COMPARISON OF MILKCLOTTING CHARACTERISTICS OF CRUDE KIWIFRUIT (*Actinidia deliciosa*) AND GINGER (*Zingiber officinale*) PROTEASES IN SOFT CHEESEMAKING

by

Bibek Shrestha

Department of Food Technology

Central Campus of Technology

Institute of Science and Technology

Tribhuvan University, Nepal

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

by

Bibek Shrestha

Department of Food Technology

Central Campus of Technology

Institute of Science and Technology

Tribhuvan University, Nepal

2021

Tribhuvan University

Institute of Science and Technology

Department of Food Technology

Central Campus of Technology, Dharan

Approval Letter

This ***dissertation*** entitled ***Comparison of Milkclotting Characteristics of Crude Kiwifruit (Actinidia deliciosa) and Ginger (Zingiber officinale) Proteases in Soft Cheesemaking*** presented by **Bibek Shrestha** has been accepted as the partial fulfilment of the requirement for the **B. Tech. degree** in **Food Technology.**

Dissertation Committee

1. **Head of Department** _____

   Mr. Navin Gautam, Asst. Prof.

2. **External Examiner** _____

   Dr. Dhan Bahadur Karki, Prof.

3. **Supervisor** _____

   Mr. Bunty Maskey, Asst. Prof.

4. **Internal Examiner** _____

   Mr. Basanta Kumar Rai, Prof.

2022

Acknowledgements

I would like to express my heartfelt gratitude to my respected guide Asst. Prof. Mr. Bunty Maskey for his kind support, excellent guidance, encouragement and constructive recommendations throughout the work.

I am really thankful to Assoc. Prof. Dr. Dil Kumar Limbu (Campus Chief, Central Campus of Technology) and Asst. Prof. Navin Gautam (Chairperson, Central Campus of Technology) for providing opportunity and making available all the facilities to carry out the best in the dissertation work.

I cannot stay from thanking all my classmates for their help in preparing me for this through all those experience and memories which will never be forgotten in my whole life. Special thanks must be bestowed to my senior Mr. Nabindra Kumar Shrestha for all the help and support he has shown throughout my work. I also want to thank my friends Ashish Koirala, Sharad Bhattarai, Manish Dahal, Sumitra Shrestha, Santosh Thapa, Raju Subba, Nishan Rai and all my beloved juniors who assisted me directly or indirectly throughout the work. I am also grateful to all my teachers, seniors, junior, staffs of library and laboratory for their co-operations and suggestions.

No words in this world can ever express the love, encouragement, inspiration and moral support given by my parents and family members during my tough days.

Date of submission: 2022

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Bibek Shrestha

Abstract

The main objective of this dissertation work was to perform comparative study on the milkclotting characteristics of crude kiwifruit *(Actinidia deliciosa)* and ginger *(Zingiber officinale)* proteases in soft cheesemaking. The effect of crude kiwifruit and ginger protease on physicochemical and microbial characteristics of soft cheese prepared were examined. Temperature and pH of the milk on milk clotting activity (MCA) and time of coagulation (TOC) were optimized by response surface methodology (RSM). The protease activity and protein concentration of the crude kiwifruit and ginger protease were also measured in optimized conditions. Finally, the cheese prepared by using crude kiwifruit and ginger protease were compared with rennet cheese for physicochemical and microbial properties.

Numerical optimization study revealed that optimum condition of milk clotting for kiwifruit and ginger were found to be 61°C (at pH 6.5) and 55°C (at pH 5.5) respectively. The optimized TOC and MCA for crude kiwifruit protease were found to be 120.513 s and 492.630 units respectively. Similarly, TOC and MCA for crude ginger protease were 1591.667 s and 241.988 units respectively. The physico-chemical analysis showed that there was non-significant difference ($P>0.05$) in protein content among cheeses made using rennet and crude plant proteases while significant difference ($P<0.05$) was observed in moisture, fat, ash, calcium, acidity, pH and cheese yield. Microbiological analysis showed the absence of coliform in all three cheeses. Similarly, significant difference ($P<0.05$) was observed in the total plate count (TPC) of all three samples while non-significant difference ($P>0.05$) was found in the count of yeast and mold.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
BSE	Bovine spongiform Encephalopathy
CBS	Customized Brewing Solutions
CCP	Colloidal Calcium Phosphate
DANIDA	Danish International Development Agency
DDC	Dairy Development Corporation
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization
FDB	Fat on Dry Basis
FDM	Fat on Dry Matter
HTST	High Temperature Short Time
IDF	International Dairy Federation
LSD	Least Significant Difference
LTLT	Low Temperature Long Time
MCA	Milk Clotting Activity
MFFB	Moisture on Fat Free Basis
MFFS	Moisture in Fat Free Substance
MT	Metric Tonnes

NDDB	Nepal Dairy Development Board
NSLAB	Non-Starter Lactic Acid Bacteria
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PPM	Parts Per Million
RCT	Rennet Coagulation Time
RO	Reverse Osmosis
SCN	Thiocyanate
TOC	Time of Coagulation
TPC	Total Plate Count
UF	Ultrafiltration
VRBA	Violet Red Bile Agar
WFFS	Water in Fat Free Substance
WHO	World Health Organization

Part I

Introduction

1.1 General introduction

Cheese has been defined as a product made from milk by coagulating the casein with rennet or 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 is a solid or semi-solid fresh or matured product produced by processing techniques involving the coagulation of milk and/or milk-based materials (provided that the whey protein/casein ratio does not exceed that of milk) and which produces an end product having physical, chemical and organoleptic properties similar to those of the product mentioned above (Upadhyay, 2003).

The most popular milk coagulating enzyme is calf rennet, which is present in the stomach of infant bovine animals (Ishak *et al.*, 2006). World cheese production has doubled in less than 25 years from 1961-1994 (Farkye, 2004). In 2020, global cheese production amounted to about 21.69 million metric tons. Similarly, in 2018 and 2019 it amounted to 20.72 and 20.98 million metric ton (Shahbandeh, 2021).

Based on the catalytic mechanism used during the hydrolytic process, plant proteases are divided into different classes. Aspartic, serine, and cysteine proteases are the main classes of milk-clotting proteases (Mamo and Assefa, 2018). The enzyme number and type varies from species to species and depends on the part of the same plant itself. Because of their easy accessibility and quick purification processes, plant proteases have become the subject of increasing interest in the cheese industry. Furthermore, the use of plant proteases in cheese manufacturing promotes the greater acceptability by the vegetarians and may improve their nutritional intake. For several years, plant extracts have been widely used in the preparation of various types of artisanal cheeses which are mainly produced in Mediterranean countries, Southern Europe, and West Africa (Ben Amira *et al.*, 2017). Several researches on plant proteases have been conducted: papaya (papain) (Maskey and Shrestha, 2020), kiwifruit (actinidin) (Karki and Ojha, 2018), ginger (zingibain) (Nafi *et al.*, 2014), artichoke (cardosin) and common fig (ficin) (Liburdi *et al.*, 2019), sunflower

and albizia seeds (Egito *et al.*, 2007a), thistle flower (Folgado and Abranches, 2020) and so on.

Actinidin is a cysteine protease found in the fruit in kiwifruit (McDowall, 1970). Kiwifruit accumulates actinidin to very high concentrations, where it constitutes up to 60% of soluble protein in the fruit (Paul *et al.*, 1995). The amount of enzyme is greater in quantity with a higher level of activity in ripe kiwi, which represents a potential use for overripe and discarded fruit (Karki and Ojha, 2018). Similarly, zingibain is the protease found in ginger. It exhibits remarkable proteolytic activity as well as good milk clotting activity (Nafi *et al.*, 2014).

1.2 Statement of the problem

The world wide increase in cheese market, unmet demands and increase in rennet prices has led us in search of possible rennet substitue (Ben Amira *et al.*, 2017). Moreover, factors like vegetarianism, religious beliefs and have drawn a great deal of attention to the use of microbial coagulants and coagulants extracted from plants as a new substitute of rennet (Chazzara *et al.*, 2007).

The drastic rise in the cheese consumption with the declination of rennet production has led to an increase in the price of calf rennet, a shortage of rennet cheese and the search for alternative milk coagulants. In addition, the use of calf rennet for cheese is absolutely opposed by some religions and beliefs. A suitable cheap non-rennet coagulating agent has to be developed to solve this issue, which could help cheese makers as well as consumers (Maskey and Shrestha, 2020). Various researches have been done but there are still some gaps left to be filled for an effective solution of the above problem. So, more research should be done for the advancement and flourishment of the cheese industry.

1.3 Objectives

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

1.3.1 General objective

The general objective of the dissertation work is to compare the milkclotting characteristics of crude kiwifruit (*Actinidia deliciosa*) and ginger (*Zingiber officinale*) proteases in soft cheesemaking.

1.3.2 Specific objectives

- To extract proteolytic enzyme from kiwifruit and ginger to be used as a coagulating agent in cheese preparation.
- To determine time of coagulation (TOC) and milkclotting activity (MCA) of protease from kiwifruit and ginger.
- To determine protease activity and protein content of the protease from kiwifruit and ginger.
- To study physicochemical properties of cheese prepared from crude kiwifruit and ginger proteases.
- To study the microbial quality of cheese prepared by crude kiwifruit and ginger proteases.
- To compare the cheese made by crude kiwifruit and ginger proteases with rennet cheese.

1.4 Significance of the study

Cheese is a ripened or unripened product made by coagulation of the proteins in milk through the action of rennet or another coagulant (IDF, 2021). The conversion of milk to cheese curd is generally made through enzymatic coagulation and rennet is being used for this purpose since ancient times (Moschopoulou, 2011). The worldwide increase of cheese production, coupled with reduced supply and increasing prices of calf rennet, has led to a search for alternative substitutes (Maskey and Shrestha, 2020). Microbial rennet are being used as substitutes but they are hard to produce and may lead to off-flavour generation with low cheese yield and quality (Liburdi *et al.*, 2019). Moreover, many microorganisms responsible for the production of microbial rennet are known to be pathogenic (Garg and Johri, 1994).

The present study is for the potential existence of proteolytic enzymes from plants with promising industrial application and excellent milk coagulating function. The work will be focused on the study of enzymes extracted from kiwifruit and ginger and their use in cheese preparation. This study is also done to bring a potential use of the overripe and

excess plant materials which can be used as crude enzyme source and ultimately for cheese preparation.

1.5 Limitations of the study

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

- Purification of the crude enzyme was not carried out.
- Storage stability of crude protease was not performed.
- Casein hydrolysis pattern of crude proteases was not performed.
- Sensory analysis was not performed.

Part II

Literature review

2.1 History and development of cheese

Early records suggest that foods such as cheese and bread in the 'Fertile Crescent' between the Euphrates and Tigris rivers in Iraq were staple foods (6000-7000 BC). Cheese is also referred in this region as the cradle of civilization (Upadhyay, 2003). The origins of cheese were lost in ancient times, but most certainly, milk was contaminated with lactic acid bacteria, which created conditions that were unfavorable for the growth of other bacteria by milk acidification (Jhonson *et al.*, 2001). During that time, cheese making served as a way to preserve milk while providing nourishment to lactose-intolerant people. Soon, people found that the flavor grew more intense the more days it sat around; different species of animal gave different tasting cheese; and adding salt preserved it longer. It took until nineteenth century for anyone to realize the chemistry behind this, but that didn't stop people from creating and experimenting on cheese (Tunick, 2014).

