, α-lactose monohydrate, anhydrous α-, and β-lactose. The β-form is of markedly higher solubility but, through mutarotation, an equilibrium mixture of the two forms exists in solution. In isolated form, lactose exists in either of the two crystalline forms,

α-hydrate and anhydrous-β or as amorphous “glass” mixtures of α- and β-lactose (Chandan, 2008).

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, colostrum and late lactation milk. Abnormal milk is not suitable for cheesemaking due to factors or conditions resulting in ‘slow starter’, slow coagulation with rennet and weak curd formation (De, 1980).

2.5.2.1 Mastitis milk

Mastitis is an infection of the udder which negatively impacts milk quality (Goff, 2003). Mastitis is the inflammation of the mammary gland caused by microorganisms, mainly bacteria and seldom fungi, yeasts, or algae. Because of the immune reaction, leukocytes enter into the mammary gland, resulting in a noticeable increase in somatic cell count (SCC) in the milk. The SCC in milk from uninfected quarters is typically <100,000 cells/mL (Brandt *et al.*, 2010).

Depending on its severity and symptoms, mastitis can be classified into acute or chronic and into clinical or subclinical (Hamann, 2005). Visible alteration in milk, such as flakes or clots, is found especially with acute clinical forms of mastitis, characterized additionally by typical symptoms such as pain, redness, and swelling of the udder. Subclinical mastitis causes no visual alterations in the milk or udder; however, alterations in milk constituents and an elevated SCC can be observed (Brandt *et al.*, 2010).

2.5.2.2 Colostrum

The milk produced 1-5 days after parturition is called colostrum, and usually contains higher nutrient contents than normal mature milk including total solids, protein and ash. The most remarkable difference between colostrum and normal milk is the protein content, especially immunoglobulins. The new born calves, lambs and kids can absorb immunoglobulins containing antibodies during their first day of life. After the first day, because of changes in the absorptive ability of the intestine, the digestive enzymes break down the globulins so that they lose their ability to provide immune protection of the neonate (Haenlein and Park, 2013).

Colostrum contains 10 times more vitamin A than mature milk. Colostrum also has a higher content of calcium, magnesium, phosphorus, and chlorine, and is lower in potassium than normal milk (Park, 2010).

2.5.2.3 Late lactation milk

Late lactated milk is poor for cheesemaking, because of its large sodium and potassium content causing more protein hydrated than in normal milk (De, 1980).

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 (Upadhyay, 2003). According to Walstra *et al.* (2006) changes may be classified as follows:

- Physical changes occurring, for instance, when air is incorporated during milking: Because of this, additional dissolution of oxygen and nitrogen occurs in milk. Milk contains many surface active substances, predominantly proteins, which can become attached to the air water interface formed. Furthermore, by contact with the air bubbles, fat globules may become damaged, i.e., lose part of their membrane.
- Chemical changes may be caused by the presence of oxygen: Several substances may be oxidized. In particular, light may induce reactions, often leading to off flavors. Composition of salts can vary with temperature.
- Biochemical changes can occur because milk contains active enzymes: Examples are lipase, which causes lipolysis; proteinases, which cause proteolysis; and phosphatases, which cause hydrolysis of phosphoric acid esters.
- Microbial changes are often the most conspicuous: The best known effect is production of lactic acid from lactose, causing an obvious decrease in pH. Numerous other changes, such as lipolysis and proteolysis, may result from microbial growth.

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 2.5.

Table 2.5 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	P
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 contains a variety of compounds that have antimicrobial activity. Their purpose is to protect the udder from infection and also to protect neonates, but they may also have a role in the preservation of raw milk during storage and transport (Fernandes, 2009).

2.5.4.1 Natural inhibitory systems in milk

The natural inhibitory system in raw milk includes lactoperoxidase, lactoferrin, and lysozyme system (FAO, 1999).

Lactoperoxidase an enzyme naturally present in raw milk that catalyses the conversion of hydrogen peroxide to water. When hydrogen peroxide and thiocyanate are added to raw milk, the thiocyanate is oxidized by the enzyme/ hydrogen peroxide complex producing bacteriostatic compounds that inhibit Gram negative bacteria, *E. coli*, *Salmonella spp*, and *Streptococci* (Goff, 2003).

Lactoferrin is also found in milk and is a glycoprotein that binds iron so that it is not available to bacteria. The chelation of iron in the milk inhibits the growth of many bacteria.

In addition to producing an iron-deficient environment, lactoferrin is thought to cause the release of anionic polysaccharide from the outer membrane of Gram-negative bacteria, thereby destabilising the membrane (Fernandes, 2009).

Lysozyme an enzyme that hydrolyses glycosidic bonds in gram positive cell walls. However, its effect as a bacteriostatic mechanism in milk is probably negligible (Goff, 2003).

2.5.4.2 Antibiotic residues

Antibiotics are often used to control mastitis in dairy cattle. But, the presence of antibiotic residues in milk is extremely problematic. In the production of fermented milks, antibiotic residues can slow down or destroy the growth of fermenting bacteria. From a human health point of view, some people are allergic to specific antibiotics and their presence in the food consumed can have serious consequences (Goff, 2003). Cheesemilk must be free from antibiotics, which totally, or partially, inhibit the starter bacteria; delayed acidification results in an abnormal composition, defects in flavour and texture and causes growth of harmful, pathogenic or food poisoning microorganisms (Fox *et al.*, 2004).

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 is a serious problem. (Walstra *et al.*, 2006).

2.5.5 Microbiological quality of milk

Milk is not only rich in nutrients but also has the properties to readily support microbial growth and potentially pathogenic organisms. Milk on the farm is exposed to many sources of potential contamination. Some of these can be water, food sources, exposure to manure, insects, contact with sick animals in housing or corral areas, udder injuries and poor milking practices milking (Chandan, 2008). Pathogens can also be present in milk produced in a hygienic manner. In short, raw milk is a potentially dangerous product, the microbiological safety of which cannot be guaranteed without the use of pasteurization or an equivalent process (Fernandes, 2009).

Most of the raw milk received by dairies in developed countries has a viable count of less than 50,000 CFU/ml. Milk is often stored in large silos for perhaps 24 h or more, then

additional growth and contamination from poorly cleaned silos can occur, so milk for cheesemaking may have levels above 10^5 CFU/ml. However, counts above 10^5 CFU/ml in milk before pasteurization could affect the quality of the cheese, as many psychrotrophs, especially *Pseudomonas* spp., produce thermostable lipases (Fox *et al.*, 2000). The temperature of the milk in the silo should be kept at $7^{O}C$ ($45^{O}F$) or less. Even at these temperatures, psychrotrophic organisms can cause proteolysis and lipolysis if milk is stored for long periods of time. Therefore, it is recommended to empty the silos, cleaned and disinfected at regular intervals (Chandan, 2008).

2.6 Pre-treatments of milk for cheesemaking

The production of cheese made from raw milk obviously involves an increased risk of survival of pathogenic bacteria and their incorporation into the cheese. Therefore, there should be other factors that control or eliminate this hazard; these include the long maturation time of many cheese varieties, competition from starter and non-starter lactic acid bacteria, low pH and water activity, and high salt content (McSweeney, 2007). 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

- Carbon dioxide treatment
- Ultrafiltration

2.6.1 Chilling and cold storage

There are different approaches to control the microbial quality of bulk raw milk during storage.The simplest way is to lower the storage temperature from 6 to 2°C causes substantial retardation of the growth of psychrotrophs up to the critical value of 10^6 CFU/ml for 3 days (Griffiths *et al.,* 1987). However, during the cold storage period endogenous milk enzymes are generally active. This is especially true for lipoprotein lipase, which can hydrolyze the triglycerides of fat globules when the milk fat globule membrane (MFGM) is damaged by temperature fluctuations or by agitation and foaming. The induced lipolysis in milk results in rancidity. Actually, lipoprotein lipase activity is only a problem in raw milk, because it is almost totally inactivated by pasteurization (Haenlein and Park, 2013).

2.6.2 Lactoperoxidase treatment

Lactoperoxidase is an enzyme found in milk. It has no inherent antimicrobial activity, but, in the presence of hydrogen peroxide (usually of microbial origin), it oxidizes thiocyanate to produce inhibitors such as hypothiocyanite. This is referred to as the lactoperoxidase (LPO) system, and it has bactericidal activity against many Gram-negative spoilage organisms, and some bacteriostatic action against many pathogens (Fernandes, 2009).

It is the second most abundant enzyme in bovine milk after xanthine oxidoreductase and it catalyzes the oxidation of SCN^- by H_2O_2 to $OSCN^-$. Both SCN^- and $OSCN^-$ are harmless to animals but inhibit most H_2O_2 producing bacteria themselves. The activity of the LPO system in milk, which includes H_2O_2 (coming mainly from bacterial metabolism) and thiocyanate anions from cyanoglucosides in animal feeds, depends on SCN^- concentration and pH. Activation of the LPO system in milk through the addition of thiocyanate ions and H_2O_2 or addition of an H_2O_2 system (e.g. glucose oxidase) can be used for the preservation of raw milk during storage or for the extension of milk shelf life, if applied prior to pasteurization. In the absence of refrigeration or heat treatment, this treatment is known as ‘cold pasteurization’ (Pruitt, 2003; Seifu *et al.*, 2005)

2.6.3 Thermization

The most commonly used technique is to apply a mild heat treatment (thermisation), by heating to around 57 - 68 °C for 15 - 20 s and then cooling rapidly to <6°C. This greatly reduces the psychrotrophic population and can extend the shelf life of raw milk by several days. However, thermisation cannot eliminate vegetative pathogens, and is therefore not a reliable control for the hazard. For example, *Listeria monocytogenes* can survive the process and could then grow during chilled storage (Fernandes, 2009).

2.6.4 Bactofugation

Bactofugation is a process which is usually applied to remove spores from products that are low pasteurized. This may involve removal of spores of *Bacillus* spp. or *Clostridium* and related species from cheese milk. The spores are quite small, but the density difference with plasma is greater than that of bacteria, and at separation temperatures of 60-65°C a substantial part can be removed, usually 90-95% (Walstra *et al.*, 2006). The sludge is subsequently sterilized to kill the spores and bacteria and is then added back to the milk (Fox *et al.*, 2004).

Bactofugation removes spores but causes losses of about 3% of milk constituents. It is used for the extension of shelf-life of pasteurized milk; however, it is an expensive method and requires two separators in a row for the removal of 99% of spores (Haenlein and Park, 2013).

2.6.5 Microfiltration

Filtration techniques such as microfiltration (pore size ~1 μm) operating at low pressure are also used to remove spores, microorganisms and somatic cells from raw milk prior to heat treatment. These milk constituents have diameters above 1 μm, similar to some of the fat globules, and therefore this process is applied to skimmed milk. The retentate and the cream are heat treated/sterilized independently before they are mixed with the permeate, the latter containing 0.1–1% of the initial counts (Elwell and Barbano, 2006; Goulas and Grandison, 2008; Pouliot, 2008). Microfiltration is very efficient at removing bacterial cells (> 99%) and is being used increasingly in the dairy industry, such as in the production of extra-long life pasteurized milk. It is not yet widely used for

cheese milk except for the removal of spores from milk for Swiss and similar cheeses. The technique has been very useful in studying the effect of the indigenous raw milk micro flora and of enzymes inactivated by pasteurization on cheese flavor (Fox *et al.*, 2000).

2.6.6 Standardization

Standardization refers to the practice of adjusting the composition of cheese milk to maximize economic return from the milk components while maintaining both cheese quality and cheese composition specifications. In practice, this means that milk composition is adjusted to achieve the most economically favorable balance of the cost of ingredients and the percent transfer of milk solid components to cheese while maintaining cheese quality. Cheese yield is mainly determined by the recoveries of protein and fat in the cheese (that is the percent of fat and protein transferred from milk to cheese) (Goff, 2003). 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

Pasteurization is a microbiocidal heat treatment aimed at reducing the number of any pathogenic microorganisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard. Pasteurization conditions are designed to effectively destroy the organism *Mycobacterium tuberculosis* and *Coxiella burnetti* (Haenlein and Park, 2013). 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 /14-40 s
3. LTLT: 61-65°C / 20-40 min

Most of the bacteria found in raw milk are heat labile and are killed by pasteurization at 72°C for 15 s; most milk for cheesemaking is subjected to this heat treatment. Pasteurization kills all potential pathogens that might be present in the milk, but spores of *Clostridium* and *Bacillus* are not killed by this treatment. Pasteurization inactivates many enzymes, including alkaline phosphatase and lipoprotein lipase. The absence of active alkaline phosphatase in

cheese is often used to determine whether milk has been properly pasteurized before cheese is made (Fox *et al.*, 2000).

2.6.8 CO_2 treatment

CO_2 can be added to raw milk as an anti-microbial agent (Hendricks and Hotchkiss, 1997; Martin *et al.*, 2003). Addition of 20–30 mmol/L at refrigeration temperatures has been proposed for the extension of storage of raw milk, while considering the pressure and temperature conditions of the treatment since these can cause precipitation of proteins (Rajagopal *et al.*, 2005). The effect of CO_2 on various species and strains is variable. In general, the lag phase of aerobic plate counts increases substantially and more dramatically than the increase in psychrotrophs lag phase. Inhibition of coliforms by at least 1 log CFU/ml is also possible. This treatment has a more pronounced effect on Gram-negative bacteria than on Gram-positive bacteria and spores (Martin *et al.*, 2003; Singh *et al.*, 2012). However, the reduction in pH caused by CO_2 may cause dissociation of the casein micelles, which can be detrimental for the efficiency of heat exchangers due to fouling (Loss and Hotchkiss, 2003).

2.7 Additives in cheese milk

The essential additives in the cheesemaking process are the starter culture and the rennet. Under certain conditions it may also be necessary to supply other components such as calcium chloride ($CaCl_2$) and saltpetre (KNO_3 or $NaNO_3$) (Bylund, 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 (Lucey and Fox, 1993). $CaCl_2$ is a common additive used in cheesemaking and its addition to cheesemilk improves the rennet coagulation and syneresis properties of the milk (McSweeney, 2007).

Ca^{++} is necessary in milk in order to be able to precipitate the para-casein produced by renneting, and the more Ca^{++}, the stronger the rennet effect, the more solid the coagulation, and the faster the curdling. There must be the fitting ratio of calcium salts and sodium potassium salts in the milk. If there is too much calcium, the coagulum will be too solid,

and if there is too little, it will be too loose and soft. The addition of $CaCl_2$, is therefore used when the milk originally contains too little calcium ions, or in order to replace the calcium which is precipitated during the milk's pasteurization, or to improve the rennet ability that was weakened by cooling and/or homogenizing the milk (Acharya, 2010).

2.7.2 Saltpetre ($NaNO_3$ or KNO_3)

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 (Farkye, 2004).