Since animal skin bags were a convenient way of strong liquids for nomadic tribes, the surplus to daily needs would have been carried in such skin bags (Scott, 1986). Under such circumstances, milk would interact with coagulating enzymes (rennet) from the stomach tissue, leading to the coagulation during storage. Unfortunately, milk is also a rich source of nutrients for bacteria, some species of which utilize milk sugar, lactose, as a source of energy producing lactic acid as a byproduct. When sufficient acid has been produced, the milk protein coagulates at the isoelectric point of casein to form a gel entrapping the fat (Fox, 1987). The acid curds would have been broken up by swaying animals during journeys, to produce curds and whey (Scott, 1986). It would have been realized quickly that the whey made a pleasant refreshing drink for immediate consumption while the curd could be consumed fresh or stored for future (Fox, 1987). The shelf life of the curd could be greatly extended by dehydration and salting. This activity gave rise to the evolution of cheese. The rennet curd can be converted into more stable curds (due to low moisture) than acid curds. Thus, rennet coagulation has become predominant in cheese manufacture (Fox and McSweeney, 2004).

Over several thousand years, cheese making has advanced from an art to near science. Cheese varieties have proliferated to suit varied conditions and requirements. It is estimated that more than 2000 varieties exist and the list may still be growing. It is possible, however, to classify the cheeses as hard and soft. 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).

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

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

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

Source: Scott (1986)

2.1.1 Scientific and technological developments

Key scientific and technological developments that have taken place during the last 100 years contributed tremendously to the growth of cheese industry. According to Johnson (2017), the key developments are given as follows:

- Isolation and characterization of the principal constituents of milk, especially those involved in cheese making.
- Refrigeration, use of commercial starters, and the use of pasteurized milk for cheese making.
- Fundamental research on the genetics of starter bacteria greatly increased the reliability of fermentation, which in turn made automation feasible.

- Membrane concentration and separation of milk offered a solution and greatly enhanced plant capacity.
- Bacterial removal systems: These systems remove bacterial spores, including spores of Clostridia and *Bacillus* that cause gassy, slitty cheese and produce off-flavors.
- Elucidation of the mechanism of coagulation i.e. conversion of liquid milk to gel.
- Development in cheese packaging technology for more storage and shelf life.
- Studies on the safety and public health aspects of cheese.
- Characterization of the maturation of cheese-biochemistry, microbiology, flavor, texture, functionality.
- Search and development of rennet substitute and starters. (a) Fungal rennet derived from *Rhizomucor miehei* (e.g. Hannilase, Marzyme, Rennilase, Fromase, etc.), *Rhizomucor pusillus* and (b) Fermentation derived chymosin from microorganisms by application of DNA recombination technology.
- Development of technology for production of enzyme modified cheeses and cheese analogues.

2.2 Cheese production

In 2020, global cheese production amounted to about 21.69 million MT. Similarly, in 2018 and 2019 it amounted to 20.72 and 20.98 million MT (Shahbandeh, 2021). This production was spread across six continents. Home-made and farmstead were not included as they do not appear in national statistics (Kindstedt, 2017).

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 Lamtang 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 Commission was formed (Khanal *et al.*, 2019). A number of

small-scale cheeses makers-initiated productions of Swiss Emmental and French Cantal type cheeses in Langtang and Ilam. In Nepal, there are 15 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) to the United States, Britain, Canada and Japan which is commonly referred to as "dog chew" (Khanal *et al.*, 2019).

2.3 Varieties and classification

Cheese manufacture is essentially a dehydration process in which the fat and casein of milk are concentrated 6-10-fold, depending on cheese variety (Kheir *et al.*, 2011). There are more than 1400 kinds of cheeses that are listed in a record of the University of Wisconsin Centre for Dairy Research (Khanal *et al.*, 2019). There are 18 common types of cheese and they are: Brick, Trappist, Camembert, Neufchatel, Roquefort, Edam, Gouda, Sapsago, Hand, Cheddar, Limburger, Provolone, Cottage, Cream, Parmesan, Romano, Swiss and Whey cheese (McSweeney *et al.*, 2017). Cheese types vary in taste, flavor, texture due to production methods, ripening period and ripening conditions (Owni and Sana, 2009).

According to Khanal *et al.* (2019), there are different schemes of cheese classification and they are: (i) classification scheme is based on texture (ii) classification scheme based on method of coagulation (iii), classification based on ripening indices. Under aforementioned scheme, there are several classes of cheese and they are:

- Extra hard verities (Grana Padano, Granone Lodigiano, Parmigiano Reggiano, Asiago, Bagozzo, etc.)
- Cheddar and related varieties (The British Territorial varieties, Cheshire, Derby, Gloucester, and Leicester, are dry-salted cheeses)
- Cheese with propionic acid fermentation (Semi-hard cheeses such as Maasdamer, Leerdamer, and Jarlsberg. Gruyère etc.)
- Gouda and related varieties (Edam, Maribo, Danbo, Colonia, Hollanda, Norvegia, and Svecia).
- Pasta filata cheese (Mozzarella, Cascaval, Kashkaval, Provolone, Kasseri, and Kasarpeyniri).

- Cheese ripened under brine (Feta, Domiati, Brinza, Beli Sir,etc.).
- Blue cheese [fermented by P. roqueforti, Bleu d'Auvergne, Cabrales, Gorgonzola, Danablu (Danish Blue), and Stilton]
- Sheeps', Goats' and Buffalo milk cheese.
- Acid curd cheese (Cream, Cottage, Quarg, some Queso Blanco

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

Table 2.2 Classification of cheese according to Codex Alimentarious

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

Source: Scott (1986)

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

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

Yak 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

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 (Hill *et al.*, 1982). Very-low-fat versions are also available. However, a surface-mold-ripened cheese, such as Brie or Camembert, contains a high proportion of fat and protein and less water (Buttriss, 2003).

The different types of soft cheese present are shown in the Table 2.3.

Table 2.3 Types of soft cheese

Soft Cheese (50-80% moisture)	Examples
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: Vedumuthu and Washam (1983)

Cottage cheese is a high-moisture, unripened soft cheese, consisting of individual grains (3–12 mm), relatively uniform in size. Although the specific origin of this cheese is unknown, the name "Cottage" implies that this cheese is originally produced on family farms (McKevith and Shortt, 2003; Tratnik *et al.*, 2001). It may be available in low-fat and nonfat forms; while regular cottage cheese only has 4% of fat by weight, low-fat cottage cheese has only 2% (Kongo and Malcata, 2016).

2.4.1 History of Soft cheese

Records indicate that soft cheeses have been consumed as far back as in ancient Rome and Greece; Europe however, is widely regarded as the birth place of the first soft, creamy cheese (Mahalo, 2007). Further documents indicate the existence of soft cheese from as early as the 1650's (Christensen, 2011).

In the late nineteenth century, around 1870–1880, post-colonial farmers in the USA started cream cheese production. In 1875, large scale production was done by Lawrence's factory (Pombo, 2020). Similarly, industrial production of cottage soft cheese started approximately in 1915 in USA and until today, different ways of production have been developed (Tratnik *et al.*, 2001).

2.4.2 Uses of Soft cheese

Soft cheese has long been considered an ideal source of protein for those on weight loss diets because it can be made in a variety of low-fat forms, it is inexpensive, and it can be used in a multitude of recipes. Billions of pounds of cottage cheese are consumed each year in the United States, probably as a result of a public's perception of cottage cheese as a health food (Kongo and Malcata, 2016).

2.5 Quality of milk in relation to cheese making

Quality of milk for cheese making may be defined as its stability to give a good cheese, under normal working conditions and with a satisfactory yield (Bylund, 2003). Milk is the most versatile of all the animals desired food commodities (Patel and Gupta, 1986). It is a starting material for cheese making and exerts a decisive influence on the course of manufacture and effects yield and quality of resultant cheese. The changes in the quality of raw milk could influence cheese making, entailing modification in the existing process of manufacture or may disrupt normal manufacturing operations or may give altered product yield, composition and quality. Hence, there is a need to use high quality milk for cheese manufacture (Upadhyay, 2003). The milk quality for cheese production is influenced by factors such as milk composition, abnormal milk, microbial quality of milk and many other factors (Nassar *et al.*, 2015).

2.5.1 The amount and composition of milk constituents

Milk composition varies depending on animal-related factors (breed, milking, lactation, gestation stage, age, parity, nutritional/health status); husbandry-related factors (milking intervals, feeding, herd size, physical activity); and environmental factors (season, temperature, weather) (Salas *et al.*, 2019). Cheese made of buffalo milk has higher fat, protein, ash and total solids than cheese made of cow milk (Mijan *et al.*, 2010). The composition of milk is closely linked to yield and properties of resultant cheese. The milk constituents of importance to cheese making are milk proteins (i.e. casein), milk fat and minerals salts particularly calcium (Scott, 1986).

2.5.1.1 Milk protein (casein) and its effect

Casein has been defined as a heterogeneous group of phosphoprotein precipitated from raw skim milk at pH 4.6 and 20°C. Casein comprises about 79 % of total milk protein and

bovine milk ranges from 2.1 to 3.0 % casein. The principle casein fractions are α_{S1}, α_{S2}, β and κ-casein. The distinguishing property of all caseins is their low solubility at pH 4.6. The common compositional factor in caseins is that they are conjugated proteins, most with phosphate groups esterified to serine residues (Walsh, 1999).

There is a linear relationship between casein content and cheese yield during cheese making (Metz *et al.*, 2001). Cheese yield quality, curd firmness and moisture retention are affected by the casein portion of milk. Milk with a low casein content, give lower cheese yield than milk with richer in casein (Nassar *et al.*, 2015).

2.5.1.2 Milk fat

In most cheese varieties, fat is a major compositional variable and major changes in its level result in concomitant 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, biochemistry, microstructure, yield, rheological and textural properties, cooking properties, cheese texture and taste (El-Gawad *et al.*, 2007). Cheese made with no fat or low-fat milk has problem of moisture retention and it dried out fat to give a hard, dry body (Pradhan, 2000).

Milk fat content is also directly correlated with cheese yield, as fat and casein make up >90% of total cheese solids. The cheese yield per kg of fat used decreased with an increase in milk fat content as high fat milk typically produces less fatty casein than milk rich in fat (Upadhyay, 2003). Cheese with high fat content is less firm and elastic while cheese with low fat content is harder and less smooth due to the increase in cross linking between the curd (Nassar *et al.*, 2015).

2.5.1.3 Milk salt

Milk salts or ash contain a large proportion of the metallic and non-metallic components. The calcium content (colloidal, soluble and ionic) of milk greatly influences the firmness of the curd and together with phosphate is important for the drainage of whey. The level of Ca^{++} retained in the curd also influences the body and textural characteristics of the cheese. Normal milk contains adequate amount of calcium which is needed for proper coagulation of milk by rennet. Variations in concentration of calcium as well as magnesium, phosphates, citrates, sodium have a direct influence on rennet clotting of milk. High

soluble phosphate, citrates and sodium, and low soluble Ca^{++} and magnesium, and low proportion of casein bound Ca^{++} have been found to give slow coagulation of milk by rennet (Kindstedt and Kosikwski, 1985).

2.5.2 Abnormal milk

The general term used to describe any type of milk that usually includes mastitis milk, colostrum and late lactation milk, which differs significantly from normal milk, is abnormal milk. Irregular milk is unsuitable for cheese making due to factors or conditions that result in 'weak starter', slow coagulation with rennet and thin curd formation. Abnormal enzyme content can affect both ripening and the development of flavor of cheese (De, 2000).

2.5.3 Changes in the milk after production

The changes in concentration of casein, calcium and phosphorus in the soluble phase of milk stored at 4^oC storage are shown in the Table 2.4.

Table 2.4 Changes in concentration of casein, calcium and phosphorus in the soluble phase of milk stored at 4^oC storage

Days	Casein fraction (mg/ml)			Minerals (ppm)	
	α_{S1}	β	γ	Ca^{++} (ppm)	P (ppm)
0	1.66	1.32	0.87	430	395
1	1.73	1.56	0.96	485	407
2	1.80	1.81	0.98	528	426
3	1.68	1.51	0.96	506	418
4	1.71	1.67	0.90	519	420
5	1.66	1.53	0.88	489	401
6	1.74	1.59	0.93	492	405
7	1.72	1.56	0.86	487	401

Source: Upadhyay (2003)

Milk is highly perishable and is subjected to physical, chemical, bacteriological and organoleptic production. Milk may have taints after processing, such as feed flavor, marijuana flavor, etc. that are naturally present in the milk. Thus, milk can display off-flavor growth, acid production, lipolysis and proteolysis, changes in casein micelles and salt balance and oxidation after production (Upadhyay, 2003) changes during the time between the production of milk and the beginning of cheese.

2.5.4 Inhibitory substances

Raw milk is known to contain natural inhibitory systems. In addition, milk may also contain residual antibiotics, detergents and sanitizers (Walstra *et al.*, 2006).

- In raw milk, the natural inhibitory mechanism involves immunoglobulin, lactoferrin, lysozyme and the peroxide system of lactoperoxidase-thiocyanate-hydrogen. These systems have been shown to be active against the multiplication and acid development of lactic *streptococci*. Under aerobic conditions, some strains of lactic acid bacteria can create ample hydrogen peroxide and self-inhibitory effects can be observed during cheese making (FAO, 1999).
- The presence of residual antibiotics in milk is highly unsatisfactory both from public health point of view and because their presence can give rise to inhibition of cheese starter, which in turn, can lead to several faults, slow whey drainage, high moisture in cheese, early and late blowing, weak and pasty body, various types of taints, cracks, open texture and sponginess (Farkye, 2004).
- Detergents and sanitizers are used for a wide range of applications at production farm, at a chilling center and at dairy factory. When applied by ‘Good practice’ they cause no residue problems in milk. However, their misuse possesses major problem (Walstra *et al.*, 2006).

2.5.5 Microbiological quality of milk

Raw milk is a complete food, which contains protein, fat, sugars, vitamins and minerals. Even though, raw milk is sterile during secretion, contamination of milk by microorganisms can take place during milk handling, storage and other pre-processing activities (De-Silva *et al.*, 2016). For cheese making, low count milk as free as possible from fault producing microorganisms is highly desirable. In addition, for public health

reasons milk must be free from pathogens and also from organisms which produce toxins involved in food poisoning (Upadhyay, 2003).

2.6 Pre-treatments of milk for cheese making

For centuries, milk for cheese-making was subjected to no pre-treatment before curdling, and many cheese varieties worldwide are still made from raw milk, particularly, but not exclusively, artisanal cheeses. However, predominantly for reasons of safety, but also for the consistency of quality, and manipulation of product characteristics, most cheese-making today involves the treatment of milk by one or more processing steps prior to addition of coagulant and starter culture (Kelly *et al.*, 2008). They have profound effect on cheese manufacturing schedule, cheese making efficiency, physio-chemical, microbiological and organoleptic characteristics of cheese and shelf life (Walstra *et al.*, 2006).

2.6.1 Chilling and cold storage

Most raw milk in developed dairy countries is cooled to 4°C and stored on the farm in refrigeration bulk tanks or in insulated or refrigerated storage tanks in cheese plants prior to its conversion into cheese. Such practice of hold in cooled raw and /or processed milk for extended period not only increases the possibility of growth of psychrotrophs, but also modifies the physiochemical status of milk components, particularly casein and minerals (Upadhyay, 2003).

2.6.2 Carbon dioxide treatment

Carbon dioxide (CO_2) can be used as a treatment for milk for preservation and technological reasons, due to its solubility in milk and inhibitory effect against a broad spectrum of micro-organisms. It has been shown that addition of CO_2 to raw milk decreased proteolysis, due to effects on both microbial growth and concomitant protease production and inhibited plasmin activity due to pH reduction; lipolysis was also retarded, probably due to reduced microbial growth. Milk treated with CO_2 has lower whey pH at drainage, shorter total make time, and altered yield due to the increased losses of calcium and fat, and increased salt retention (Kelly *et al.*, 2008).

2.6.3 High pressure treatment

High pressure treatment principally exerts effects on macromolecules with complex structures, changing their structure and properties, which intern cause various changes. Rennet coagulation time, for example, may be reduced by treatment at 100–300 MPa. Studies showed that HP treatment increased wet curd yield, by up to 25% after treatment at 600–800 MPa and increased moisture retention. It has been found that HP treatment can modulate the negative effects of excessive heat treatment on cheese-making properties of milk (Kelly *et al.*, 2008).

2.6.4 Bactofugation

Bactofugation normally separates the milk into a fraction that is more or less free from bacteria, and a concentrate (bactofugate). It has principally been used in the cheese industry where its high-cleaning capabilities have been used to remove spores from cheese milk that could cause latent fermentation in semi-hard cheeses (Faccia *et al.*, 2013).

2.6.5 Standardization

Standardisation can remove any major imbalance in milk composition and thereby assist in maintaining the uniform quality of a cheese (Phelan, 2007). Since fat and protein are the two components that constitute the main body of cheese, it is reasonable to standardize the milk at the ratio of fat/protein, which gives good quality of cheese and uniform quality of production during all seasons (Scott, 1986).

2.6.6 Pasteurization

To destroy all pathogenic and harmful microorganisms and to inactivate the phosphatase and xanthine oxidase enzymes present in the milk, pasteurization of milk is performed. By insolubilizing part of the serum protein, pasteurization also increases the yield of cheese (Banks, 2011). There are three systems of pasteurization practiced in most counties namely: Flash heating(no holding) to temperature of 75-95℃, HTST: 71-75℃/14-40 s and LTLT: 61-65℃ / 20-40 min (Scott, 1986).

2.6.7 Homogenization

Homogenization of milk results in improvement in the appearance of product, texture, functionality, fat recovery and moisture content leading to increased yield of product (Suthar *et al.*, 2018; Rowney *et al.*, 1999).

2.6.8 Lactose hydrolysis

According to Upadhyay, (2003) lactose hydrolyzed milk has following effects on the manufacture for cheese :

- Faster acid production with reduced milk ripening and cheddering time.
- Increase the moisture content of cheese slightly.
- Whey obtained in cheese making process could be used for production of syrup as it contains increased level of glucose and galactose.

2.7 Additives in cheese milk

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

2.7.1 Calcium salt

In the secondary phase of rennet action, calcium plays an important role. Therefore, for successful coagulation, the calcium balance between soluble, colloidal and complex is very important. The lack of calcium balance and disturbance is due to chilling and long-term cold storage at 4-5°C, causing β-casein dissociation, severe milk heating during pasteurization, and water-based milk dilution (Lucey, 1993). Calcium may be added to milk in different forms such as calcium chloride ($CaCl_2$) up to 0.02%, dibasic calcium phosphate is recommended for use with pepsin rennet (<0.01%), lime water and calcium lactate (Upadhyay, 2003).

2.7.2 Cheese colour

In order to give cheese an appealing and appetizing look, it is common practice to add extra color to pale colored milk. Two essential colors are present in milk, but they are lost

in whey: riboflavin and carotenoids. The color of annatto cheese of vegetable origin is commonly used at a rate of 88 g/1000 kg (Kosikowski, 1982).

2.7.3 Inhibitory salts

Inhibitory salts (saltpeter) are applied to milk to avoid the growth of gas-producing species such as coliform/aerogenes and butyric acid bacteria in the manufacture of less acidic cheese (such as Edam, Gouda and Swiss cheese). To prevent the growth of spores, a concentration of 10-100 ppm of nitrite or 2-5 ppm of nitrate is necessary. The key drawback of the use of nitrate in cheese making is color defects and potentially carcinogenic effects. Lysozyme has been introduced to suppress clostridia species as a substitute for saltpeter (Farkye, 2004).

2.7.4 Salt

Salt has three major functions in cheese: it acts as a preservative, contributes directly to flavour and is a source of dietary sodium. Salt together with the desired pH and calcium level has large effect on water binding capacity of casein matrix, its rheological and textural characteristics and its cooking properties (McSweeney, 2007).

2.7.5 Rennet

The conversion of milk to cheese curd is usually made through enzymatic coagulation. Rennet is the enzymatic preparation of two main acid proteolytic enzymes (chymosin and pepsin) secreted in the fourth stomach (abomasum) of unweaned ruminants (calves, lambs) (Moschopoulou, 2011). The main purpose of adding rennet to cheese milk is to cause the milk to coagulate, which is necessary for whey exudation. However, action of rennet in cheese-making does not end here, but it also plays a part in breaking down the casein during cheese ripening (Dalgleish, 1987).

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

2.7.7.1 Calf rennet

Rennet was produced mainly from the abomasa of young calves, up until the end of the twentieth century (Andrén, 2011). In a slightly acidic salt solution, the stomachs are cut into strips that are collected. Since, heating to 55-60°C kills rennet enzymes, the rennet extracts cannot be preserved by heating. The extract's pH value is adjusted to about 5.5, and salt solution to 15-20%, to obtain the best possible preservation. The lawn must be shielded from light and stored in a cold position. Under these conditions, it will only lose about 1% of its activity. The enzymes are destroyed by alkali and strong acid. Most of the coagulation activity of calf rennet is caused by the enzyme chymosin (rennin). Part of the coagulation activity, however, is caused by another enzyme: bovine pepsin. As the calf matures the amount of pepsin increases and chymosin (rennin) decreases (Acharya, 2010).

2.7.7.2 Microbial rennet

Chymosin produced by recombinant DNA technology was first applied to cheese for evaluation in 1988. Several microorganisms such as proteinases, which can replace the calf rennet, are regarded as producers of rennet. However, there were two major disadvantages to microbial enzymes, i.e. the existence of high levels of unspecific and heat-stable proteases, leading to the production of bitterness after storage in cheese; and low yield. The development of enzymes that are fully inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases has resulted in extensive research in this field (Fox, 1987). Microorganisms like *Rhizomucor pusillus* and *Cryphonectria parasitica* (Egito *et al.*, 2007), *Thermomucor indicae-seudaticae* N31, *Aspergillus oryzae* (Hashem, 2000) are extensively used for rennet production in cheese manufacture.