2.7.3 Cheese colour

Colour is a very important attribute of foods and serves as an index of quality, although in some cases, this is cosmetic. The principal indigenous pigments in milk are carotenoids which are obtained from the animal's diet, especially from fresh grass and clover. Colours such as carotene and orleana, an anatto dye, are used to correct these seasonal variations in countries where colouring is permitted (Fox *et al.*, 2000).

2.7.4 Starters

The starter culture is a very important factor in cheesemaking. According to Fox *et al.* (2000) two principal types of culture are used in cheesemaking:

- Mesophilic cultures with a temperature optimum between 25°C and 40°C such as *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Leuconostoc* spp.
- Thermophilic cultures, which develop at up to 50°C such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus.*

The acidification of milk is the key step in cheesemaking. Acidification is essential for the development of both flavor and texture; it promotes coagulation; and the reduction in pH inhibits the growth of pathogens and spoilage organisms. It is normally obtained from the

fermentation of lactose by starter bacterial cultures to produce lactic acid, although some fresh cheeses, such as cottage cheese, can be acidified by the direct addition of acid and do not require starter. Most cheeses are now made from selected starter, giving predictable and desirable results. *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus* and *Lactobacillus delbrueckii* are the primary species of starter bacteria used in cheese manufacture (Fernandes, 2009). According to Acharya (2010) three characteristic abilities of starter cultures are of primary importance in cheesemaking:

- Produce lactic acid,
- Break down the protein and,
- Produce carbon dioxide (CO_2)

Direct acidification using acid (usually lactic acid or HCl) or acidogen (GDL) is an alternative to biological acidification and is used commercially to a significant extent in the manufacture of Cottage, Quark, Feta-type cheese from ultrafiltration (UF) concentrated milk and Mozzarella (Farkye, 2004). Direct acidification is more controllable than biological acidification and, unlike starters, is not susceptible to bacteriophage infection. However, enzymes from starter bacteria are essential in cheese ripening and hence chemical acidification is used mainly for cheese varieties for which texture and functionality are more important than flavor (Haenlein and Park, 2013).

2.7.5 Salt

Salt inhibits or retards the growth and activity of microorganisms, including pathogenic and food poisoning microorganisms, and hence increases the safety of cheese. It inhibits the activity of various enzymes in cheese. It affects the syneresis of cheese curd, resulting in whey expulsion and thus in a reduction in the moisture of cheese, which also influences the activity of microorganisms and enzymes. Salt causes changes in cheese proteins that influence cheese texture, protein solubility, and probably protein conformation. It affects cheese flavor directly and indirectly via its influence on microorganisms and enzymes in cheese (Fox *et al.*, 2000).

2.7.6 Rennet

'Rennet' is a general term for proteinase preparations used to coagulate milk. Most proteinases will coagulate milk under suitable conditions of pH and temperature (McSweeney, 2007). Rennet is extracted from the stomachs of young calves and marketed in the form of a solution with a strength of 1:10 000 to 1:15 000, which means that one part of rennet can coagulate 10,000 – 15,000 parts of milk in 40 min at 35°C. Bovine and porcine rennet are also used, often in combination with calf rennet (50:50, 30:70, etc.). Rennet in powder form is normally 10 times as strong as liquid rennet (Bylund, 1995).

Rennet consists of mainly rennin and pepsin, principally the former being responsible for milk clotting and the latter for proteolysis (although both possess the two characteristics). Rennin is liable to heat and alkali besides other physical and chemical agents and gets inactivated at 70°C in 14 min at pH 6.8-7.0 (Acharya, 2010). The prime enzyme used in cheese making is chymosin (rennin) (EC 3.4.23.4, isoelectric pH ≈ 4.65). It is an aspartate-proteinase, hence an endopeptidase, which means that it can split proteins into relatively large fragments (Walstra *et al.*, 2006).

2.7.6.1 Calf rennet

Animal rennet is prepared by extracting dried (usually) or salted (called vells) gastric tissue with 10% NaCl and activating and standardizing the extract. Calf rennet contains about 60-70 RU/ml and is preserved by making the extract up to 20% NaCl by the addition of sodium benzoate or sodium propionate. A rennet unit (RU) is the amount of rennet activity that will coagulate 10 ml of milk (usually low-heat skim milk powder reconstituted in 0.01 % $CaCl_2$ and perhaps adjusted to pH 6.5) in 100 s (Fox *et al.*, 2000). Rennet should be protected from light and kept cold. Under these conditions, it will only lose about 1% of its activity (strength). The enzymes are destroyed by alkali and strong acid (Acharya, 2010).

2.7.6.2 Microbial rennet

All the well-known microbial coagulants used for cheesemaking are of fungal origin. Most of the bacterial proteases described as milk clotting enzymes have been found to be unsuitable, mainly because they have too high a proteolytic activity. Of the two microbial coagulants used for cheesemaking, *Rhizomucor miehei* is predominant. *Rhizomucor pusillus*

coagulant is similar to the *Rhizomucor miehei* product; it was used in the past, but has no advantages over *Rhizomucor miehei* coagulant (Law and Tamime, 2010).

2.7.6.3 Factors affecting the rennet activity

The factors affecting rennet activity have been discussed by (Acharya, 2010); (Adhikari *et al.*, 2000) and (De, 1980).

1. pH

   In connection with the splitting of the κ-casein in milk (the primary phase), the chymosin optimum pH is at 5.4. 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 (De, 1980).

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

   The casein which is converted by the rennet enzyme (para-casein) can only precipitate if the milk contains free calcium ions. Variations in the content of free calcium ions in the milk will cause changes in coagulation time, firmness of the curd, and whey exudation (Acharya, 2010).

2.8 Plant proteases as rennet substitute

Plant proteases, obtained from different tissues including fruits, flowers, stems, latex, etc., possess attractive catalytic properties with diverse optimum conditions of pH and temperature to clot milk. Flowers from cardoon plant species (*Cynara cardunculus and Cynara scolymus*) contain high amount of milk clotting proteases (cardosins and cynarases) with similar catalytic properties to chymosin (Yegin and Dekker, 2013). However, proteases from other catalytic groups are also considered as an attractive option to diversify the characteristics of cheeses produced. Cysteine proteases (EC. 3.4.22) such as papain, bromelain, zingibain, and actinidin from papaya, pineapple, ginger, and kiwi, respectively,

and serine proteases (EC. 3.4.21) from melon (i.e., cucumisin) (Mazorra-Manzano *et al.*, 2018; Setiasih *et al.*, 2018) . The most successful plant rennets are from the flowers of the Cardoon thistle (*Cynara cardunculus*) which grows wild in Spain and Portugal (McSweeney, 2007).

Many enzymes extracted from higher plants have been tried for clotting cheese milk. However, attempts to use them have been unsuccessful. Most plant proteases are strongly proteolytic and cause extensive digestion of the curd, which has resulted in reduced yields and bitter flavors cheese (Wong, 1999).

2.8.1 Production of plant proteases

Proteases used as milk coagulants have been identified and studied by almost every part of the plant, whether it is seeds, flowers or latex. These enzymes can be obtained from their natural source or by in vitro cultures to ensure a continuous supply of plant proteases (Gonzalez-Rabade *et al.*, 2011).

2.8.1.1 Production from natural sources

These enzymes have been extracted from their natural source by aqueous maceration of various plant organs such as flowers, seeds, roots and leaves. There are several different ways of preparing the aqueous extract of the plant material (Roseiro *et al.*, 2003). The crude extract thus obtained can be further purified to obtain a partially purified enzyme or a pure enzyme depending on the degree of purification. Precipitation with ammonium sulfate is an efficient way to produce substantial amounts of active proteases (Barros *et al.*, 2001).

2.8.1.2 In vitro production

The plant cells are totipotent and thus are able to produce the same chemical compounds in vitro and in vivo. The yield of enzymes differs between the two processes. Generally, the yield of plant proteases obtained in vitro is lower than in vivo conditions (Gonzalez-Rabade *et al.*, 2011). Different plant parts produce proteases with different activities (Pérez *et al.*, 2013). Different in vitro techniques such as callus and cell suspension cultures have been used to produce proteases. For example, cell suspension culture of *Centaurea calcitrapa* (Raposo and Domingos, 2008), and callus culture of *Silybum marianum* (Cimino *et al.*, 2006) and *Cynara cardunculus* (Oliveira *et al.*, 2010).

In vitro techniques have several advantages. The production of enzymes in vitro has the potential to overcome the low enzyme yield and the difficulties of extraction from natural sources. The problems due to climate and season conditions, and heterogeneity of the product obtained from plant parts can also be solved by in vitro techniques (Gonzalez-Rabade *et al.*, 2011).

2.8.2 Classification of proteases

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bond of proteins i.e. proteases conduct proteolysis and they begin protein catabolism. Proteases are also called as proteolytic enzyme or proteinases. Proteinases constitute a large family and divided as endopeptidases and exopeptidase on the basis of cleavage site at which they breakdown peptide chain. Endopeptidases are categorized according to the reactive groups at the active site involved in catalysis viz., serine proteinase, cysteine proteinase, aspartic proteinase and metalloproteinase (Barrett, 1994), however the plant proteases used as milk coagulants have been reported only from first three types and none from metalloproteases (Bruno *et al.*, 2006).

2.8.2.1 Serine protease

Serine proteases are one of the largest groups of proteolytic enzymes, found in eukaryotes and prokaryotes. They exhibit different types of activities including exopeptidases, endopeptidase, oligopeptidase and omegapeptidase (Rawlings and Barrett, 1994). Serine proteases possess a serine residue in their active site and share a number of biochemical and physiological features. In humans, they are divided in major groups, including the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase groups (Borgono *et al.*, 2007).

2.8.2.2 Aspartic protease

Aspartic acid proteases, also known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2) and the enzymes from pararetroviruses (A3). Most aspartic proteases show maximal activity at low pH (pH 3 to 4) (Kaur, 2011). Aspartic proteases with milk clotting activity have been reported in artichoke (*Cynara*

scolymus L.) (Llorente *et al.*, 1997); milk thistle (*Silybum marianum* L. Gaertn.) (Vairo-Cavalli *et al.*, 2005); *Onopordum turcicum* (Tamer, 1993); rice kernels (Asakura *et al.*, 1997); *Centaurea calcitrapa* (Domingos *et al.*, 2000).

2.8.2.3 Cysteine protease

Cysteine proteases also known as thiol proteases are found in both prokaryotes and eukaryotes (e.g. bacteria, parasites, plants, invertebrates and vertebrates). The catalytic mechanism of these enzymes involves a cysteine group in the active site. Cysteine proteases comprise a family of enzymes, consisting of papain and related plant proteases such as chymopapain, caricain, bromelain, actinidin, ficin, aleurain (Turk *et al.*, 1997). Cysteine proteases have great potential in the food, biotechnology and pharmaceutical industries due to their property of being active over a wide range of temperatures and pH (Gonzalez-Rabade *et al.*, 2011).

2.8.3 Partial purification of protease by ammonium sulphate precipitation

Salt precipitation can be a very powerful tool to purify proteins by precipitation. Ammonium sulfate is usually the salt of choice since it is cheap, very soluble in water, and is able to become much more hydrated than almost any other ionic solvent. At low salt concentrations (<0.15M) the addition of more salt in general tends to increase the solubility of proteins as ions shield the protein molecules from the charges of other molecules; this trend is termed 'salting-in'. At some point the ionic strength becomes too high and starts to have a negative effect on the solubility of proteins, termed 'salting-out'. This happens because the dissolved salt competes with the proteins for scarce water molecules, increasing the surface tension of water and therefore causing the protein to fold tighter. The reduction in protein surface area means less protein water interactions which allows for more hydrophobic interactions between protein molecules, causing aggregation and subsequently precipitation (Wingfield, 1998).

Proteins in solution can also be fractionated out since they will precipitate out as a function of salt concentration. In this way it is possible to purify specific proteins by adding a specific amount of ammonium sulfate to precipitate out non-desirable proteins, recovering the supernatant, and then adding a bit more ammonium sulfate to precipitate out the desired protein and then save that pellet of precipitated protein. Since salt precipitation only affects

the solubility of proteins and does not denature them, the recovered fraction can be stored in the salt solution for prolonged periods of time without having to worry about bacterial contamination since the high salt content inhibits any microbial growth or protease activity. Because ammonium sulfate precipitation only reduces the solubility of proteins and does not denature them proteins can be concentrated by removing the remaining ammonium sulfate solution then the protein pellet can be resolubilized in standard buffers or a lower concentration of ammonium sulfate. Hydrophobic interaction chromatography can be used to further purify the protein solution (Mukherjee, 2019).

2.8.4 Applications of proteases

Proteases are proteolytic enzymes which catalyze the hydrolysis of proteins based upon their structures or properties of the active site (Raju *et al.*, 2013). Proteases from plant sources offer a high potential as processing aids in production of food (e.g. production of novel dairy products, meat tenderizers and protein hydrolyzates) and medicine (e.g. digestive and anti-inflammatory agents) (Huang *et al.*, 2008; Katsaros *et al.*, 2010). Now proteases can be produced in industries that allow cost effective use of these proteases in industrial processes (Elizabeth *et al.*, 2003). Kiwi fruit extract has the potential to be employed as an efficient and low-cost milk clotting agent in the production of dairy products (Grozdanovic *et al.*, 2013).

Ha *et al.* (2013) reported from their study that kiwi fruit protease extract was found to be more effective at hydrolysing myofibrillar and collagen proteins than the asparagus protease and suggesting that this enzyme might have potential for targeting specific meat applications. The addition of the correct dosage of protease in dough can improve the extension of freshness, increase the quantity of fermentation sugar as proteases cause the cleavage of peptide bonds, they have ability to give new amino groups (David and Misca, 2012).

Piero *et al.* (2011) and Puglisi *et al.* (2014) worked on actinidin and showed that actinidin does exhibited milk clotting activity, which was correlated with the enzyme concentrations, indicating that the activities of actinidin enzymes depends upon both pH and temperature. Moreover, the enzyme dependence on pH and temperature and the stability profiles were fully suitable with the chemical-physical conditions adopted during the cheese-making procedure. The applicability of the kiwi aqueous solution as plant coagulant as well as its

utilization in the manufacture of mozzarella cheese, reported that aqueous solution exhibited high levels of milk clotting activity probably due to the presence of the clotting enzyme actinidin, might be a promising vegetable coagulant which would replace the calf rennet.

2.8.5 Enzymatic activities of plant protease

MCA is the most important property of proteases used in cheese production. It is the ability of the enzyme to hydrolyse specifically the κ-casein from milk (Jacob *et al.*, 2011). The ratio of the milk clotting activity (MCA) to proteolytic activity (PA) of proteases is a very important index to evaluate their potential as rennet substitutes. A higher MCA/PA ratio enhances the ability of the protease to form curd, which is accompanied with higher cheese yield and less bitterness. In contrast, a decreased ratio would greatly decrease the curd recovery and firmness. Formation of peptides with bitter taste could highly affect the sensory attributes of the final product. Due to the highest MCA/PA ratio relative to other proteases, chymosin has been regarded as the most efficient coagulant (Afsharnezhad *et al.*, 2019).