2.8 Plant enzymes as rennet substitute

There are many advantages associated with the use of plant coagulants in cheese making: they are safe, inexpensive, easy to prepare, allow simple processes and are used to manufacture cheese for ecological markets (Roseiro *et al.*, 2003). Plant proteases are classified into various groups based on their catalytic mechanism used during hydrolytic process. The main classes for milk clotting proteases are aspartic, serine and cysteine proteases (Amira *et al.*, 2017). Plant proteases employed for cheese production in various areas of the world include papain, bromelain, ficin, oryzasin, cucumisin, *Withania*

coagulans, sodom apple and *Jacaratia corumbensis* (Duarte *et al.*, 2009; Mohamed *et al.*, 1997; Nawaz, 2007; Aworh and Muller, 1987).

In general, the major drawback of most plant rennet is the development of an increased bitterness and the appearance of cheese texture defects during storage and/or ripening (Egito *et al.*, 2007). These defects are mainly due to excessive proteolytic activities and low milkclotting activity / protease activity ratios. For this reason, the evaluation of enzyme activities and their comparison with those of commercial rennet (chymosin) is an important first step in selecting a suitable plant coagulant (Aworh and Muller, 1987).

2.8.1 Actinidin (kiwifruit protease)

Actinidin is a cysteine protease found in the fruit in kiwifruit, which catalyzes the hydrolysis of peptide bonds containing basic amino acids such as Lys and Arg in position P1 (McDowall, 1970). Kiwifruit contains very high amount of cysteine protease: actinidin (Uchikoba and Kaneda, 1996). This enzyme consist of 220 amino acids with an apparent molecular weight of about 23.0 kDa (Kamphuis *et al.*, 1985). Actinidin prefers β-casein, followed by κ-casein, which is hydrolyzed into small number of larger peptides (Sharma and Vaidya, 2018). It has no or limited proteolytic effect on globular proteins such as immunoglobulin 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 hydrolyze collagen and fibrinogen perfectly at neutral and mild basic pH. Moreover, this enzyme can digest pure α-caesin and major subunits of micellar casein especially in acidic pH (Chalabi *et al.*, 2014). The enzyme has been extensively characterized biochemically with the substrate specificity, kinetic parameters and catalytic site characteristics being well defined (Watts and Brocklehurst, 2004).

The optimal temperature and pH of immobilized actinidin is 40-60°C and 5.0-7.5 respectively (Alirezaei *et al.*, 2011; Homaei and Etemadipour, 2014). The pH and temperature dependency of the enzyme present in kiwifruit and its stability profile are compatible with the physiochemical criteria necessary during the cheese making procedure (Karki and Ojha, 2018).

2.8.1.1 Kiwifruit

Kiwifruit belongs to the genus *Actinidia* (Actinidiaceae) and is derived from a deciduous woody, fruiting vine. It is composed of different species and cultivars that exhibit a variety of characteristics and sensory attributes (Singletary, 2012). Utilizing kiwi juice as a milk coagulant for cheese production can be done to produce 100% lacto-vegetarian friendly cheese along with providing a viable product diversification alternative (Piero *et al.*, 2011). Kiwifruit has protein of two types: soluble and insoluble proteins. The largest percentage of the soluble protein, which is the most important enzyme, is the actinidin enzyme (Kazem and Habeeb, 2020).

The small kiwifruit stores a treasure of nutritional surprise. One medium size kiwifruit contains 42 calories and no saturated fat or cholesterol (Cervoni, 2019). Kiwifruit not only is rich in vitamin C, but also a good source of nutrients such as folate, potassium and dietary fibre (Kazem and Habeeb, 2020). This fruit's nutrient content and biologically active phytochemicals has stimulated investigations into its anti-oxidant and anti-inflammatory actions which might help prevent cardiovascular disease, cancer and other degenerative disorder (Singletary, 2012).

2.8.2 Zingibain (Ginger protease)

Ginger protease or zingibain, which was first reported as a new source of protease in 1973, exhibits remarkable proteolytic activity. It has good milk clotting activity and is also used in preparation of ginger milk curd in south of China (Nafi *et al.*, 2014).

Ginger proteases are separated into 2 factions (GPI and GPII). The complete amino acid sequence for GPII contains 221 amino acids, while about 98% of the amino acid sequence for GPI has been determined. GPII is a cysteine protease with two predicted glycosylation sites at Asn 99 and Asn 156 (Murtala *et al.*, 2017). Ginger proteases, all with molecular weight around 31 kDa, were found to exist in 3 forms with isoelectric point values around 5.58, 5.40 and 5.22 respectively (Huang *et al.*, 2011).

Protease extracted from common ginger *(Zingiber officale)* shows an optimum activity at 35-55°C with a broad pH range of 5 to 8 (Murtala *et al.*, 2017). The enzyme is considered as cysteine protease due to its inhibition by NEM and Hg^{2+} and Cu^{2+} and the fact it retains its activity in presence of monocations except Li^{+} and some detergents (Nafi

et al., 2013). They are also capable of hydrolyzing isolated α_{S1}-, β-, and κ-casein, of which α_{S1}-caesin is most susceptible to the enzyme; κ-casein is hydrolyzed with higher specificity than α_{S1}- and β-casein. In addition, ginger proteases exhibit a similar affinity for κ-casein and higher specificity with increasing temperature. Ginger proteases are also capable of hydrolyzing native collagen, showing preferences for the peptide bonds where Pro is in the P2 position. Ascorbic acid protects the activity of ginger proteases and Cu^{2+}, Hg^{2+}, Cd^{2+} and Fe^{2+} inhibit their activity whereas Ca^{2+}, EDTA, and dithiothreitol activate enzymes (Huang *et al.*, 2011).

2.8.2.1 Ginger

Ginger is a herbaceous perennial plant but largely grown as annual. Its height varies from 30- 90 cm. The underground stem (rhizome) grows horizontally, thick, flattened, branched and covered with small scale leaves and fibrous roots. It is an important spice crop of the world which originates from India. Beside used as a spice, ginger is also used for the production of oil, oleoresin, essence, soft drinks and non-alcoholic beverage. Ginger has medicinal properties as a carminative and stimulation of gastro-intestinal tract (Teferra *et al.*, 2015). Ginger (*Zingiber officinale*) has also been used as a peptic drug in Chinese medicine and in various food dishes from ancient times. It has also been applied as a meat tenderizing agent, the meat tenderizing component being the ginger protease (Hashim *et al.*, 2011). It was also used as a digestive aid and anti-nausea remedy and to treat bleeding disorders, rheumatism, baldness, toothache, snakebite, and respiratory conditions (Bhatt *et al.*, 2013).

2.8.3 Evaluation of enzymatic activities of plant proteases

Milkclotting activity 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). Concerning cheese production using plant rennet, the initial goal was always the production of coagulants with a maximum specific coagulant activity. This activity depends on several factors, such as the plant source, the part in the plant, as well as the type and the concentration of protease (Silvestre *et al.*, 2012). In order to detect the optimum of activity, different comparison studies were carried out between extracts of different parts in the same plant. Results revealed that the highest clotting activity was observed in the extract of latex followed by extracts of stems, leaves, and flowers of

Calotropis gigantea, in descending order (Rajagopalan *et al.*, 2014). In fact, aqueous extracts from flowers have been widely used as substitutes of animal rennet in some artisanal Italian Spanish and Portuguese cheeses (Aquilanti *et al.*, 2011).

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

Within the frame of research for a suitable substitute for calf chymosin, comparative studies of coagulant activities and MCA/PA ratios of plant extracts (or purified proteases) were established. The specific activities of crude extracts obtained from seeds of *Helianthus annus* and *Albizia lebbeck* were evaluated, showing very low values of 156×10^{-3} and 591×10^{-3} U/mg for *A. lebbeck* extracts (crude and concentrated) and 5.8×10^{-3} and 39×10^{-3} U/mg for *H. annus* extracts (Egito *et al.*, 2007b).

2.8.3.1. MCA/PA

Manzano *et al.* (2013) compared the MCA of three crude extracts obtained from kiwi (*Actinidia chinensis*), 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, Ben Amira *et al.* (2017) varied the pH of *Cynara cardunculus* rennet from 3 to 6 and evaluated enzymatic activities of the crude extracts. They demonstrated that MCA/PA ratio increased with pH drop and reached a maximum value of 28.71 for extract at pH 3, which exceeded that of chymosin (23.59). The lowest ratio attributed to the extract at pH 6 was mainly related to its high PA as well as to its low MCA. The effect of increasing pH buffer on rennet activity could be explained by the fact that, at high pH, the extraction level of several other compounds, including non-proteolytic enzymes, is more important. These compounds may promote the development of extraneous reactions and interfere with enzyme tests, thus causing underestimation of MCA. In addition, the high content of phenolic compounds involves their swift oxidation

to form pigments, which may attach to native enzymes, thus leading to their inactivation (Barros *et al.*, 2001).

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

2.9 Cheese technology

The objective of the various cheese manufacturing procedures is to establish conditions suitable for the biochemical and physical changes which are responsible for the development of the characteristic body and flavor (Harper and Hall, 1976). Two cheeses, even from the same batch of production may not be identical (Pradhan, 2000).

2.9.1 Basic principles of cheese making

According to Nielsen and Ullum (1989), the basic cheese making 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 figures

At the beginning, the three-dimensional casein network which is formed during coagulation encloses all the other milk constituents. When the coagulum contracts, water

and the constituents dissolved in the water are squeezed out, whereas fat globules and bacteria retained in the fine-meshed casein network (Acharya, 2010).

The retention figures of some milk constituents in cheese are shown in the Table 2.5.

Table 2.5 Retention figures of some milk constituents in cheese

Milk constituents	Retention figure
Protein	~75% (not higher than 88%)
Fat	~88-95%
Lactose	~3-5%
Ash	~30-40%
Citric Acid	~10%
Bacteria	~90% concentrated in curd grains

Source: Acharya (2010)

2.10 Soft cheese manufacturing method

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

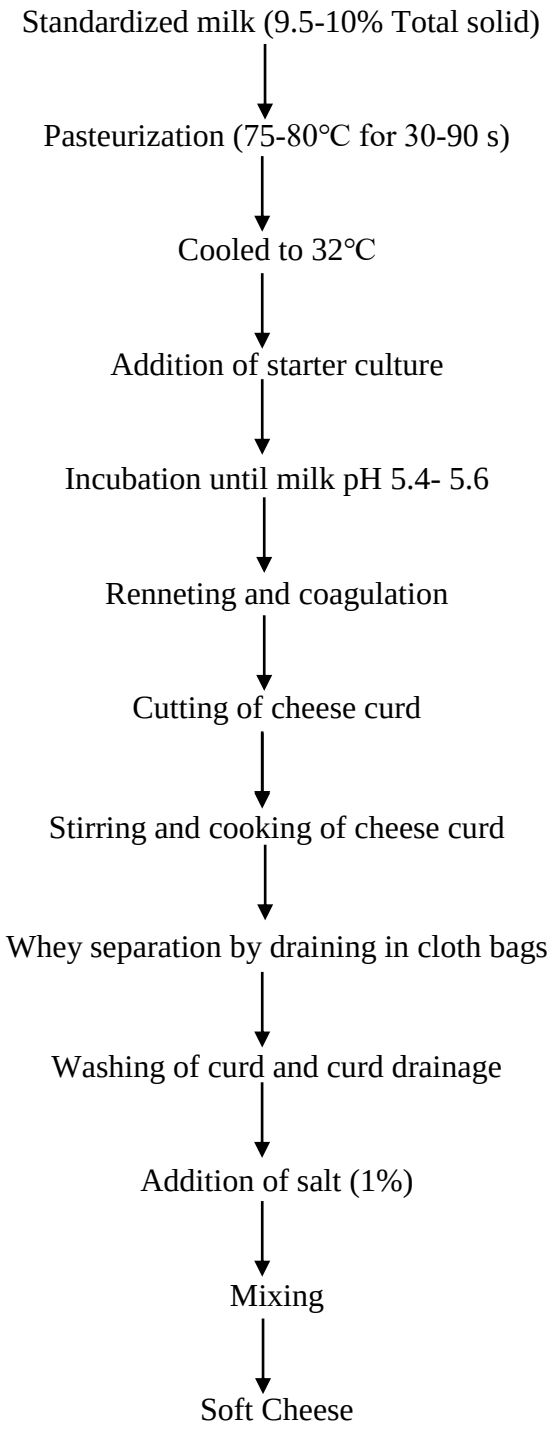

Fig. 2.1 Processing steps for soft cheese making

Source: Jain *et al.* (2019)

2.11 Manufacturing steps involved in soft cheese

2.11.1 Milk

Milk as a starting material for cheese making exerts a decisive influence on the course of manufacture and effects yield and quality of resultant cheese. So, good quality fresh milk must be used for cheese production (Upadhyay, 2003).