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

2.8.6 Kiwi fruit

Kiwi fruit (*Actinidia deliciosa*) is native to Southern China (Ferguson, 1984) and was originally called "Yang Tao" in China and "Chinese Gooseberry" in rest of the world. Its cultivation was spread from China in the early 20th century, when Isabel Fraser introduced seeds to New Zealand (Dadlani *et al.*, 1971). Kiwi fruit is an oval berry about the size of a hen's egg with a light brown hairy skin and emerald-green flesh with numerous tiny black seeds embedded in a juicy pericarp. Kiwi fruit have persistent long, hard, bristle like hairs, which are partially removed during grading and packaging. The green color of the flesh of *A. deliciosa* is due to the presence of chlorophyll, which is retained during fruit maturation and ripening. The flesh has a combination of tangy, sweet, and sour flavors (Padmanabhan and Paliyath, 2016).

Kiwi fruit is very popular in human diet due to its pleasant taste and high content of vitamin C, minerals (potassium, phosphorus, iron) and low calorific value. Kiwi fruit are good sources of folate, potassium, and contain high amounts of vitamin E in the seeds. Kiwi fruit also contain different pigments including chlorophylls, carotenoids, lutein and anthocyanins (Jung *et al.*, 2005).

Kiwi fruit is known to contain a highly active proteolytic enzymes and dominated one is actinidin (Kaur *et al.*, 2010). Its inactive forms, also called thaumatin like protein and an unusual protein called kiwellin, literally has unknown function. Actinidin is the predominant enzyme in Kiwi fruit, similar to the thiol proteolytic enzymes papain (papaya), ficin (fig) and bromelain (pine apple) (Boland, 2013).

According to Motohashi *et al.* (2002), kiwi fruit contain a number of valuable anti-cancer bioactive materials that are prooxidant (at higher concentrations) and antioxidant (at lower concentrations). Composition of kiwi fruit has been shown in the Table 2.6.

Table 2.6 Composition of kiwi fruit (per 100 g)

Kiwi fruit	Nutritional value (per 100 g)
Carbohydrates	14.66 g
Sugar	8.99 g
Dietary fiber	3 g
Fat	0.52 g
Protein	1.14 g
Vitamin C	92.7 mg
Calcium	34 mg
Water	83 g

Source: NDB (2011)

2.8.6.1 Kiwi protease (Actinidin)

The name actinidin, which derives from Actinidia, the genus of the source plant, was first proposed by Arcus (1959). Actinidin (EC. 3.4.22.14) is a cysteine protease found in the fruit of Chinese gooseberry (*Actinidia chinensis*) or kiwi fruit, which catalyses the hydrolysis of peptide bonds containing basic amino acids such as Lys and Arg (Piero *et al.*, 2011). The amount of enzyme is greater inquantity with a higher level of activity in ripe kiwi, which represents a potential use for overripe and discarded fruit (Karki and Ojha, 2018).

Actinidin is a single polypeptide chain globular protein with a sequence of 220 amino acid residues, a molecular weight of 23.0 kDa. The polypeptide is folded into a two-domain structure. The L-domain (domain I) consists of residues 19-115 and 214-220 and the R-domain (domain II) residues 1-18 and 116-213 (Boland, 2013).

Actinidin has the ability to form milk clots so that the enzyme is fully compatible with conditions used in cheese manufacture. Analysis of product produced by hydrolysis by using actinidin showed that the preferred substrate for this enzyme is β-casein, followed by k-

casein, which is hydrolysed into a small number of larger peptide (Piero *et al.*, 2011). It has no or limited proteolytic effect on globular proteins such as immunoglobulins including IgG, rabbit IgG, chicken IgG, bovine serum albumin (BSA), and whey proteins (α-lactalbumin and β-lactoglobulin). In contrast to globular proteins, actinidin can hydrolyse collagen and fibrinogen perfectly at neutral and mild basic pH. Moreover, this enzyme can digest pure α-casein and major subunits of micellar casein especially in acidic pH (Chalabi *et al.*, 2014).

2.9 Cheese technology

The technology of cheesemaking has two overriding goals: firstly, to establish the parameters that make a given cheese desirable (flavour, body, texture, melt and stretch properties); and second, to develop a manufacturing and ripening protocol that will routinely reproduce these parameters every time this cheese is made (Law and Tamime, 2010).

Cheesemaking is a rather simple process in itself, but it involves complex chemical and physical phenomena. It is essentially a concentration process, beginning with the coagulation of the major milk protein, casein and then proceeding with manufacturing steps designed to control the chemistry of the casein molecules (Johnson and Lucey, 2006). Factors that influence these interactions are the following:

- pH,
- Dissolution of colloidal calcium phosphate,
- Proteolysis,
- Temperature

2.9.1 Basic principles of cheesemaking

According to Nielsen and Ullum (1989), the basic cheesemaking principles are concentration, preservation and ripening.

1. Concentration: Coagulation, whey exudation (in cheese vat cutting, cooking, stirring, during pressing and during salting), evaporation during storage.
2. Preservation: hygiene, pasteurization, concentration, acidification, salting, addition of saltpeter, surface treatment, cooling.
3. Ripening: changes in solids (protein, lactose, fat).

2.9.2 Retention of constituents of milk

The amount of active coagulant retained in the curd is of great importance for proteolysis during maturation and the development of the texture, flavour and functionality of the cheese. A number of factors affect retention of coagulant activity including moisture content of cheese, cooking temperature, pH at whey drainage, ionic strength, amount of coagulant added to milk, casein content of milk, the size of the casein micelles and pH of the cheese during ripening (McSweeney, 2007). According to Acharya (2010) the retention figures of some milk constituents in cheese are shown in Table 2.7.

Table 2.7 Retention figures of some milk constituents in cheese

Milk Constituents	Retention Figure
Protein	~75% (not higher than 88%)
Fat	~83-90%
Lactose	~3-5%
Ash	~30-40%
Citric acid	~10%

Source: Acharya (2010)

2.10 Manufacturing steps involved in soft cheese

The production of all varieties of cheese involves a generally similar procedure, in which various steps are modified to give a product with the desired characteristics (Fox *et al.*, 2000). The general steps involved in cheesemaking are:

1. Selection, standardization and, in most cases, pasteurization of the milk.
2. Acidification, usually via the in situ production of lactic acid by selected bacteria.
3. Coagulation of the milk by acidification or limited proteolysis.
4. Dehydration of the coagulum to yield cheese curd, by a range of techniques, some of which are variety specific.
5. Forming the curds into characteristic shapes.

6. For most varieties, ripening (maturation) of the curd during which the characteristic flavour and texture of the cheese develop.

2.10.1 Selection of Milk

To obtain a satisfactory result in the manufacture of cheese, it is necessary that the milk used is of the best quality both bacteriologically and chemically.

According to Acharya (2010), standards requirements for high-quality milk are:

- Few bacteria.
- Good ability to starter culture.
- Good ability to rennet.
- Milk must be of natural composition.
- Good smell and taste.

2.10.2 Pre-treatments of milk

Various pre-treatments such as standardization, pasteurization, filtration/centrifugation are performed to improve the characteristics of soft cheese.

- Standardization

Standardization normally means adding skim milk or skim milk solids, or removing cream to increase the ratio of protein to fat (P/F) (Goff, 2003). Standardization of cheese milk controls the composition and particularly the fat in dry matter ratio of the cheese, maximizes cheese yield and helps to control cheese quality (McSweeney, 2007). Different cheese varieties have a characteristic fat in dry matter content (Fox *et al.*, 2000).

- Pasteurization

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

- Filtration/centrifugation

Removal of dirt particles is done by filtration or centrifugation. Bactofugation is sometimes applied to reduce the number of spores of *Clostridium tyrobutyricum* (to about 3%). The removal of the sediment obtained, containing the spores, causes about 6% reduction in cheese yield. Therefore, the sediment is UHT heated and added again to the cheese milk (Walstra *et al.,* 2006)

2.10.3 Conversion of milk to cheese Curd

After the milk has been standardized and pasteurized or otherwise treated, its temperature is adjusted to a value in the range 30–35°C, depending on the variety, and transferred to vats (or kettles), which vary in shape (hemispherical, rectangular or vertical or horizontal cylinders), may be open or closed and may range in size from a few hundred litres to 30,000 L or more (Bennett and Johnston, 2004), where it is converted to cheese curd by a process which involves three basic operations: acidification, coagulation and dehydration.

2.10.3.1 Acidification

The acidification of milk is the crucial step in cheesemaking. Acidification is essential for the development of both flavor and texture; it promotes coagulation; and the reduction in pH inhibits the growth of pathogens and spoilage organisms. It is normally obtained from the fermentation of lactose by bacterial starter cultures to produce lactic acid, although some fresh cheeses, such as cottage cheese, can be acidified by the direct addition of acid and do not require starter. In the past, acidification was achieved by the development of the resident micro flora of the milk. However, this process is difficult to control and tends to give a variable product that may suffer from taints and inconsistent flavors. As a result, most cheeses are now made using selected starter, that gives predictable and desirable results. *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* are the primary species of starter bacteria used in cheese manufacture (Fernandes, 2009).

Direct acidification method involves addition of lactic acid/phosphoric acid to cold milk (2°C–12°C) to achieve a pH of 5.2 followed by addition of glucono-δ-lactone, which is slowly hydrolyzed to gluconic acid, resulting in a gradual reduction in pH to 4.6-4.8 (Makhal and Kanawjia, 2008). Direct acidification is more controllable than biological acidification, and, unlike starters, it is not susceptible to phage infection. In addition to

acidification, the starter bacteria helps in cheese ripening, and hence chemical acidification is used mainly for cheese varieties for which texture is more important than flavor (Fox *et al.*, 2000).

2.10.3.2 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. 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). The vast majority of cheese varieties (representing about 75% of total production) are produced by rennet coagulation, but some acid coagulated varieties, such as Quarg and Cottage cheese, are of major importance. The acid heat coagulated cheeses are of relatively minor importance (Fox *et al.*, 2000).

2.10.4 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).

2.10.4.1 Cutting the coagulum

After a few minutes of rennet addition, the precipitation of para-casein will be taken place. At the beginning, the coagulum is very soft but it gradually becomes firmer. The coagulum hardens faster at higher temperature and lower pH values. The rennet coagulum is cut (horizontal and vertical) with cheese knives into pieces, thereby, increasing the surface area of the curd for easy expulsion of whey. The mode of cutting the coagulum varies with the variety of cheeses, as it directly determines the rate and extent of moisture removal and the final cheese texture. The smaller the size of the curd piece, the greater is the moisture expulsion and harder is the texture (lower moisture) of cheese obtained (Acharya, 2010).

2.10.4.2 Cooking

The combination of heat and the developed acidity (decreasing pH) causes syneresis with the consequent expulsion of moisture, lactose, acids, soluble minerals and salts, and whey

proteins (Goff, 2003). Cooking temperature in cheese vat influences moisture removal from curd during cheese making and differences in moisture content could be a significant impact on cheese. Thus differences in cooking temperature may affect chemical composition and sensory characteristics of cheese (Abdalla and Mohamed, 2009).

2.10.4.3 Drainage

The process is performed to separate whey from curd and to aggregate or coalesce the curd particles. It takes about 15 min. The dipping is a traditional approach in which all the curd mass was collected in a cheese cloth or scooped into perforated moulds to separate it from whey (Acharya, 2010).

2.10.5 Salting

Salting is the last manufacturing operation. Salting promotes syneresis but it is not a satisfactory method for controlling the moisture content of cheese curd which is best achieved by ensuring that the degree of acidification, heating and stirring in the cheese vat are appropriate to the particular variety (Fox *et al.*, 2000).

The salt content directly affects the taste of cheese, provides sodium, which is important for regulating blood pressure and safe body cell function, and serves as a preservative. Salt decreases the activity of water inside the cheese matrix and consequently regulates the growth of bacteria, the activity of enzymes, the level of protein hydration and aggregation, and the rheological and cooking properties of cheese (Islam, 2006).

2.10.6 Ripening or ageing

Ripening is the natural process of microbial and biochemical reactions that occurs in a cheese after its manufacture and during storage. Ripening gives different cheeses their unique flavors, textures, and appearances. Except for some soft cheeses (e.g., cottage cheese, cheese, quark, etc.) almost all cheeses are held under controlled conditions to develop distinct attributes. Ripening essentially results from the action of microorganisms present within the curd mass and on its surface (Gunasekaran and Ak, 2003).

During ripening, further moisture loss occurs, and a complex combination of microbial and enzymic reactions take place, involving milk enzymes, the coagulant, and proteases and peptidases from the starter culture and non-starter organisms, which remain viable although

their growth is inhibited. Ripening conditions vary with cheese variety. Soft, high-moisture cheeses are ripened for relatively short periods, whereas hard, strongly flavored cheeses may ripen for more than a year (Fernandes, 2009).

2.11 Soft cheese manufacturing method

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

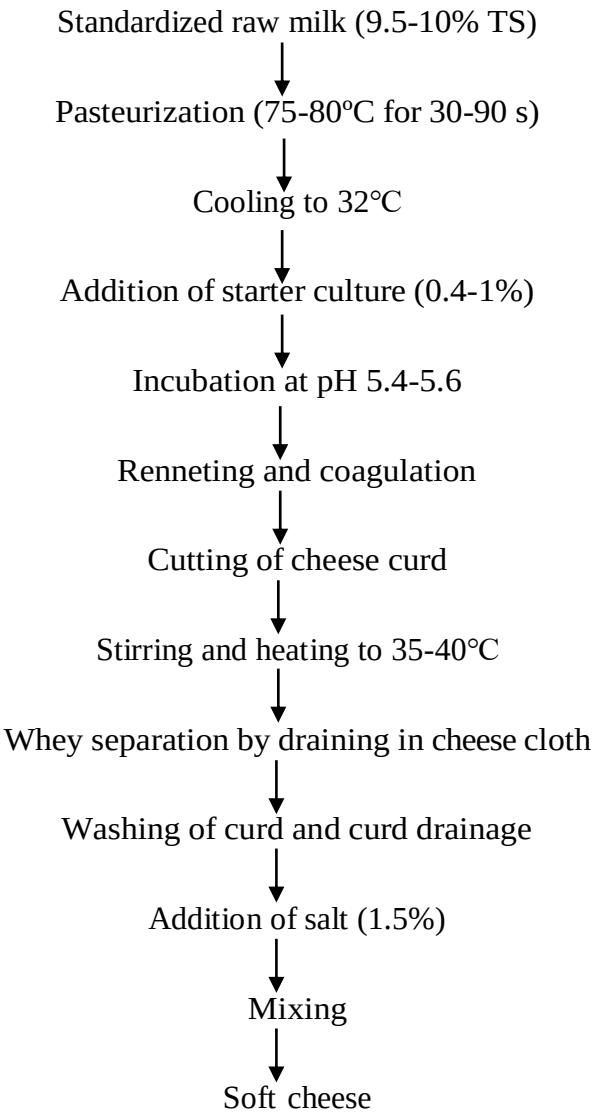

Fig. 2.1 Processing steps for soft cheesemaking

Source: Jain *et al.* (2019)

Part III

Materials and methods

3.1 Materials

3.1.1 Milk

Raw cow milk of local breed was purchased from Bijayapur, Dharan-14.