2.11.2 Pre-treatments of milk

Various pre-treatments are performed to improve the characteristics of cheese.

1. Standardization

   Milk fat and protein are main constituents of cheese contributing to texture, taste, functionality of cheese, so the product is highly influenced by their concentration in milk. So, casein to fat ratio is used to prepare and judge the quality of milk for cheese preparation. The common proportion of casein to fat is ranging from 0.64-0.72 (Rasovic *et al.*, 2013; Rowney *et al.*, 1999)
2. Heat treatment

   Pasteurization of milk is performed to kill all pathogenic and harmful microorganisms and to inactivate phosphatase and xanthine oxidase enzymes present in the milk. Pasteurization also increases the yield of cheese by insolubilizing part of the serum protein (Banks, 2011).

2.11.3 Renneting and cooking

There is a dual function of the coagulant used in cheese making. Its primary function is to coagulate milk, producing curd that is converted into cheese afterwards. Furthermore, a small amount of the coagulant is taken into the cheese. During aging, this residual coagulant remains proteolytically active, playing an important role in texture and flavour production (Nunez *et al.*, 1991). Rennet is added to the acidified milk when it reaches pH 5.4-5.6 at 31-35ºC. After the coagulum is completely formed, the curds are stirred and cooked with whey to a temperature of about 60-70ºC (Phadungath, 2005).

2.11.4 Curd treatment

After coagulation, the purpose of curd treatment in the cheese vat is to encourage the contraction of the casein network and the subsequent whey exudation without losing too much whey fat and curd (Harper and Hall, 1976).

3. Cutting the Coagulum

   Stirring soon after the coagulum is cut is more detrimental to a coagulum that is cut firm than to one that is cut soft (Johnson *et al.*, 2001). Similarly, if the coagulum is cut and agitated too soon, the curd will be prone to tearing and fracturing. Therefore, cutting too soon or too late can result in an increase in fat loss, especially if the subsequent rate of stirring is too fast for the resiliency of the curd. After the coagulum has reached the desired firmness, it is cut. The objective of cutting is to divide the large mass of curd into smaller, uniform particles in order for the moisture to be more easily expelled (Harper and Hall, 1976).

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

4. Stirring

   During stirring, the curd is agitated gently just enough to keep it from matting (Harper and Hall, 1976). Increment in stirring rate is done when the outer covering of the curd looks like membrane. The curd is very soft and delicate after cutting and must be handled carefully to avoid undue crushing and loss of fat and curd dust. When sufficient whey is released, the agitation can become faster so that the curd can be stirred without splitting (Fagan *et al.*, 2017). The stirring phase usually lasts for about 15-25 minutes (Nielsen and Ullum, 1989).

5. Heating/cooking/scalding

   Cooking aids in more discharge of whey from the curd due to contraction and pressure exerted on the curd grains (Fagan *et al.*, 2017) Increase in cooking temperature also triggers the metabolism of the starter bacteria (present in the curd) which in turn increases the production of lactic acid and drops pH (Ong *et al.*, 2017). During cheese making, the decrease in level of lactose has a huge effect on the growth of lactic acid bacteria. Therefore, different techniques are imposed to adjust the level of lactose in the curd (Düsterhöft *et al.*, 2017). Soft cheese with higher moisture content

necessitates a lower scalding temperature resulting in more whey in the curd (FAO, 1999).

Cheeses made with pasteurized milk are preferable to those made with high-temperature milk. The melting and stretching properties of high-temperature milk cheese would also be lower than those of pasteurized milk cheeses (Ghosh and Singh, 1990).

2.11.5 Whey draining and salting

Once cooking is finished, whey is then separated by various methods from the curd; the conventional method includes allowing the hot curd (75-90ºC) to drain overnight in cloth bags, while the modern methods use a centrifugal separator for soft cheese operating at 70-85ºC or ultrafiltration at 50-55ºC. The hot curd is cooled down to 10-20ºC after whey separation, then mixed with salt (0.5-1%) (Phadungath, 2005).

Salting of curd is a traditional and integral part of the manufacture of most cheese varieties. Salt should be allowed to dissolve properly otherwise it accumulates around the rind for some time (Scott *et al.*, 1998). According to the type of cheese, the salting method is also different. Salting is done on the surface of moulded curd for blue veined cheese, brine salting is done for Edam, directly immersing the cheese for Gouda Cheese in brine and dry salting is done for the Cheddar and cottage cheeses. To turn the curd into a compact mass of sufficient size, cheese salted by brine is kept to handle it easily (Guinee and Fox, 2004).

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).

2.11.6 Storage and packaging

Soft cheese has high moisture content so are easily perishable. Optimal packaging could minimize quality changes resulting in increased shelf life as well as quality maintenance. Different types of cheese have to be packed in different packaging concepts. Most fresh soft cheese is packed in air atmosphere due to short shelf life required. Vacuum packaging,

plastic packaging and even metal container packaging are used in cheese packaging. Soft cheese is generally stored at 4°C±0.5°C and lasts for 15 days in vacuum packaging (Brasava *et al.*, 2011).

Part III

Materials and methods

3.1 Materials

3.1.1 Milk

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

3.1.2 Rennet

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

3.1.3 Kiwifruit and ginger

The kiwifruit (*Actinidia deliciosa*) and ginger (*Zingiber officinale*) were collected from market in Dharan-02.

3.2 Methods

3.2.1 Extraction of plant protease

3.2.1.1 Crude kiwifruit and ginger protease extract

Ripe kiwi fruit was peeled with a spoon (gauging out the pulp). The pulp obtained was squeezed in a muslin cloth to obtain juice (from 200 g kiwi pulp, 170 ml juice was obtained). The juice obtained was centrifuged at 3000 rpm for 10 min. The obtained supernatant was filtered through a muslin cloth to remove suspended particles and clear juice was obtained (Karki and Ojha, 2018).

Fresh ginger rhizome was peeled, crushed and squeezed (from 107 g peeled ginger, 48 ml juice was obtained) through a muslin cloth to obtain crude enzyme (Saranya *et al.*, 2016).

3.2.1.2 Milk clotting activity

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

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

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

3.2.1.3 Protease activity

Protease activity was determined using protocol given by Cupp-Enyard (2008). This uses casein as protease substrate. 0.05 ml of enzyme extract was dissolved in 5 ml sodium acetate buffer 10 mM (pH 7.5) and 5 ml calcium acetate buffer 10 mM (pH 7.5). For each sample 455 µL Casein 65%(w/v) were preheated in a thermal bath at 60, 65 and 70ºC for 10 min and then 20 µL of these were added. After 10 min of reaction, the reactions were stopped by the addition of 455 µL trichloroacetic acid 110 mM. and were kept in the thermal bath for another 30 min. Each reaction has its negative control, which did not have enzyme during pre-incubation, but it was added after the trichloroacetic acid addition.

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

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

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

3.2.1.4 Protein determination

Protein content of the crude plant protease was determined using the procedure given by Lowry *et al.* (1951). In principle, the peptide bond in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored compound. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate component of Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this product. Quantitative analysis of protein was done spectrophotometically by measuring color against a standard, λ= 660 nm.

3.2.2 Optimization of plant protease

3.2.2.1 Experimental design

The experimental design, data analysis and model building were performed using "Design Expert" software (Version 12.0.3, Stat-Ease Inc., USA). The soft cheese was prepared with variations in: pH and temperature of milk during enzyme addition as shown in Table 3.1 (kiwifruit protease) and Table 3.2 (ginger protease). The independent variables and their levels were selected on the basis of literature and preliminary experiments. The temperature and pH of immobilized kiwi protease was 35-75°C and 5.5-6.5 respectively (Alirezaei *et al.*, 2011; Homaei and Etemadipour, 2014). Similarly, the temperature and pH of immobilized ginger protease was 35-55°C and 5.5-6.5 (Murtala *et al.*, 2017). A two-factor central composite rotatable design was employed. The response variables were time of coagulation (TOC) and milk clotting activity (MCA) of the plant enzyme.

The different constraints of optimization of kiwifruit protease and ginger protease are given in Table 3.1 and Table 3.2.

Table 3.1 Different constraints for optimization of kiwifruit protease

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

Table 3.2 Different constraints for optimization of ginger protease

Name	Goal	Range
Temperature of milk	To be in range	35-55°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 for each.

3.2.2.2 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. Data were fitted to

the selected models and the statistical significance of the terms was examined by analysis of variance 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 good the model predicts a model value) and Fisher'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 have in explaining the behaviour variation. Then the effect of predictors on the response was interpreted using the models.

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

3.2.3 Preparation of soft cheese

Cheese was prepared with rennet and plant proteases by following the process shown in Fig. 3.1. The cheese prepared using calf rennet was labelled as cheese A, crude kiwifruit protease was labelled as cheese B and prepared using crude ginger protease cheese C.

Milk was heated until it reached the temperature of 75-80℃ followed by stirring for 1 min. The addition of rennet for cheese A was done at temperature (37℃) after the milk attained pH (5.4-5.6) by using 2% citric acid solution to decrease pH of milk. The milk was stirred gently and when curd was formed it was cut with a knife for easy whey separation. The whey and curd was than cooked at 38-42℃ for 15-20 min. The curd was then drained using filter cloth. The draining process was done for 15 min and the drained curd was mixed with 1% common salt. The cheese was stored in refrigerator at below 5℃ (Jain *et al.,* 2019). Similar, process was followed for cheese B and C with their optimum conditions.

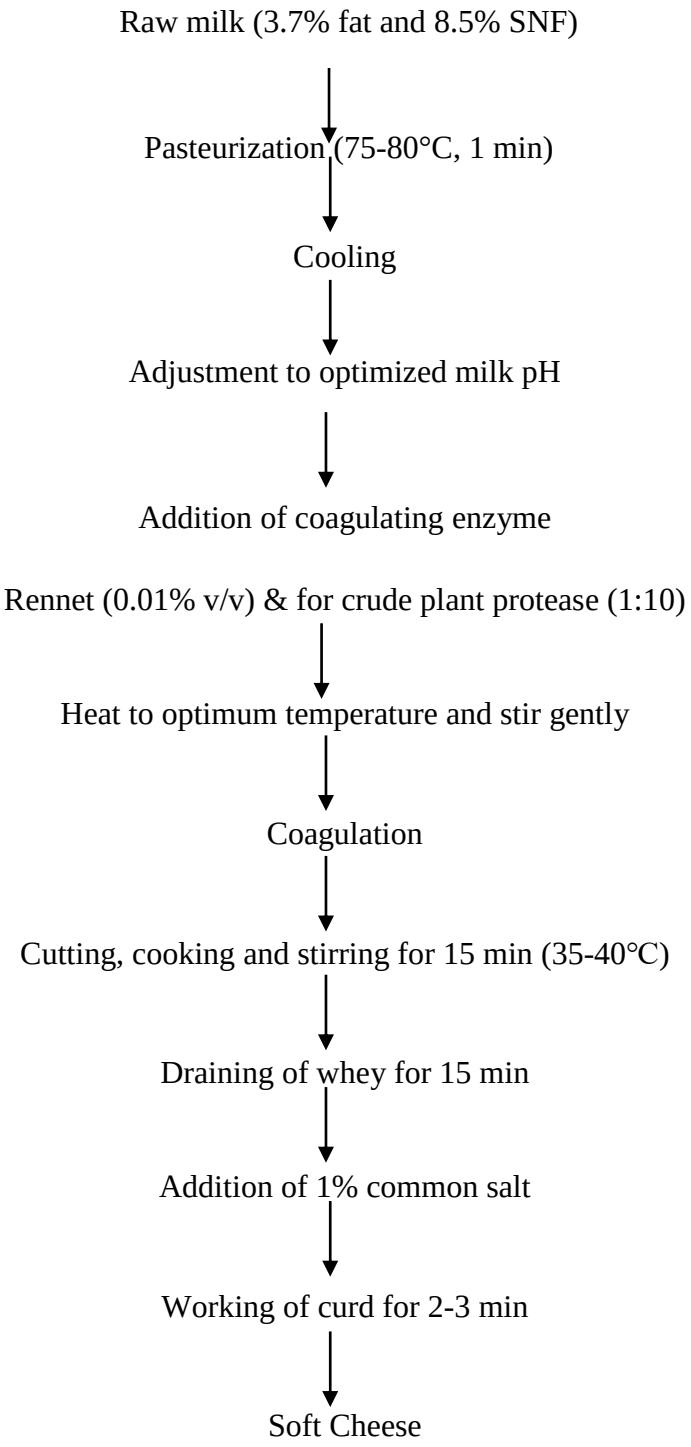

Fig. 3.1 Preparation steps of soft cheese with slight modification (Jain *et al.*, 2019)

3.2.4 Physicochemical analysis of milk and cheese

3.2.4.1 Determination of fat in milk and cheese

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

3.2.4.2 Determination of SNF in milk

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

3.2.4.3 Determination of pH in milk and cheese

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

3.2.4.4 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).

3.2.4.5 Determination of acidity in milk and cheese

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

3.2.4.6 Determination of calcium in cheese

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

3.2.4.7 Determination of moisture in cheese

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

3.2.4.8 Determination of ash in cheese

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

3.2.4.9 Theoretical yield and actual yield

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

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

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

The 0.93×milk fat assumes that some 93% of milk fat is retained in the cheese. The value for casein – 0.1 approximates to a theoretical loss of 4% casein and casein retention

of approximately 96%. The 109 is a ‘constant’ to allow milk salts retention of whey protein and lactose.

Actual yield was calculated by weighing the curd after pressing as described by Razzaq (2003). The percentage of cheese yield was calculated as follow:

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

3.2.5 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 were statistically processed by R Studio Version 1.3.959 (Team, 2020). Statistical significance was tested by using Tukey’s Honestly Significant Difference (HSD) method at 5% level of significance.

Part IV

Results and discussion

In this research work, plant proteases were extracted from kiwifruit and ginger. The impact of pH and temperature of milk on time of coagulation (TOC) and milk coagulating activity (MCA) were analyzed by response surface methodology (RSM). The cheeses, prepared from rennet (A), crude kiwifruit protease (B) and crude ginger protease (C) were analyzed for physio-chemical and microbiological qualities.

4.1 Numerical optimization for time of coagulation and milk clotting activity

The measured expansion of the time of coagulation and milk clotting activity for crude kiwifruit protease varied from 1-2115 s and 0.57-1200 units respectively (Appendix A). Similarly, for crude ginger protease it varied from 40-10800 s and 1.11-300 units respectively (Appendix A). Table B.1 and B.2 show the coefficients of the model and other statistical attributes of time of coagulation (TOC) whereas Table B.3 and B.4 show that of MCA for enzyme extracted from kiwifruit. Similarly, Table B.5 and B.6 show the coefficients of the model and other statistical attributes of time of coagulation (TOC) whereas Table B.7 and B.8 show that of MCA for enzyme extracted from ginger.

The coded equations for the extracted proteases are:

For Kiwifruit protease,

$$TOC = 147.10 + 397.10A - 492.93B - 399.80AB + 207.30A^2 + 272.19\ B^2 \quad \ldots 4.1$$

$$MCA = 326.23 - 245.49A + 477.49B - 353.80AB \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4.2$$

For ginger protease,

$$TOC = 4416.33 + 2261.33A - 3746.67B \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4.3$$

$$MCA = 48.71 - 68.31A + 51.37B - 73.60AB \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4.4$$

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

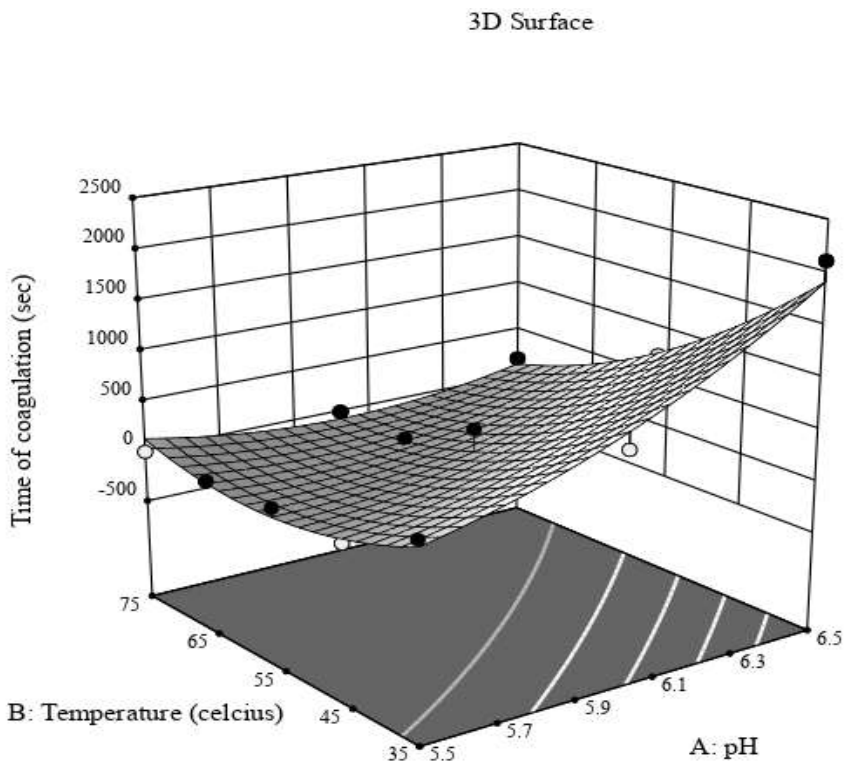

Fig. 4.1 Response surface plot of TOC of crude kiwi protease as a function of pH and temperature of milk.

In the equation 4.1, TOC for crude kiwi protease had significant ($P<0.05$) positive effect of pH of milk and significant ($P<0.05$) negative effect of temperature of milk. The quadratic terms of pH of milk and temperature of milk both had significant ($P<0.05$) positive effect on TOC as given in Table B.2. Similarly, the interaction term of pH of milk and temperature of milk (AB) had significant ($P<0.05$) negative effect on TOC. In Fig. 4.1, TOC increased gradually with the increase in pH and only slight increase was seen in TOC with the increase in temperature. Also, the combined increase of pH and temperature of milk caused a slight increase in TOC.

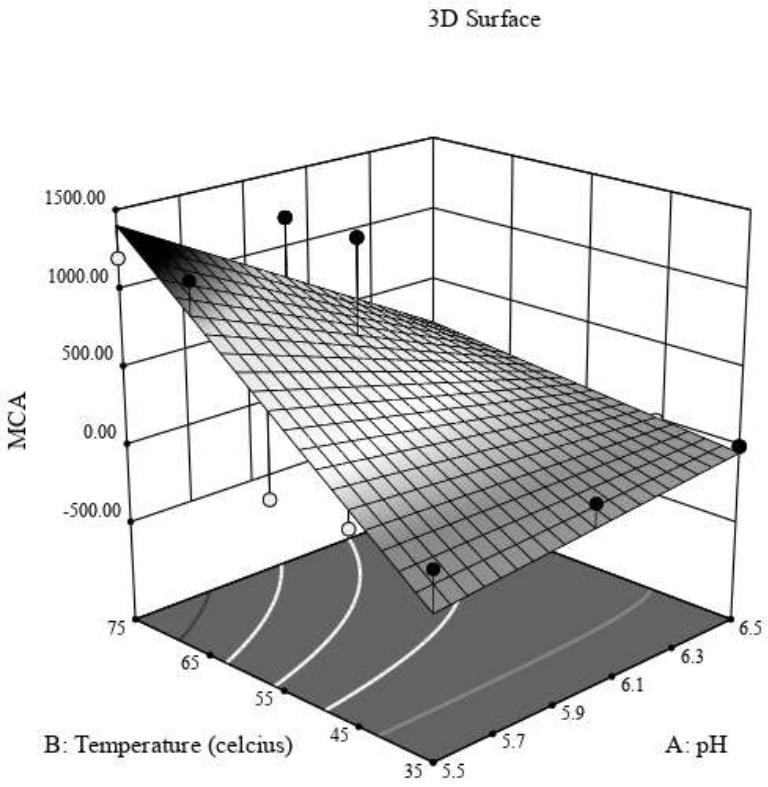

Fig. 4.2 Response surface plot of MCA of crude kiwi protease as a function of pH and temperature of milk.

In the equation 4.2, MCA for crude kiwi protease had significant ($P<0.05$) negative effect of pH of milk and highly significant ($P<0.05$) positive effect of temperature of milk. The interaction term of pH of milk and temperature of milk (AB) had significant ($P<0.05$) negative effect on MCA as given in Table B.4. In Fig. 4.2, MCA increased drastically with the increase in milk temperature and only slight MCA increase was seen with the increase in pH. Also, with the combined increase of pH and temperature of milk there is only a slight increase in MCA.