3.1.2 Rennet

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

3.1.3 Kiwi fruit

The kiwi fruit (*Actinidia deliciosa*) was purchased from the local market of Dharan.

3.1.4 Glucono delta lactone

The glucono delta lactone (GDL) was used from laboratory of CDFT.

3.2 Methods

3.2.1 Preparation of crude plant protease

3.2.1.1 Extraction of crude protease from kiwi fruit

The kiwi fruit (*Actinidia deliciosa*) was peeled and the pieces of kiwi fruit was blended in a juice extractor (Model: Havel's max grind 14000). The pulp obtained was centrifuged at 4000 rpm for 10 min with sodium phosphate buffer (pH 7.0) in the ratio 1:1 (w/v) under cool condition (4°C) (Setiasih *et al.*, 2018). The obtained supernatant was filtered through a muslin cloth to remove suspended particles and clear juice was obtained. The supernatant was labelled as "Crude enzyme" (Mazorra-Manzano *et al.*, 2013).

3.2.1.2 Partial purification of kiwi enzyme

Partial purification of enzyme was carried out by using ammonium sulphate fractionation method as described by Sharma and Vaidya (2018). The crude extract was precipitated by ammonium sulphate $(NH_4)_2SO_4$ using different saturations (30-80%) (Raju *et al.*, 2013). The solution obtained was centrifuged at 16,000 rpm for 15 min in refrigerated centrifuge

(Model: D3024R DLAB, UK) at 4ºC (Pal *et al.*, 2016). The precipitate was recovered by centrifugation. The supernatant was discarded and the pellet from each saturation was dissolved in sodium phosphate buffer (pH 7.0) and each pellet was extracted in 0.4 ml of sodium phosphate buffer. The extract was dialysed with the same buffer overnight under refrigeration at 4°C to remove the remaining salt (Andevari *et al.*, 2019; Sharma and Vaidya, 2018).

3.2.1.3 Specific activity

Specific activity was calculated by dividing the total enzyme activity with total protein (Blaber *et al.*, 2004).

$$\text{Specific activity} = \frac{\text{Total enzyme activity}}{\text{Total protein}}$$

3.2.1.4 Purification fold

Purification fold was calculated by dividing the specific activity of partially purified enzyme with specific activity of crude enzyme (Blaber *et al.*, 2004).

$$\text{Purification fold} = \frac{\text{Specific activity of partially purified enzyme}}{\text{Specific activity of crude enzyme}}$$

3.2.1.5 Percent yield / Recovery of enzyme

Percent yield / Recovery was calculated by dividing the total enzyme activity of partially purified enzyme with total activity of crude enzyme (Blaber *et al.*, 2004).

$$\text{Percent yield} = \frac{\text{Total enzyme activity of partially purified enzyme}}{\text{Total activity of crude enzyme}} \times 100$$

3.2.1.6 Milk clotting activity

The milk clotting activity was determined following the procedure described by IDF (1992). 60 g of skimmed milk powder (HiMedia) 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 0.1 ml per 1.0 ml of milk (0.1:1 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 curd formation (s); S = volume of the milk (ml); E = volume of the enzyme (ml).

3.2.1.7 Protease activity

Protease enzymatic activity was determined using protocol given by Cupp-Enyard and Aldrich (2008). 5 ml of 0.65% casein solution was added to four vials. Enzyme of varying concentrations (0, 0.1, 0.3, 0.5) ml was added and mixed by swirling and incubated at 37ºC for 10 min. 5 ml of the TCA reagent was added to each tube to stop the reaction. Distilled water was added to each vial such that sample volume becomes 1 ml. After 30 min incubation, each of the test solutions and the blank was filtered using 0.45um polyethersulfone syringe filter and 2ml filtrate was added to each test tube. 5 ml of sodium carbonate (5.3 g/100 ml) was added to regulate pH drop created by the addition of the 1 ml of Folin's reagent. Added Folin's reagent will react primarily with free tyrosine. The vial was swirled and incubated at 37°C for 30 min. The absorbance of the sample was measured by a double beam spectrophotometer (Agilent Cary 60 UV-vis) using a wavelength of 660 nm and was calculated by the following reaction:

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

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.

3.2.1.8 Protein estimation

Protein content of the kiwi fruit juice was determined using the procedure given by Bradford (1976). For this, 1 ml of enzyme extract was mixed with 3 ml of bradford reagent in a test tube. Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G250 in 50 ml of 95% ethanol. The solution is then mixed with 100 ml of 85% phosphoric acid and made up to 1 L with distilled water. The tube was incubated at room temperature in dark for 30 min. The contents of the tube were read in a double beam spectrophotometer (Agilent Cary 60 UV-vis) at 595 nm and compared with a calibration.

For calibration curve; 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard solution (200 µg/ml of Bovine Serum Albumin) was pipetted out in labelled test tubes. A tube with 1 ml of distilled water serves as blank. The volume in each test tube was made up to 1 ml with distilled water. 3 ml of bradford reagent was added to all the test tubes including the blank. The contents of the tubes were mixed by shaking the tubes and were incubated at room temperature in dark for 30 min. The absorbance of all the tubes were measured in a double beam spectrophotometer (Agilent Cary 60 UV-vis) at 595 nm and a standard curve was plotted by taking concentration of BSA along X-axis and absorbance at 595 nm along Y-axis.

3.2.1.9 Experimental design

The experimental design, data analysis and model building were performed using "Design Expert" software (Version 13.0, Stat-Ease Inc., USA). The soft cheese was prepared with variation in: (a) pH of milk and (b) temperature of milk during enzyme addition as shown in the Table 3.1. The independent variables and their levels were selected on the basis of literature and preliminary researches. The optimal temperature and pH of kiwi protease was 35-75°C and 5.5-6.5 respectively (Piero *et al.*, 2011). The two-factor central composite rotatable design was employed. The response variables were time of coagulation (TOC) and milk clotting activity (MCA) of the kiwi protease.

Table 3.1 Different constraints for optimization of kiwi enzyme

Name	Goal	Range
Temperature of milk	To be in range	35-75°C
pH of milk	Target = 6.5	5.5-6.5
Time of Coagulation (TOC)	To be minimized	To be determined
Milk Clotting Activity (MCA)	To be maximized	To be determined

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

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

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

3.2.1.10 Analysis of data

A second order quadratic model was employed to correlate the independent process variables. The second order polynomial coefficient for each term of equation was determined through multiple regression analysis using Design Expert software. Data were fitted to the selected models and the statistical significance of the terms was examined by ANOVA for each response. The adequacy of the model was tested considering R^2 (coefficient of determination 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 a measure of degree of fit as it is the ratio of explained variation to the total variation. When R^2 approaches to unity, a better empirical model fits the actual data. The smaller the value of R^2, the less relevance the dependent variables in the model in explaining the behaviour variation. Then the effect of predictors on the response was interpreted using the model.

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

3.2.2 Preparation of soft cheese

A general process for the preparation of soft cheese is shown in Fig. 3.1. The cheese prepared using rennet by direct acidification was labelled as cheese A. Similarly, cheese prepared using kiwi protease was labelled as cheese B. Milk was heated until it reached the temperature of 75-80°C followed by stirring for 1 min. For cheese A, The rennet was added at the rate of (2.5 g/100 L) after the milk attained pH 5.6 by food grade (GDL) i.e. glucono delta lactone (2%) addition at temperature of 37°C for optimum activity. For cheese B, pH 6.5 and temperature 55°C of the milk were adjusted. The milk was stirred gently and allowed to coagulation for 40 min. The curd was then cut by a stainless-steel knife to separate the whey and it was further cooked at 50-55°C for 1 h. The whey and the curd were separated

and the curds were drained using cheese cloth. The draining process was performed for 3 h and the drained curds were mixed with 1.5% common salt. The cheese was stored in refrigerator at below 5°C (Jain *et al.*, 2019).

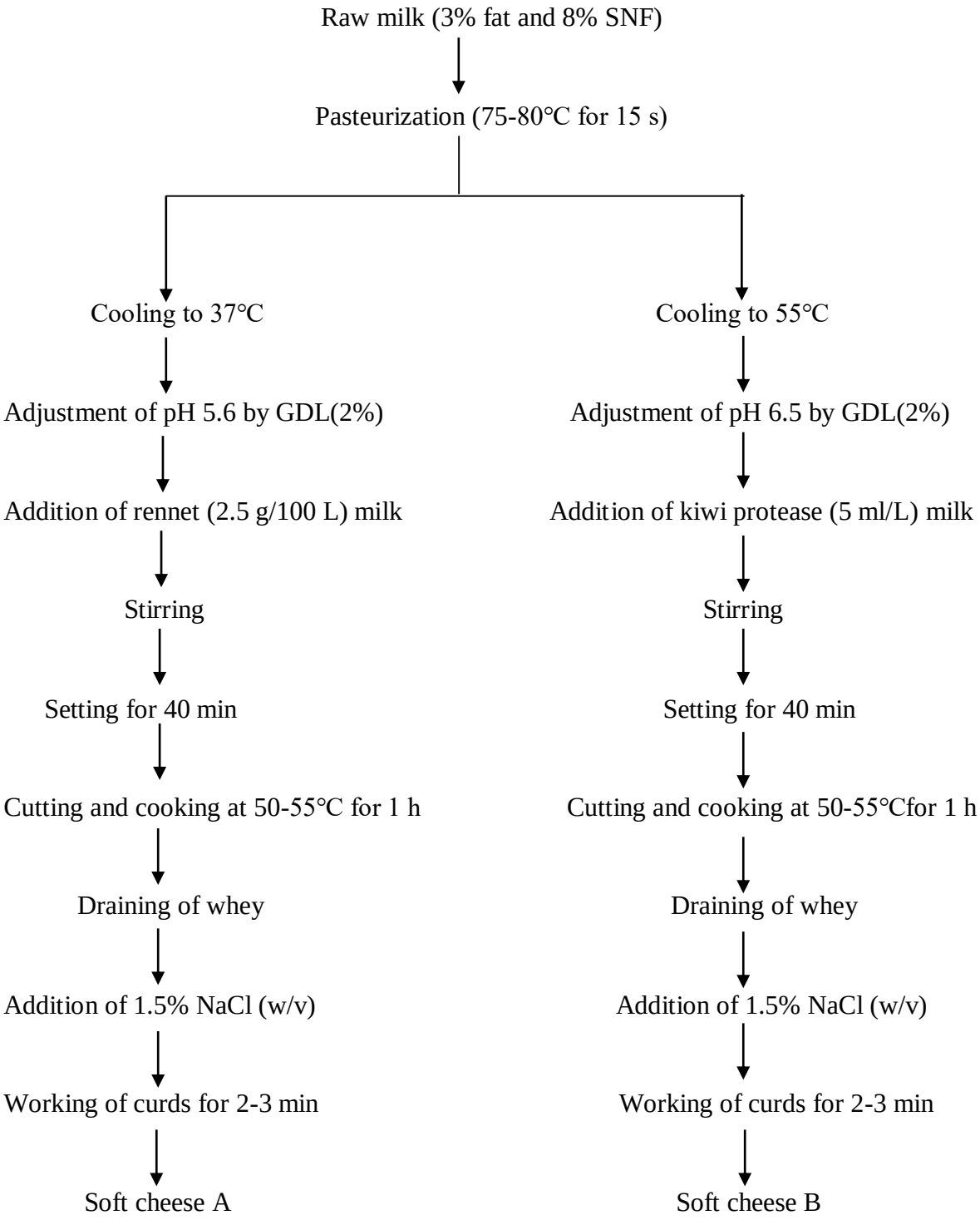

Fig. 3.1 Preparation steps of soft cheese

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 were determined by Gerber method as per AOAC (2005).

3.2.3.2 Determination of SNF in milk

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

3.2.3.3 Determination of pH in milk and cheese

The pH in milk and cheese were determined as per AOAC (2005).

3.2.3.4 Determination of protein in milk and cheese

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

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 AOAC (2005).

3.2.3.8 Determination of ash in cheese

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

3.2.3.9 Theoretical yield and actual yield

Theoretical yield was calculated using Van Slyke yield equation given by Emmons and Modler (2010)

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

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 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 (AOAC, 2005).

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 using paired t-test in MS-excel at 5% level of significance in IBM SPSS statistics version 26.

Part IV

Results and discussion

In this research work, protease was extracted and partially purified from kiwi fruit. The impact of pH and temperature of milk on time of coagulation (TOC) and milk coagulating activity (MCA) were analyzed by response surface methodology. The cheeses prepared from rennet (A) and partially purified kiwi protease (B) were analyzed for their physico-chemical properties as well as microbiological properties. Various sensory attributes (texture, spreadability, flavor, aftertaste and overall acceptability) of prepared cheeses were analyzed.

4.1 Partial purification of kiwi fruit enzyme

The crude enzyme from kiwi fruit was subjected to ammonium sulphate precipitation, at different concentrations ranging from 30-80% saturation (Raju *et al.*, 2013). The protease precipitation at 50% saturation, gave maximum milk clotting activity (841.65 U/ml) as shown in Table 4.1. Duarte *et al.* (2009), Setiasih *et al.* (2018) and Andevari *et al.* (2019) reported the highest activity of protease enzyme precipitated with 50% ammonium sulphate.

Table 4.1 Activity of kiwi protease at different concentrations of ammonium sulphate

Ammonium sulphate precipitation (%)	Time of coagulation (s)	Milk clotting activity (U/ml)
30	45	533.33
40	35	685.71
50	30	841.65
60	36	666.67
70	47	510.63
80	92	260.86

4.2 Numerical optimization for time of coagulation and milk clotting activity

The measured values of the time of coagulation (TOC) and milk clotting activity (MCA) for partially purified kiwi protease varied from 19-80 s and 300-1263.15 units respectively (Appendix A). Table B.1 and B.2 show the coefficients of the model and other statistical attributes of TOC whereas Table B.3 and B.4 show that of MCA.

$$TOC = 27.59 + 5.17A - 25.83B - 3.25AB + 1.95A^2 + 16.95B^2 \quad \text{.....4.1}$$

$$MCA = 868.24 - 97.39A + 432.32B - 98.56B^2 \quad \text{.....4.2}$$

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

In the quadratic equation 4.1, TOC had significant ($P<0.05$) positive effect of pH of milk (A) but significant ($P<0.05$) negative effect of temperature of milk (B) at 95% level of confidence. The quadratic term of pH of milk (A^2) had not-significant ($P>0.05$) positive effect and the quadratic term of temperature of milk (B^2) had significant ($P<0.05$) positive effect on TOC as given in Table B.2. The interaction term of pH of milk and temperature of milk (AB) had significant ($P<0.05$) negative effect on TOC.