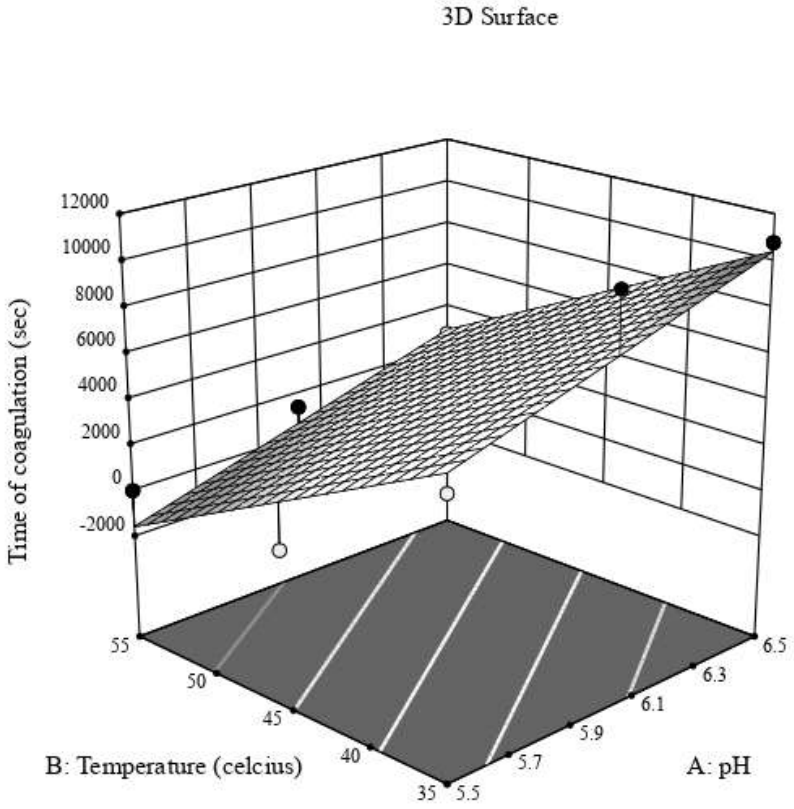

Fig. 4.3 Response surface plot of TOC of crude ginger protease as a function of pH and temperature of milk.

In the equation 4.3 for TOC of crude ginger protease, pH of milk had significant ($P<0.05$) positive effect on the TOC whereas temperature of milk had highly significant ($P<0.05$) negative effect on TOC at 95% confidence level as given in Table B.6. In Fig. 4.3, TOC decreased drastically with the increase in temperature and TOC increased drastically with the increase in pH. With the combined increase in pH and temperature there was only a slight increase in the TOC.

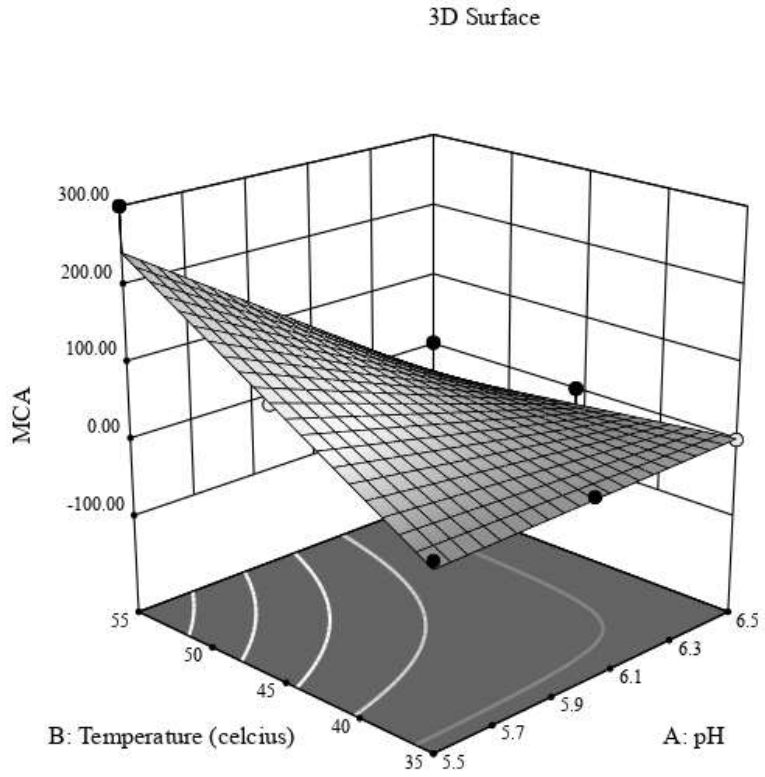

Fig. 4.4 Response surface plot of MCA of crude ginger protease as a function of pH and temperature of milk.

In the equation 4.4 for MCA of crude ginger protease, pH of milk had highly significant ($P<0.05$) negative effect on MCA whereas temperature of milk had non-significant ($P>0.05$) positive effect on MCA. Similarly, the interaction term of pH of milk and temperature of milk (AB) had non-significant ($P>0.05$) negative effect on MCA as given in Table B.8. In Fig. 4.4, MCA drastically increased with the increase in temperature and no significant change was seen in MCA with the pH increase. Also, slight decrease in MCA was seen with the combined increase in pH and temperature.

4.1.1 Protease activity of crude proteases

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

Table 4.1 Protease activity of crude kiwi fruit and ginger protease

Crude protease	OD (at 660 nm)	Protease activity (units/ml enzyme)
Kiwifruit	0.588±0.182	0.7727±0.028
Ginger	0.040±0.020	0.0508±0.003

From the Table 4.1, protease activity of crude kiwifruit protease is 0.7727 units/ml enzyme which is lower than the result given by Nafi *et al.*(2013) and Kazem and Habeeb (2020). And the protease activity of ginger is 0.0508 units/ml enzyme which is lower than the result given by Murtala *et al.* (2017). The variation in data of protein content can be attributed to the difference in variety, climatic condition, soil condition, maturity stages of plants and experimental conditions.

4.1.2 Protein concentration

Crude proteases of kiwifruit and ginger were subjected to protein determination. The results are calculated using equation deduced from standard curve (Fig. D.2) and are presented in Table 4.2.

Table 4.2 Protein concentration of crude kiwifruit and ginger protease

Crude Protease	OD (at 660 nm)	Protein concentration (mg/ml)
Kiwifruit	1.502± 0.01	15.018±0.7
Ginger	0.115±0.01	56.3±0.52

From the Table 4.2, protein concentration of crude kiwifruit protease extracted is 15.018 mg/ml which is higher than seen by Kazem and Habeeb (2020). Similarly, protein concentration of crude ginger protease is 56.3 mg/ml which is lower than seen by Murtala *et al.* (2017). This variation may be due to the different variety of the plant itself or its maturity stage.

4.1.3 Optimization of crude enzyme

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 4.3 and Table 4.4.

Table 4.3 Different constraints for optimization of crude kiwi protease

Name	Goal	Lower Limit	Upper Limit
Temperature of milk	is in range	35	75
pH of milk	is target = 6.5	5	6.5
Time of coagulation	Minimize	58	159
Milk clotting activity	Maximize	150.9	413.8

Under the assumptions by Design Expert Software (version 12), the optimum operating conditions for minimum time of coagulation and maximum milk clotting activity of crude kiwifruit enzyme were found to be 6.5 pH and 61.43℃ of milk temperature. The responses predicted by the software for these optimum conditions reported MCA of 492.630 units at 120.513s of coagulation time.

Table 4.4 Different constraints for optimization of crude ginger protease

Name	Goal	Lower Limit	Upper Limit
Temperature of milk	is in range	35	55
pH of milk	is target = 6.5	5.5	6.5
Time of coagulation	Minimize	58	159
Milk clotting activity	Maximize	150.9	413.8

Under the assumptions by Design Expert Software (version 12), the optimum operating conditions for minimum time of coagulation and maximum milk clotting activity of crude ginger enzyme were found to be 5.5 pH and 55℃ of milk temperature. The responses predicted by the software for these optimum conditions reported MCA of 241.988 units at 1591.667 s of coagulation time.

4.1.4 Verification of the model

Within the scope of the variables investigated in Central Composite Rotatable Design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. The conditions and the results of the confirmatory experiments are presented in Table 4.5.

Table 4.5 Predicted and actual values of the responses at the optimized condition

Response	Conditions			Predicted value	Mean Observed value
	Crude Protease	Temperature of milk	pH of milk		
TOC	Kiwifruit	61	6.5	508.182	498.67
MCA	Kiwifruit	61	6.5	32.56	24.07
TOC	Ginger	55	5.5	1869.32	1876.67
MCA	Ginger	55	5.5	15.89	16.4

4.2 Physicochemical properties

4.2.1 Chemical composition of raw milk

The proximate composition of raw cow milk is given in Table 4.6.

Table 4.6 Proximate composition of raw cow milk

Parameters	Cow milk
Moisture (%)	87.1 ± 0.17
Fat (%)	3.7 ± 0.05
Protein (%)	3.2 ± 0.02
Ash (%)	0.7 ± 0.01
pH	6.5 ± 0.1

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

The resulted presented in Table 4.3 revealed that the moisture, fat, protein, ash and pH in cow milk were 87.1%, 3.7%, 3.2%, 0.7% and 6.5 respectively. The values are similar to those reported by Walstra *et al.* (2006) and any variation may be due to cow breed, milking conditions and milking time.

4.2.2 Chemical composition of soft cheese

The chemical composition of the soft cheese made from rennet (A), crude kiwifruit protease (B) and crude ginger protease(C) are shown in Table 4.7.

Table 4.7 Chemical composition of soft cheeses A, B and C.

Parameters	A	B	C
Moisture (%)	$48.6^{a} \pm 0.06$	$51.63^{b} \pm 0.03$	$52.03^{c} \pm 0.03$
Fat (%,wb)	$27.4^{b} \pm 0.45$	$25.96^{a} \pm 0.30$	$26.50^{ab} \pm 0.4$
Protein (%,wb)	$20.1^{a} \pm 0.28$	$20.52^{a} \pm 0.61$	$20.72^{a} \pm 0.20$
Ash (%,wb)	$2.75^{a} \pm 0.13$	$3.48^{b} \pm 0.05$	$3.33^{b} \pm 0.15$
pH	$5.63^{b} \pm 0.005$	$5.86^{c} \pm 0.057$	$4.86^{a} \pm 0.057$
Acidity (%, lactic acid)	$0.2^{ab} \pm 0.01$	$0.17^{a} \pm 0.01$	$0.26^{b} \pm 0.05$
Calcium (mg/100 g)	$631.3^{b} \pm 1.21$	$657.8^{c} \pm 2.97$	$621.4^{a} \pm 2.47$

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

The moisture percentage of cheese B is in the line with the findings of Karki and Ojha (2018) but is lower than the findings of Sharma and Vaidya (2018). For cheese A, the results coincide with Mijan *et al.* (2010), Karki and Ojha (2018), Phadungath (2005); who reported moisture content of cows' milk cheese as 47.7% and 54%, respectively. And cheese C has similar readings as Hashim *et al.* (2011) i.e. 52%. Among the three cheeses,

the average moisture was more in C. Variation in moisture might be attributed to the activity of coagulant and difficulty in whey drainage, resulting from clogging of the drainage screen by fine particles. Similar views were expressed by Hill *et al.* (1982). The difference between the moisture of cheese might be due to change in proteolysis rate (Sharma and Vaidya, 2018). Analysis of variance (Table F.1) regarding moisture content revealed that significant differences ($P<0.05$) was observed between all three samples. Moisture content in soft cheeses made using crude kiwifruit and ginger protease were higher than that of rennet. The variation could be attributed to the molecular forces involved in the coagulation of casein by crude enzymes, which resulted into a greater water binding capacity of protein matrix of cheese. The significant difference might be due to the longer coagulation time for crude enzymes that results in more moisture retention in the final product (Jhonson *et al.*, 2001).