Similarly, the quadratic equation 4.2 shows that pH of milk (A) had significant ($P<0.05$) negative effect but temperature of milk (B) had significant ($P<0.05$) positive effect on MCA at 95% level of confidence. The quadratic term of temperature of milk (B^2) had significant ($P<0.05$) negative effect on MCA as given by Table B.4.

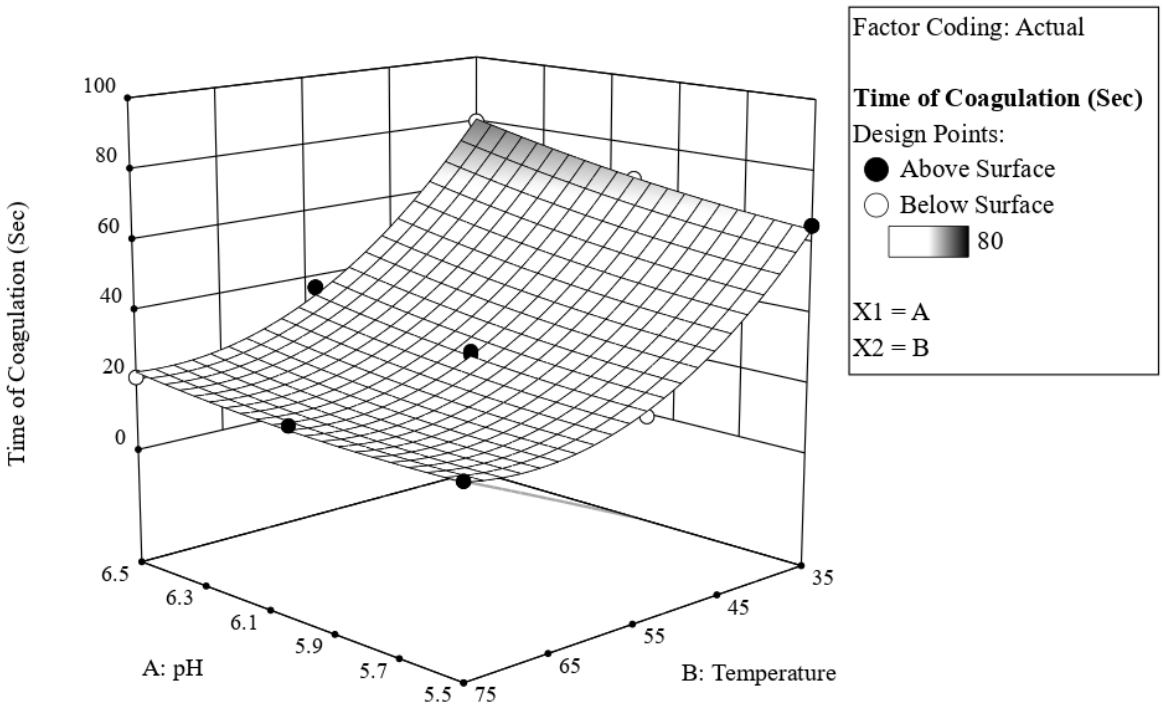

Fig. 4.1 Response surface plot of TOC of kiwi protease as a function of pH and temperature of milk.

In the Fig. 4.1, TOC increased gradually with the increase in pH and decreased in TOC was seen with the increase in temperature.

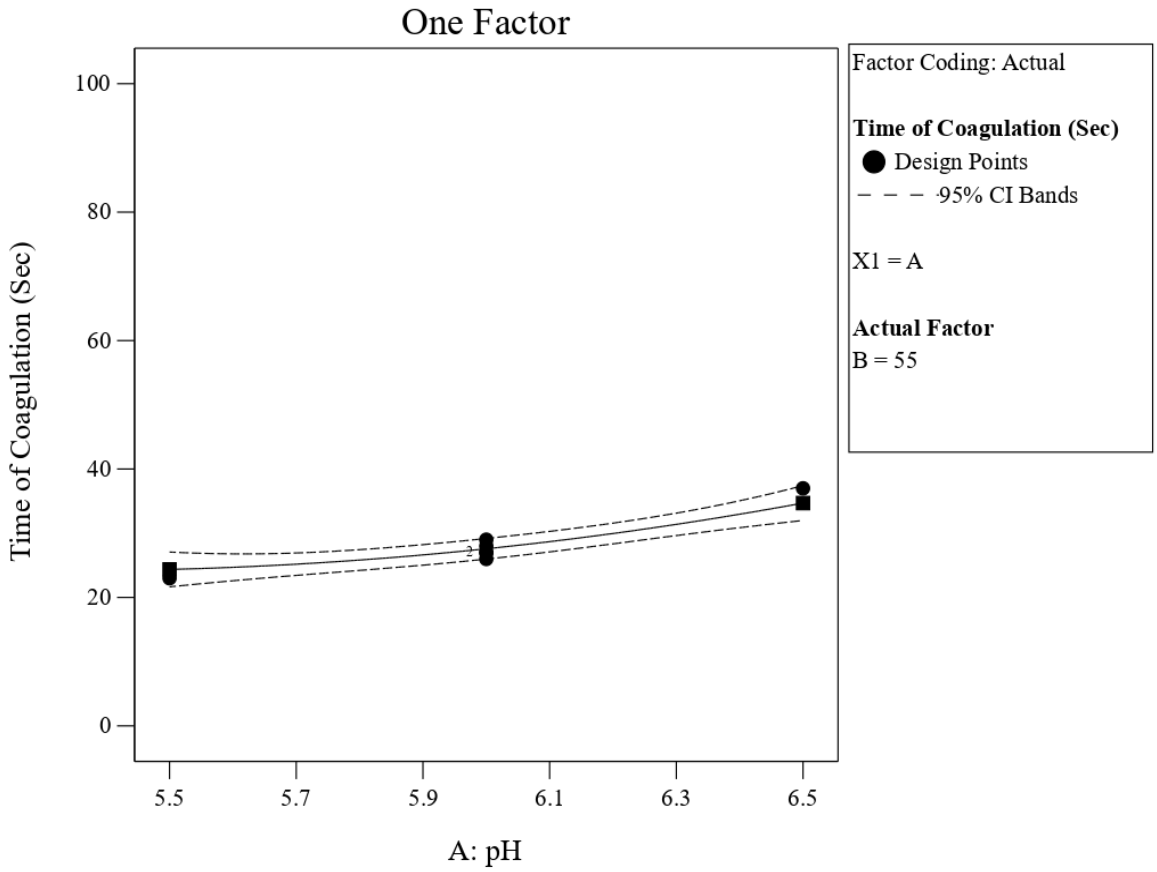

Fig. 4.2 Single factor graph of TOC of kiwi protease for individual factor A: pH

From Fig. 4.2, it can be concluded that increase in pH led to the increase of TOC of purified kiwi protease.

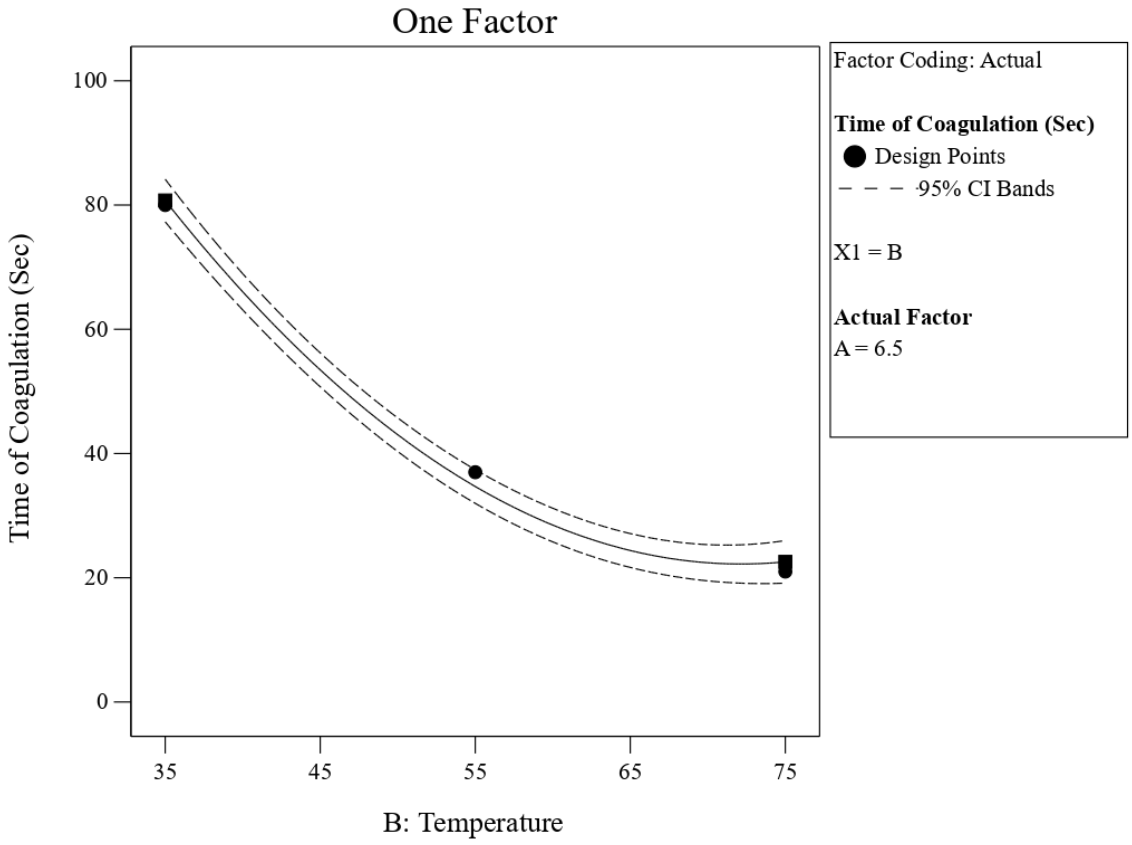

Fig. 4.3 Single factor graph of TOC of kiwi protease for individual factor B: temperature

From Fig. 4.3, it can be concluded that increase in temperature led to the decrease in TOC of purified kiwi protease.

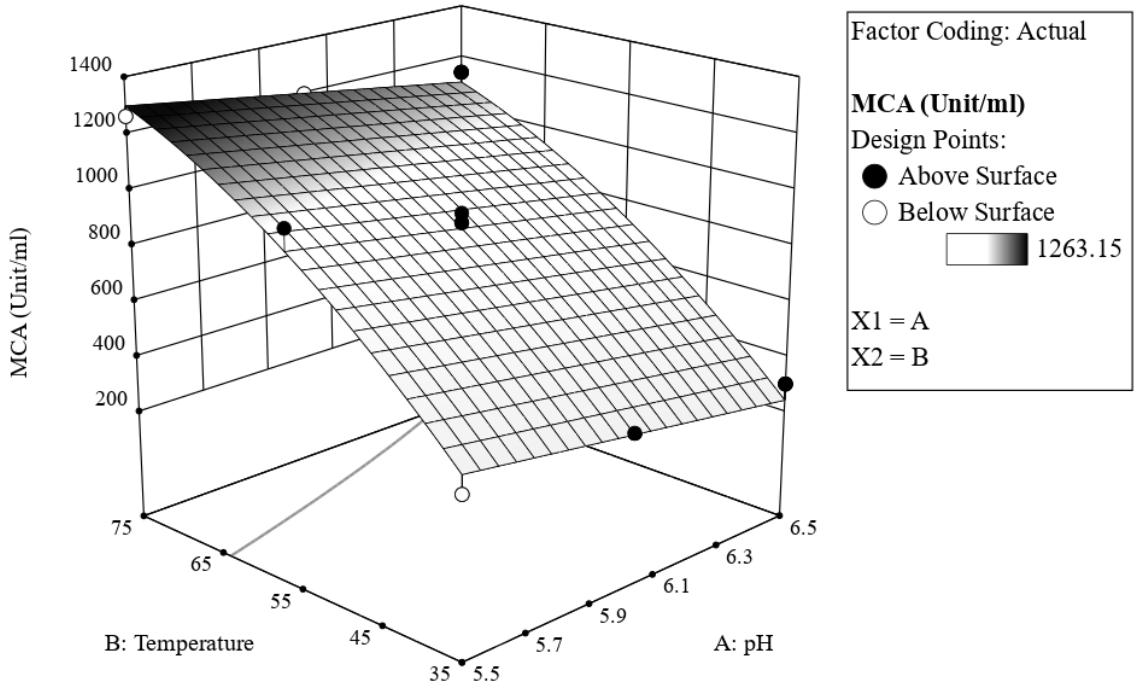

Fig. 4.4 Response surface plot of MCA of kiwi protease as a function of pH and temperature of milk

In the Fig. 4.4, MCA increased with the increase in milk temperature and decreased with the increase in pH. Also, with the combined increase of pH and temperature of milk there was gradual increase in MCA.

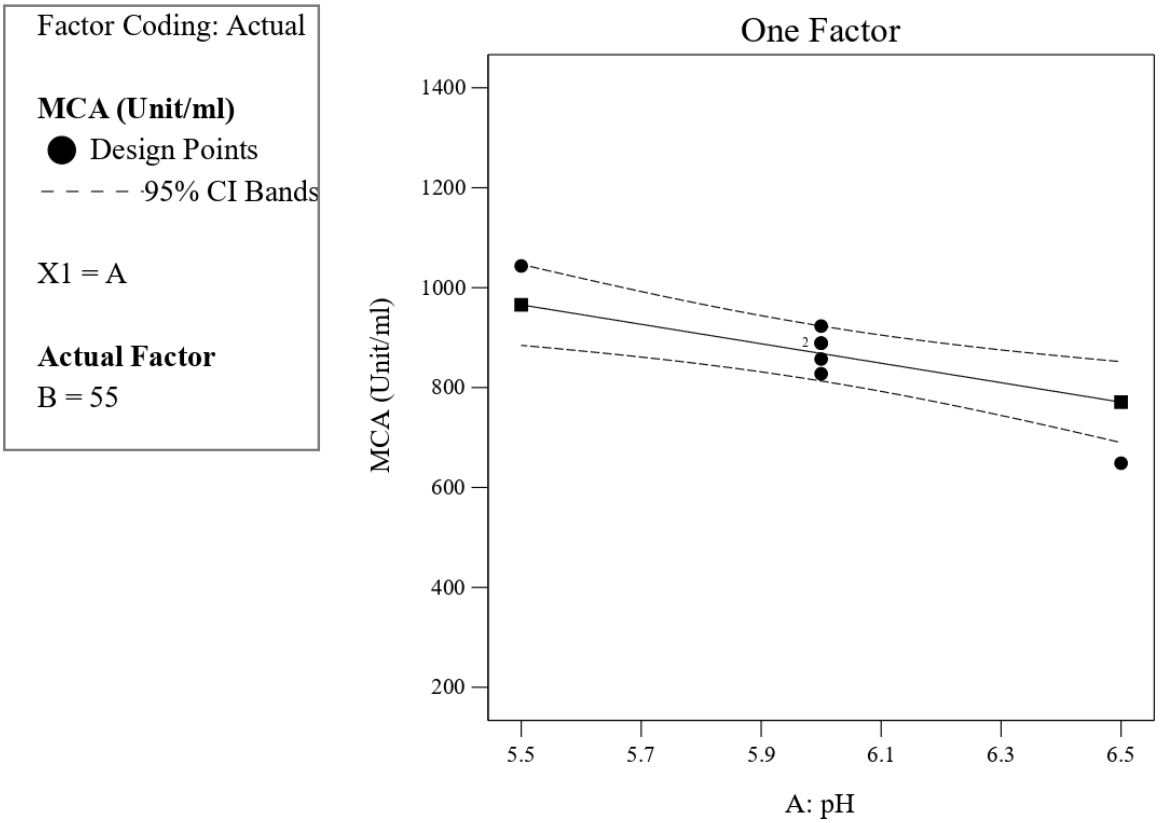

Fig. 4.5 Single factor graph of MCA of kiwi protease for individual factor A: pH

Fig. 4.5 revealed that MCA of purified kiwi protease decreased with the increase in pH.

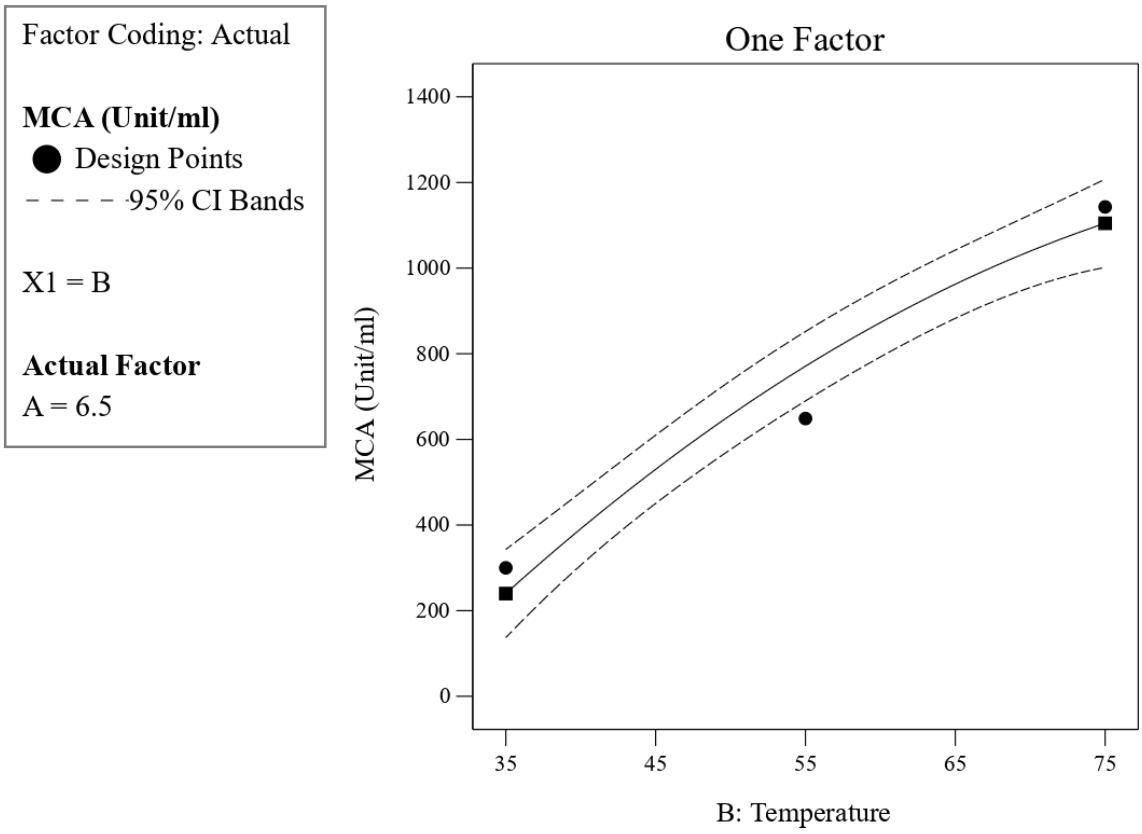

Fig. 4.6 Single factor graph of MCA of kiwi protease for individual factor B: temperature

Fig. 4.6 showed that MCA of partially purified kiwi increased with increase in temperature.

4.2.1 Optimization of partially purified enzymes

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

Table 4.2 Different constraints for optimization of partially purified kiwi protease

Name	Goal	Lower Limit	Upper Limit
Temperature of milk	is in range	35	75
pH of milk	is target = 6.5	5.5	6.5
Time of coagulation	Minimize	19	80
Milk clotting activity	Maximize	300	1263.15

Under the assumptions by Design Expert (version 13), the optimum operating conditions for minimum TOC and maximum MCA of enzyme were found to be 6.5 pH and 55°C of milk temperature. The responses for these optimum conditions reported MCA of 841.65 units at 30 s of coagulation time.

4.2.2 Verification of model

Within the scope of the variables investigated in Central Composite Design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. The conditions and the results of the confirmatory experiments are presented in Table 4.3.

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

Response	Conditions			Predicted value	Mean Observed value
	Enzyme concentration	Temperature of milk	pH of milk		
Time of Coagulation (TOC)	10	55	6.5	30.25	31.90
Milk Clotting Activity (MCA)	10	55	6.5	841.65	868.23

For the conformation of achieved optimal conditions, conformation tests were performed. The test results are given in Table 4.4 and it shows that the predicted results from the optimal conditions were similar to the results obtained through confirmation test.

4.2.3 Protein concentration, protease activity, specific activity, purification fold and percentage yield of kiwi protease

Crude and partially purified proteases were subjected to protease activity (PA) and protein determination. The results for PA were calculated using equation deduced from standard curve (Fig. D.1) and the results for protein concentration were calculated using equation deduced from standard curve (Fig. D.2). In kiwi fruit, 78 percent protease enzyme yield of 3.6 purification fold and 2.06 U/mg of protein specific activity was found with 50% ammonium sulphate fractionation which is in agreement with the data given by Sharma and Vaidya (2018). The results of protein concentration, protease activity, specific activity, purification fold and percentage yield are tabulated in Table 4.4.

Table 4.4 Protein concentration, protease activity, specific activity, purification fold and percentage yield of kiwi protease

Purification step	Protein (mg/ml)	Protease activity (U/ml)	Specific activity (U/mg)	Purification fold	% Yield
Crude enzyme	3.40	1.89	0.55	1	100
50% Ammonium sulphate precipitation	0.72	1.49	2.06	3.74	78

4.3 Physicochemical properties

4.3.1 Chemical composition of raw milk

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

Table 4.5 Proximate composition of raw cow milk

Parameters	Cow milk
Moisture (%)	87.3±0.16
Fat (%)	3.84±0.03
Protein (%)	3.44±0.04
Ash (%)	0.75±0.01
Acidity (% L.A.)	0.14±0.01
pH	6.7±0.1

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

The resulted presented in Table 4.5 revealed that the moisture, fat, protein, ash, acidity and pH in cow milk were 87.3%, 3.8%, 3.4%, 0.7%, 0.14% and 6.7 respectively. The values are similar to those reported by (Walstra *et al.*, 2006) and any variation may be due to cow breed, milking conditions and milking time.

4.3.2 Chemical composition of soft cheese

The chemical composition of the soft cheeses made from rennet and kiwi protease has been shown in Table 4.6.

Table 4.6 Chemical composition of rennet and kiwi protease soft cheeses

Parameters	Cheese samples	
	Rennet	Kiwi protease
Moisture (%)	48.63^{a}±0.06	52.07^{b}±0.14
Fat % (wb)	27.83^{b}±0.43	25.33^{a}±0.35
Protein % (wb)	19.46^{a}±0.02	19.18^{a}±0.02
Ash % (wb)	2.67^{a}±0.13	3.20^{b}±0.06
pH	5.67^{a}±0.005	6.45^{b}±0.01
Acidity (% lactic acid)	0.23^{b}±0.017	0.16^{a}±0.01
Calcium (mg/100 g)	627.43^{a}±1.22	637.33^{b}±2.44

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

The moisture percentage of kiwi protease cheese is in the line with the findings of Karki and Ojha (2018) and Kheir *et al.* (2011) but lower than the findings of Sharma and Vaidya (2018). For rennet cheese, The results were similar to Mijan *et al.* (2010) who reported moisture content of cows' milk cheese as 47.7% but higher than that of Islam (2006).

The variation in moisture might be due to the difference in milk composition, activity of coagulant and processing techniques. Between two cheeses the average moisture was more in kiwi protease cheese. 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 Maskey and Shrestha (2021). Significant differences ($P<0.05$) were observed between two samples regarding moisture content (Appendix G.1). Moisture content in soft cheese made by using kiwi protease was higher than that of rennet. Longer coagulation time for plant proteases results in more moisture retention in the final product which might be the reason for significant difference (Johnson *et al.*, 2001).

The fat level of kiwi protease cheese correlated with findings of Dimitreli *et al.* (2017) but slightly higher than the findings of Abdalla and Mohamed (2009) who found fat level to be 25.1%. For rennet cheese, fat level is similar with findings of Mijan *et al.* (2010) and Nawaz *et al.* (2011). The results were higher than the findings of Islam (2006) who found fat level to be 23.5%. Between two sample cheeses the average fat was highest in rennet cheese. The fat is one of the leading factors in determining the characteristic body, texture and flavor of cheese (Abd El-Gawad *et al.*, 2007). The lower value of fat content recorded in kiwi protease cheese because it took more time for coagulation as compared to rennet and higher fat loss during whey drainage. This may be responsible for the retention of less fat in the final product (Khan and Masud, 2013). Analysis of variance (Appendix G.2) regarding fat contents revealed that significant difference ($P<0.05$) was found between rennet and kiwi protease cheeses.

The protein content of both cheeses are in line with the findings of Mijan *et al.* (2010). Plant proteases are responsible for the formation of nitrogenous products of intermediate size, such as 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 *et al.*, 2004). The slight variation might be due to the cheesemaking 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 (Mijan *et al.*, 2010). Between two samples, the average protein content was more in rennet cheese. Analysis of variance (Appendix G.3) regarding protein contents revealed that non-significant difference ($P>0.05$) was recorded between two samples.

The ash contents of rennet cheese was similar to the findings of Mijan *et al.* (2010) but slightly lower than the findings of Kheir *et al.* (2011). The possible reason for the less ash contents may be the seasonal variation in the composition of milk. Between two samples the average ash contents was more in kiwi protease cheese. Analysis of variance (Appendix G.4) regarding ash contents revealed that significant difference ($P<0.05$) was found between two samples.

The pH of kiwi protease cheese was similar to the findings of Dimitreli *et al.* (2017). The pH of milk and milk products is measured to ensure the quality of foodstuff (Razzaq, 2003). The texture and firmness of soft cheese are greatly influenced by the pH (Phadungath, 2005). Between two samples the average pH was more in kiwi protease cheese. The possible variation may be due to the processing techniques, effect of coagulants, incubation and coagulation time and temperature. Analysis of variance (Appendix G.5) regarding pH revealed that there were revealed that significant difference ($P<0.05$) was found between two samples.

The titratable acidity of soft cheeses is similar to the findings of Nawaz *et al.* (2011) but lower than Mijan *et al.* (2010). The longer coagulation time of vegetable rennet possibly favoured microbial growth and consequently, a higher acidity was reached in curd from vegetable rennet (Kheir *et al.*, 2011). Analysis of variance (Appendix G.6) regarding titratable acidity revealed that significant difference ($P<0.05$) was found between two samples.

Between two samples, the calcium content was more in kiwi protease cheese. The calcium content of soft cheeses is similar to the findings of Karki and Ojha (2018). According to Fox *et al.* (2004), the reduction of calcium results in greater number of fat globules within the protein matrix. Analysis of variance (Appendix G.7) regarding calcium content revealed significant difference ($P<0.05$) between two samples.

4.3.3 Theoretical and actual yield

The theoretical and actual yield of soft cheeses A and B is presented in Appendix F.1 and shown in Fig. 4.7.

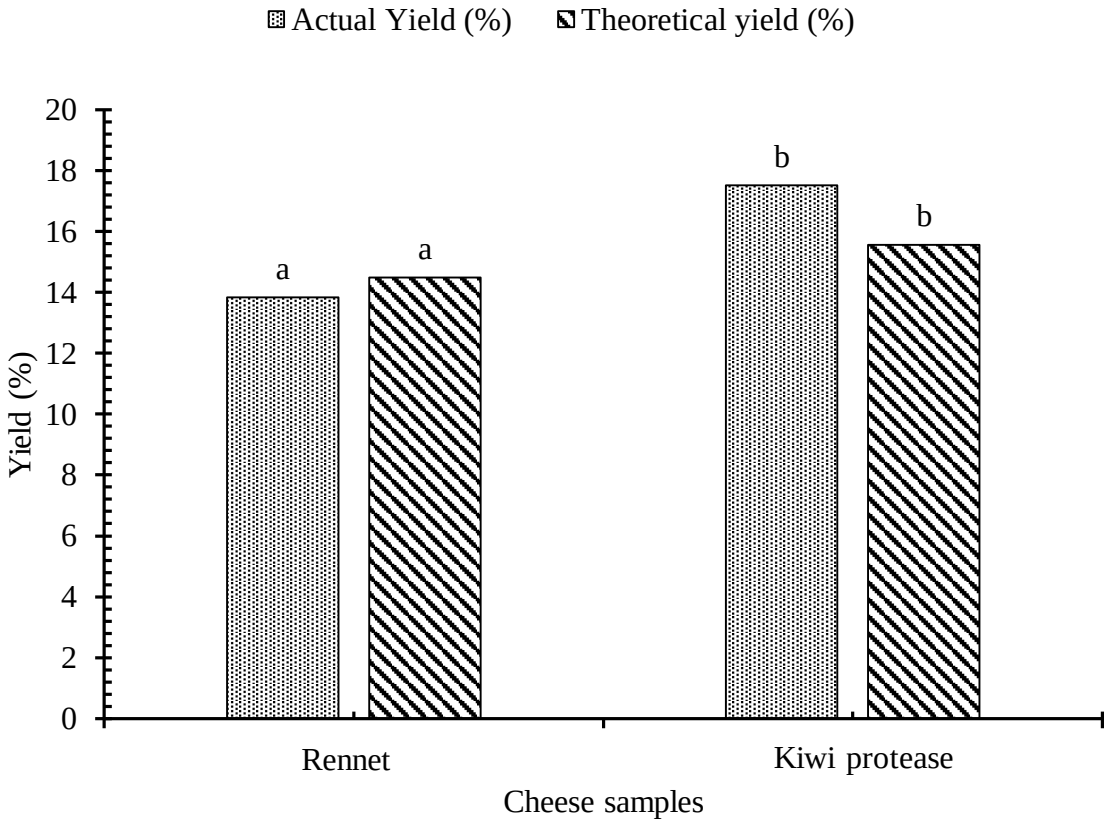

Fig. 4.7 Theoretical and actual yield of soft cheeses

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

Among both samples, the average theoretical yield was more in kiwi protease cheese. The slight variation in theoretical yield might be due to the moisture content in final cheese as same milk was used for both the samples. Analysis of variance Appendix G.8) regarding theoretical yield revealed that there was significant difference ($P<0.05$) among two samples.

Actual yield was lower than the theoretical yield. The yield reduction may be due to poor cheesemaking technique resulting in low casein and/or fat retention Mehaia (2006). Among both samples the average actual yield was more in kiwi protease cheese. The higher actual yield in cheese prepared with kiwi fruit protease may be attributed to the longer coagulation time resulting in more moisture content which increased the yield. Analysis of variance (Appendix G.9) regarding actual yield revealed that there was significant difference ($P<0.05$) among two samples.

4.4 Microbiological analysis

The result of microbiological analysis of soft cheeses are presented in Fig. 4.8 and in (Appendix F.2). It showed the average value for TPC and yeasts and molds of samples.

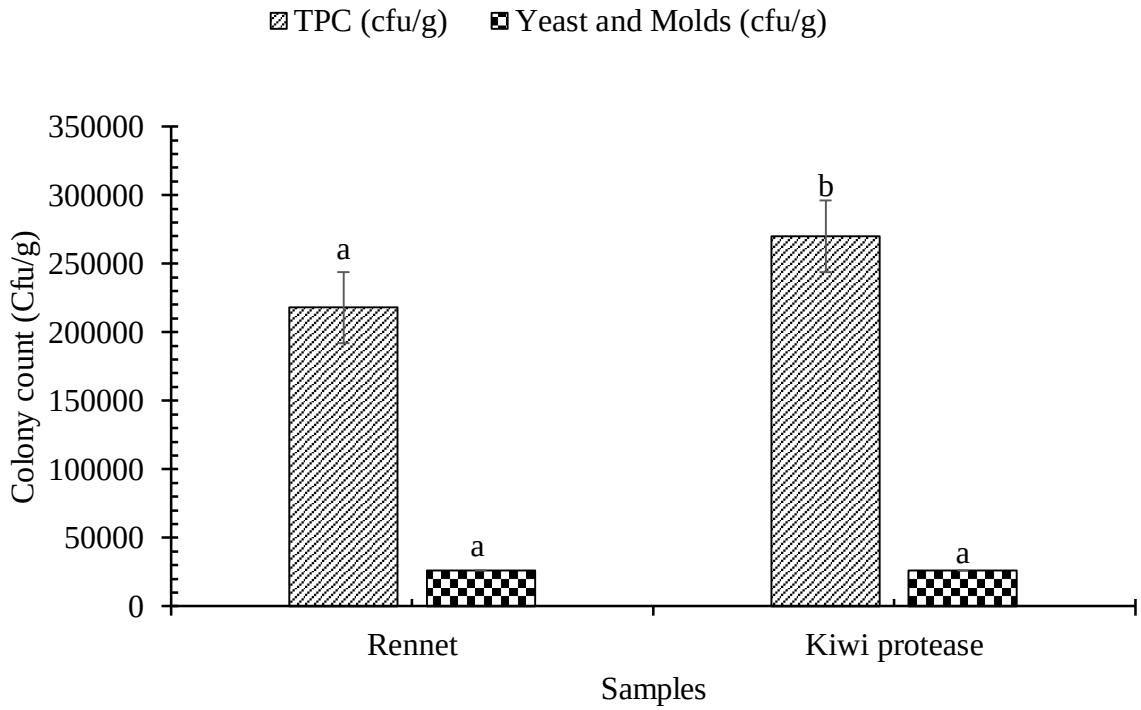

Fig. 4.8 Microbiology of soft cheese

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

Significant difference (P<0.05) was found for TPC (Appendix G.10) but non-significant difference (P>0.05) was found for yeast and molds count (Appendix G.11) of samples. Coliform were not detected in both samples. It might be due to heat treatment during cheesemaking. However, the presence of microbes was detected which might be due to handling contamination or oxygen trapped in the package (Robinson, 2002). The results were similar to Kheir *et al.* (2011).

4.5 Sensory evaluation of cheese

The sensory scores (5-point hedonic scales) of the soft cheeses are shown in (Appendix F.3). Graphical representation is given in Fig. 4.9.

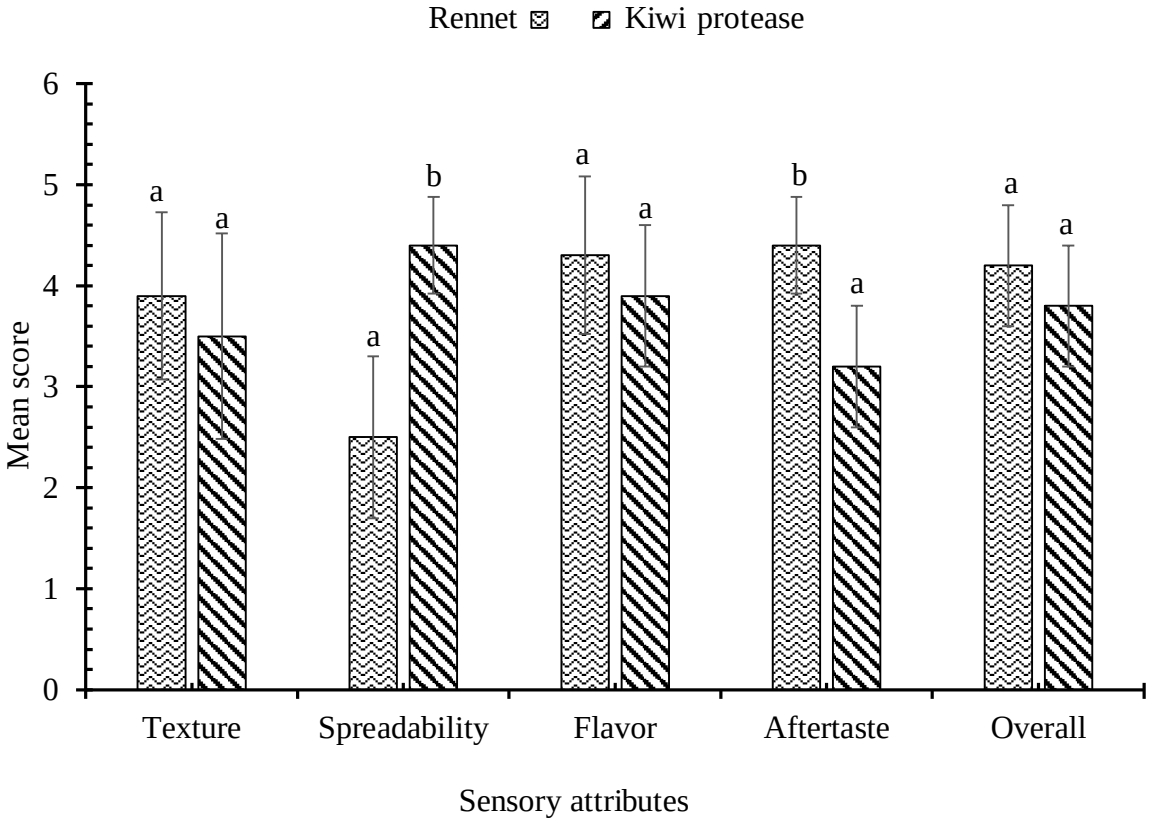

Fig. 4.9 Graphical view of mean sensory scores of soft cheeses.

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

In terms of texture the cheeses had similar scores where rennet cheese was found to be slightly better. As soft cheeses are meant for use as a spread, so its texture must naturally be smooth without any grainy feel. If the texture is not smooth then it is not soft cheese (Maskey

and Shrestha, 2020). Analysis of variance (Appendix G.12) regarding texture revealed that non-significant difference ($P>0.05$) between two samples.

Kiwi protease cheese had significantly higher score ($P<0.05$) for spreadability as compared to rennet cheese (Appendix F.2). The average spreadability was highest for kiwi protease cheese. This may be due to the variation in moisture content (Karki and Ojha, 2018). Analysis of variance (Appendix G.13) regarding spreadability showed that there was significant difference between two samples at 5% level of significance.

In terms of flavour the cheeses had similar scores where rennet cheese was found to be slightly better which is in agreement with Kheir *et al.* (2011) who reported that the flavour of cheese made with rennet scored higher compared to cheese with kiwi protease. Analysis of variance (Appendix G.14) regarding flavor revealed that non-significant difference ($P>0.05$) was observed between two samples.

The rennet cheese had higher score for aftertaste than kiwi protease cheese (Table F.2). Analysis of variance (Appendix G.15) regarding aftertaste showed that there was significant difference between two samples at 5% level of significance. A slight bitter taste was observed in kiwi protease cheese which may be the reason for low taste score. The 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 rennet cheese was slightly higher than that of kiwi protease cheese. Both cheeses were almost similar in texture and flavor. However, the rennet cheese became more acceptable in terms of aftertaste ($P<0.05$). Aftertaste is a very important attribute since it determines consumer preference. The results showed that kiwi protease cheese was slightly bitter which is in agreement with Roseiro *et al.* (2003) who reported that plant proteases are considered too proteolytic, leading to bitter flavour. With regard to spreadability, kiwi protease cheese was much better than rennet cheese as soft cheeses are meant for use as a spread which increases the acceptability of kiwi protease cheese. Analysis of variance (Appendix G.16) regarding overall acceptance revealed that there was non-significant difference ($P>0.05$) was recorded between two samples

Part V

Conclusions and recommendations

5.1 Conclusions

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

1. The optimum time of coagulation (TOC) and milk clotting activity (MCA) of partially purified kiwi protease were 841.65 units and 30 s respectively.
2. Numerical optimization study revealed that the optimum condition for maximum milk clotting activity and minimum time of coagulation for partially purified kiwi protease were pH 6.5, temperature 55ºC at enzyme concentration 0.5%.
3. The specific activity of partially purified kiwi protease increased from 0.55 U/mg to 2.06 U/mg, with degree of purity of 3.74 fold in comparison with crude kiwi protease and yield of 78%.
4. The physico-chemical analysis showed that there was non-significant difference ($P>0.05$) in protein among cheeses made using rennet and kiwi protease while significant differences were observed in moisture, fat, ash, calcium, acidity, pH and cheese yield.
5. Coliforms were not detected in both cheeses. Significant difference ($P<0.05$) in total plate count was observed in both cheeses while non-significant difference was found in the count of yeast and mold.
6. Cheese produced by using kiwi protease at optimised pH and temperature was found similar to rennet cheese in terms of all sensory attributes except spreadability and aftertaste.

5.2 Recommendations

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

1. Complete purification of kiwi protease using three phase partitioning system.
2. Use of other plant proteases such as bromelain, zingibain and calotropin to prepare soft cheese.

Part VI

Summary

The main objective of this research work is to extract and partially purify proteolytic enzyme from kiwi fruit (*Actinidia deliciosa*) to be used as milk coagulant in cheesemaking. The milk clotting activity (MCA), proteolytic activity (PA) and protein content of the partially purified protease were determined and the optimized conditions of the partially purified protease for maximum MCA and minimum TOC was determined using response surface methodology. The soft cheese thus obtained was compared with rennet cheese for physico-chemical, sensory and microbiological quality.

The present work showed that the partial purification of crude extract using ammonium sulphate fractionation at 50% saturation gives 78 percent protease yield of 3.74 purification fold and 2.06 U/mg of protein specific activity. The enzyme showed optimum temperature at 55°C and pH 6.5 which gives maximum milk clotting activity (841.65 U/ml) with minimum time of coagulation (30 s). Increase in the specific activity was observed in the partially purified enzyme than that of crude enzyme.

The physicochemical analysis showed that protein content of cheese samples was non-significantly different ($P>0.05$) but significant difference ($P<0.05$) was observed in number of parameters like moisture, fat, ash, pH, acidity and calcium in the cheese produced by kiwi protease compared to rennet cheese. Microbiological analysis showed that coliforms were not detected in both cheese samples. However, TPC of about 2.4×10^5 CFU/g and yeasts and molds count of 2.6×10^4 CFU/g was observed which may be due to handling contamination. Non-significant difference ($P>0.05$) in sensory attributes (texture, flavor and overall acceptability) were observed between kiwi protease and rennet cheeses.

This study indicate that partially purified kiwi protease can be used to produce soft cheese with a slightly bitter taste, but acceptable other sensory attributes. Therefore, kiwi fruit protease could be considered as promising alternative of natural calf rennet for the coagulation of milk and production of soft cheese.

References

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Appendices

Appendix A

Table A.1 Product responses by independent variables

Std. no.	Factor 1	Factor 2	Response 1	Response 2
	A: pH	B: Temperature (℃)	Time of Coagulation (s)	Milk Clotting Activity (MCA) U/ml
1	5.5	55	23	1043.47
2	6	55	27	888.88
3	6	35	70	342.85
4	6	55	26	923.07
5	6	55	29	827.58
6	6.5	35	80	300
7	5.5	75	19	1263.15
8	6	55	27	888.88
9	6	75	20	1200
10	5.5	35	65	369.23
11	6	55	28	857.14
12	6.5	55	37	648.64
13	6.5	75	21	1142.85

Appendix B

Table B.1 Model summary statistics for time of coagulation

Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	10.40	0.7937	0.7524	0.5885	2159.08	
2FI	10.75	0.8017	0.7357	0.1710	4349.78	
Quadratic	**1.63**	**0.9965**	**0.9939**	**0.9733**	**140.03**	**Suggested**
Cubic	1.14	0.9988	0.9970	0.9707	153.65	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	5228.15	5	1045.63	393.02	< 0.0001	significant
A-pH	160.17	1	160.17	60.20	0.0001	
B-Temperature	4004.17	1	4004.17	1505.04	< 0.0001	
AB	42.25	1	42.25	15.88	0.0053	
A^2	10.48	1	10.48	3.94	0.0875	
B^2	793.34	1	793.34	298.19	< 0.0001	
Residual	18.62	7	2.66			
Cor Total	5246.77	12				

Table B.3 Model summary statistics for milk clotting activity

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**82.90**	**0.9449**	**0.9339**	**0.8927**	**1.338E+05**	**Suggested**
2FI	86.96	0.9454	0.9272	0.7937	2.572E+05	
Quadratic	71.57	0.9712	0.9507	0.7454	3.175E+05	
Cubic	34.06	0.9953	0.9888	0.9419	72460.45	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	1.210E+06	3	4.032E+05	97.20	< 0.0001	significant
A-pH	56912.77	1	56912.77	13.72	0.0049	
B-Temperature	1.121E+06	1	1.121E+06	270.32	< 0.0001	
B^2	31382.11	1	31382.11	7.56	0.0225	
Residual	37335.39	9	4148.38			
Cor Total	1.247E+06	12				

Appendix C

Table C.1 Solutions of optimization result

Number	pH	Temperature	Time of coagulation	MCA	Desirability	
1	**6.500**	**55.401**	**30.245**	**841.519**	**0.785**	**Selected**
2	6.500	58.670	29.932	846.887	0.785	
3	6.500	58.011	30.706	833.708	0.785	
4	6.500	59.347	29.181	860.148	0.785	
5	6.500	59.560	28.950	864.311	0.784	
6	6.500	62.694	26.019	922.592	0.770	

Appendix D

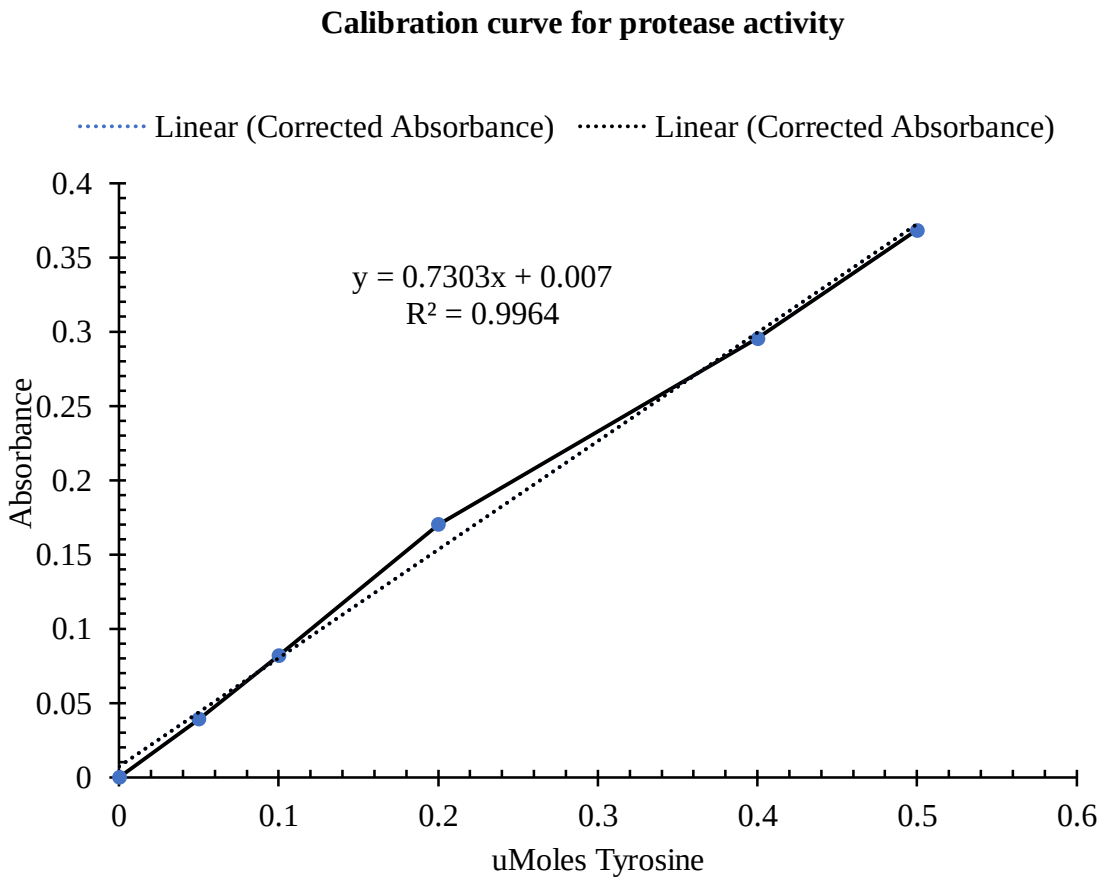


Fig. D.1 Standard curve of L-tyrosine for protease activity

Calibration curve for protein determination

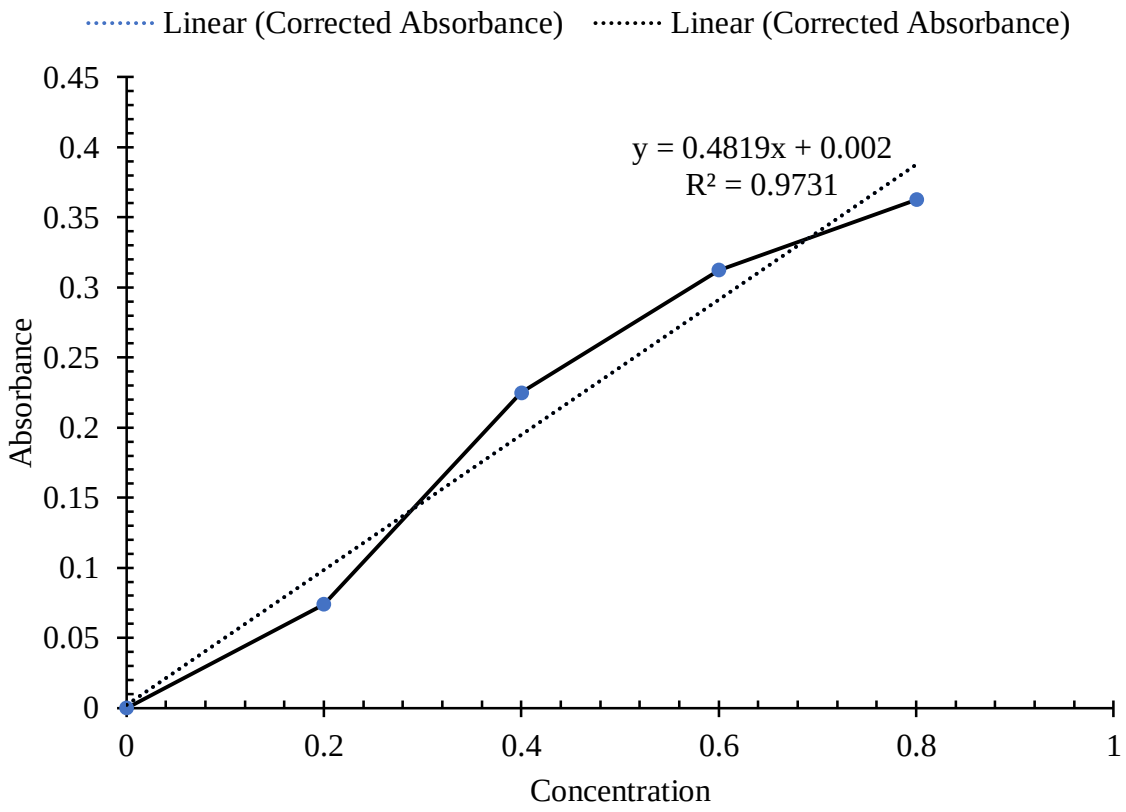


Fig. D.2 Standard curve of protein concentration for protein determination

Appendix E

Sensory Evaluation Card

Date: _____

Name: _____

Product: Soft 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					

Comments (if any) ……...

……

_____

Signature

Appendix F

Table F.1 Theoretical and actual yields of soft cheese

Source of Variation	Actual Yield	Theoretical Yield
A	$13.83^{a} \pm 0.05$	$14.48^{a} \pm 0.03$
B	$17.51^{b} \pm 0.04$	$15.56^{b} \pm 0.06$

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

Source of variation	Texture	Spreadability	Flavor	Aftertaste	Overall
A	$3.90^{a} \pm 0.83$	$2.50^{a} \pm 0.80$	$4.3^{a} \pm 0.78$	$4.40^{b} \pm 0.48$	$4.20^{a} \pm 0.6$
B	$3.50^{a} \pm 1.02$	$4.40^{b} \pm 0.48$	$3.9^{a} \pm 0.7$	$3.20^{a} \pm 0.6$	$3.80^{a} \pm 0.6$

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

Table F.3 Microbiological analysis of soft cheese

Sample	Coliform (CFU/g)	TPC (CFU/g)	Yeast & Mold (CFU/g)
A	ND	$218.67^{a} \pm 6.21$	$26.33^{a} \pm 3.05$
B	ND	$270^{b} \pm 6.01$	$26^{a} \pm 3.61$

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

Appendix G

Statistical analysis (T- test Tables)

Table G.1 T-test: Two-sample assuming unequal variances for moisture content

Variate: Moisture Content

	Variable 1	*Variable 2*
Mean	48.63333333	52.07333333
Variance	0.003333333	0.147433333
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-15.34498271	
P(T<=t) one-tail	0.002109995	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.004219989	
t Critical two-tail	4.30265273	

Table G.2 T-test: Two-sample assuming unequal variances for fat

Variate: Fat

	Variable 1	*Variable 2*
Mean	27.83333	25.33667
Variance	0.022033	0.015633
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	22.28141	
P(T<=t) one-tail	1.2E-05	
t Critical one-tail	2.131847	
P(T<=t) two-tail	2.4E-05	
t Critical two-tail	2.776445	

Table G.3 T-test: Two-sample assuming unequal variances for protein

Variate: Protein

	Variable 1	*Variable 2*
Mean	19.46667	19.18333
Variance	0.062433	0.002633
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	1.923885	
P(T<=t) one-tail	0.097134	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.194268	
t Critical two-tail	4.302653	

Table G.4 T-test: Two-sample assuming unequal variances for ash

Variate: Ash

	Variable 1	*Variable 2*
Mean	2.666667	3.206667
Variance	0.008233	0.064133
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-3.47684	
P(T<=t) one-tail	0.020073	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.040147	
t Critical two-tail	3.182446	

Table G.5 T-test: Two-sample assuming unequal variances for pH

Variate: pH

	Variable 1	*Variable 2*
Mean	5.676667	6.456667
Variance	0.002533	0.003633
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-17.204	
P(T<=t) one-tail	3.35E-05	
t Critical one-tail	2.131847	
P(T<=t) two-tail	6.7E-05	
t Critical two-tail	2.776445	

Table G.6 T-test: Two-sample assuming unequal variances for acidity

Variate: Acidity

	Variable 1	*Variable 2*
Mean	0.236666667	0.166666667
Variance	3.33333E-05	3.33333E-05
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	14.8492424	
P(T<=t) one-tail	5.98806E-05	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.000119761	
t Critical two-tail	2.776445105	

Table G.7 T-test: Two-sample assuming unequal variances for calcium

Variate: Calcium (mg/100g)

	Variable 1	*Variable 2*
Mean	627.4333	637.3333
Variance	12.20333	3.443333
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-4.33496	
P(T<=t) one-tail	0.011323	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.022646	
t Critical two-tail	3.182446	

Table G.8 T-test: Two-sample assuming unequal variances for theoretical yield

Variate: Theoretical yield

	Variable 1	*Variable 2*
Mean	14.48	15.56
Variance	4.73E-30	0
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-8.6E+14	
P(T<=t) one-tail	6.76E-31	
t Critical one-tail	2.919986	
P(T<=t) two-tail	1.35E-30	
t Critical two-tail	4.302653	

Table G.9 T-test: Two-sample assuming unequal variances for actual yield

Variate: Actual yield

	Variable 1	*Variable 2*
Mean	13.83	17.51
Variance	0.01	0.04
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	-28.5052	
P(T<=t) one-tail	4.74E-05	
t Critical one-tail	2.353363	
P(T<=t) two-tail	9.48E-05	
t Critical two-tail	3.182446	

Table G.10 T-test: Two-sample assuming unequal variances for TPC

Variate: TPC

	Variable 1	*Variable 2*
Mean	218.6666667	270
Variance	42.33333333	37
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-9.982337343	
P(T<=t) one-tail	0.000282932	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.000565863	
t Critical two-tail	2.776445105	

Table G.11 T-test: Two-sample assuming unequal variances for yeast and molds

Variate: Yeast and Molds

	Variable 1	*Variable 2*
Mean	26.33333	26
Variance	9.333333	31
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0.090909	
P(T<=t) one-tail	0.466647	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.933295	
t Critical two-tail	3.182446	

Table G.12 T-test: Two-sample assuming unequal variances for texture

Variate: Texture

	Variable 1	*Variable 2*
Mean	3.9	3.5
Variance	0.766667	1.166667
Observations	10	10
Hypothesized Mean Difference	0	
df	17	
t Stat	0.909718	
P(T<=t) one-tail	0.187845	
t Critical one-tail	1.739607	
P(T<=t) two-tail	0.375689	
t Critical two-tail	2.109816	

Table G.13 T-test: Two-sample assuming unequal variances for spreadability

Variate: Spreadability

	Variable 1	*Variable 2*
Mean	2.5	4.4
Variance	0.722222222	0.266667
Observations	10	10
Hypothesized Mean Difference	0	
df	15	
t Stat	-6.041987916	
P(T<=t) one-tail	1.12762E-05	
t Critical one-tail	1.753050356	
P(T<=t) two-tail	2.25525E-05	
t Critical two-tail	2.131449546	

Table G.14 T-test: Two-sample assuming unequal variances for flavor

Variate: Flavor

	Variable 1	*Variable 2*
Mean	4.3	3.9
Variance	0.67777778	0.544444444
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	1.14415511	
P(T<=t) one-tail	0.13377355	
t Critical one-tail	1.73406361	
P(T<=t) two-tail	0.26754709	
t Critical two-tail	2.10092204	

Table G.15 T-test: Two-sample assuming unequal variances for aftertaste

Variate: Aftertaste

	Variable 1	*Variable 2*
Mean	4.4	3.2
Variance	0.266667	0.4
Observations	10	10
Hypothesized Mean Difference	0	
df	17	
t Stat	4.64758	
P(T<=t) one-tail	0.000115	
t Critical one-tail	1.739607	
P(T<=t) two-tail	0.00023	
t Critical two-tail	2.109816	

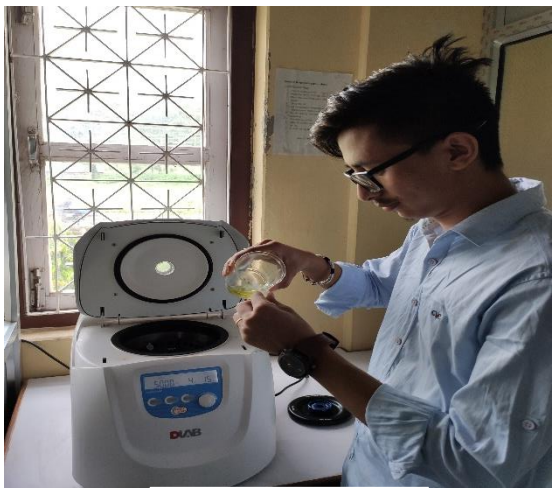
Table G.16 T-test: Two-sample assuming unequal variances for overall

Variate: Overall

	Variable 1	*Variable 2*
Mean	4.2	3.8
Variance	0.4	0.4
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	1.414214	
P(T<=t) one-tail	0.087182	
t Critical one-tail	1.734064	
P(T<=t) two-tail	0.174363	
t Critical two-tail	2.100922	

Color plates

P1 Protease extraction



P2 Centrifugation

P3 Pellets after centrifuge

P4 Clotting of milk in vials

P5 Performing proteolytic activity

P6 Cheese curd

P7 Cheese after pressing

P8 Sensory analysis of cheese