The fat level of cheese A is similar with findings of Mijan *et al.* (2010), Owni and Sana (2009). The results were slightly higher than the findings of Ghosh and Singh (1990) and Islam (2006) i.e. 24.8% and 23.5%, respectively. For cheese C, results were slightly higher than Hashim *et al.* (2011) and for cheese B, the results seen were slightly lower than Karki and Ojha (2018). Fat content decreased in plant cheese as compared to rennet cheese, might be due to change in proteolysis rate (Sharma and Vaidya, 2018). Among three samples, the average fat was highest in A and lowest in B. The fat is one of the leading factors in determining the characteristic body, texture and flavor of cheese (El-Gawad *et al.*, 2007). The lower value fat contents recorded in cheese B and C might be due to its higher time of coagulation as compared to rennet and higher fat loss during whey drainage. This may be responsible for the retention of fat in the final product (Karki and Ojha, 2018; Khan and Masud, 2013). Analysis of variance (Table F.2) regarding fat contents revealed that significant difference ($P<0.05$) was found between A and B, while, non-significant difference ($P>0.05$) was seen between B and C, A and C.

The protein content of all the cheeses are in line with the findings of Hashim *et al.* (2011), Mijan *et al.* (2010) and Owni and Sana(2009). The variation in protein content might be due to the cheese making techniques and the quality of milk used, as the milk quality changes with the lactating stage of animal, nutrition, breed and age of milking animal (Mijan *et al.*, 2010; Razzaq, 2003). Among three samples, the average protein contents were more in C which may be due to comparatively lower proteolytic activity.

Analysis of variance (Table F.3) regarding protein contents revealed that non-significant difference ($P>0.05$) was recorded among all stages.

The ash contents of cheese A was similar to the findings of Mijan *et al.* (2010). Khan and Masud (2013) and Patel and Gupta (1986) who reported that the ash content of cheese ranged from 2.50 to 3.20%. The possible reason for the less ash contents may be the seasonal variation in the composition of milk animal (Mijan *et al.*, 2010; Razzaq, 2003). Among three samples, the average ash content was more in B. Higher value was recorded in case of plant protease cheese as compared to rennet, which is probably due to the remnants of plant materials in the crude enzyme (Maskey and Shrestha, 2020). Analysis of variance (Table F.4) regarding ash contents revealed that significant difference ($P<0.05$) was found between all three samples.

pH is the most vital indicator of milk quality and safety. pH of food such as milk and milk products are measured to ensure the quality of foodstuff (Razzaq, 2003). The functional properties and properties like texture, firmness, etc. of soft cheese are greatly influenced by the pH (Phadungath, 2005; Rowney *et al.*, 1999). Analysis of variance (Table F.6) regarding pH revealed that there were significant difference ($P<0.05$) between all three samples. Among the samples, the average pH was most in B and least in C. The possible variation may be due to the processing techniques, effect of coagulants, incubation and coagulation time and temperature. The pH of the cheese may vary due to the strength of coagulants (Mohamed *et al.*, 1997).

The titratable acidity of soft cheeses is similar to the findings of Nawaz (2007) and lower than Mijan *et al.* (2010) and Owni and Sana (2009). The longer coagulation time of vegetable protease possibly favoured microbial growth and consequently, a higher acidity was reached in curd from vegetable protease (Kheir *et al.*, 2011). Analysis of variance (Table F.5) regarding titratable acidity revealed that non-significant difference ($P>0.05$) was seen between A and B, A and C, while significant difference ($P<0.05$) was found between B and C.

The calcium content of soft cheese B is similar to Karki and Ojha (2018) i.e. 663 mg/100 g of cheese. And the calcium content of A and C ranges from 621-631mg/100 g of cheese. Among three samples, the calcium content was most in B which may be due to the descendent of calcium from crude kiwifruit protease into the cheese (Karki and Ojha,

2018). Analysis of variance (Table F.7) regarding the calcium content revealed a significant difference (P<0.05) among all samples.

4.2.3 Theoretical and actual yield

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

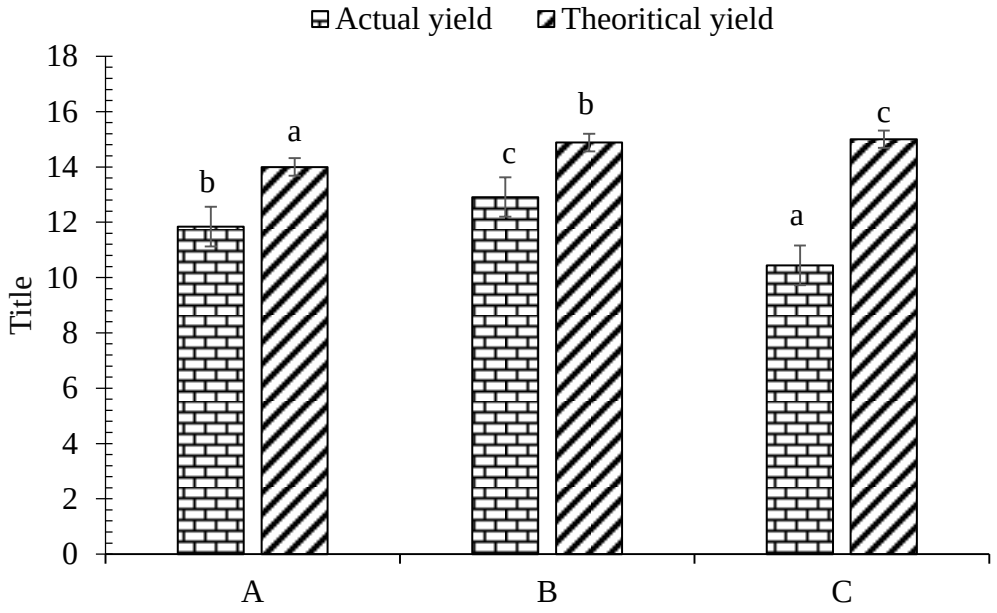

Fig. 4.5 Theoretical and Actual yield of soft cheeses

*Bars with different alphabets are significantly different at p<0.05.

4.2.3.1 Theoretical yield

Theoretical yield of cheese can be estimated from the milk fat and casein or protein contents of milk by using Van Slyke Equation of cheese yield. The theoretical yield of the cheeses is shown in Table E.1. Among them, the average theoretical yield was more in C. The slight variation in the yield is due to the moisture content in the final cheese as same milk is used for all the cheeses (Maskey and Shrestha, 2020). Analysis of variance (Table F.8) regarding theoretical yield revealed that there was significant difference (P<0.05) among all treatments.

4.2.3.2 Actual yield

Cheese yield efficiency is of vital importance in cheese making operation. The actual yield of soft cheeses is shown in Table E.1. Actual yield of all samples is higher than the findings of Karki and Ojha (2018) and lower than Aworh and Muller (1987) and Owni and Sana (2009). The variation may be due to difference in milk composition and processing techniques. Actual yield is lower than the theoretical yield. The yield reduction may be due to poor cheese making technique resulting in low casein and/or fat retention (Gawad and Ahmed, 2011). Among three treatments, the average actual yield was more in B. Cheese made with plant protease has higher yield than rennet cheese which may be due to the differences in cheese making procedures, especially the use of high temperatures during cheese manufacture procedure (Maskey and Shrestha, 2020). Whey protein denatured by heat may have been enclosed in the cheese curd of plant protease cheese, thus contributing to higher yield (Aworh and Muller, 1987). Analysis of variance (Table F.9) regarding actual yield revealed that there was significant difference ($P<0.05$) among all samples.

4.3 Microbiological analysis

The result of microbiological analysis of cheese samples are presented in Fig. 4.6 and in Table E.2, F.10 and F.11. It shows the average value for TPC and yeasts and molds of the sample.

In the TPC of three samples, significant difference ($P<0.05$) was found for A and B, B and C, whereas no-significant difference was found for A and C. For yeast and mold, all the samples were non-significantly different ($P>0.05$) from each other at 5% level of significance.

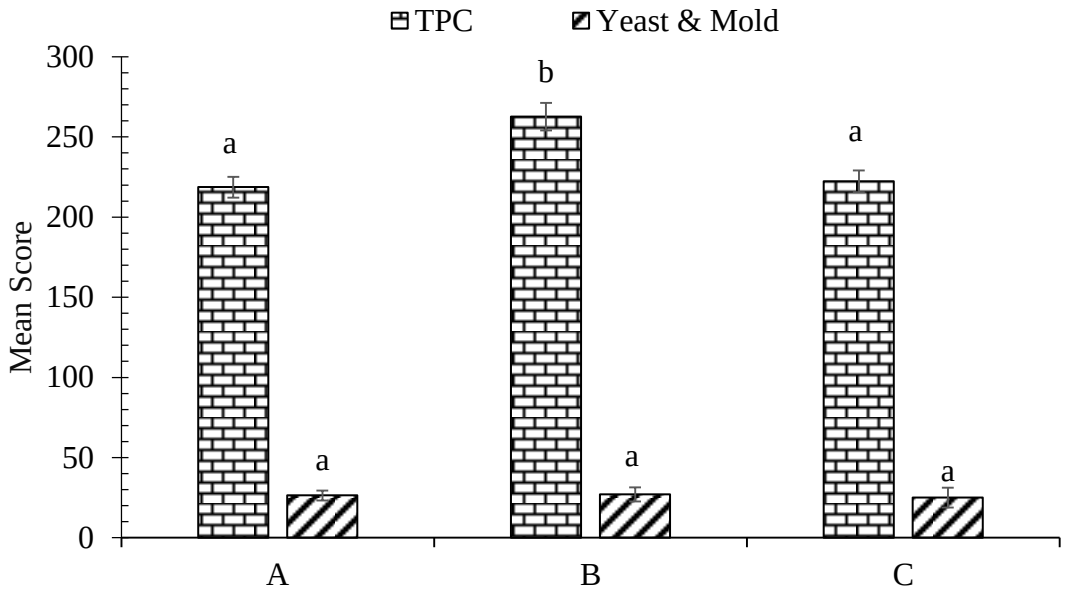

Fig. 4.6 Microbiology of soft cheese

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

Coliforms were not detected in all cheese samples. But the presence of TPC and yeasts & molds were detected. The results were similar to Maskey and Shrestha (2020). Heat treatment at 61.1℃ for 16.5 s reduced the coliform count by 93.8%. Also, low heat treatment at 57.2, 58.9, 60 and 61.1℃ for 16.5 s reduced the coliform count by 57.3, 75.0, 81.5 and 93.8%, respectively (Kosikowski and Fox, 1968).

Part V

Conclusions and recommendations

5.1 Conclusions

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

1. Optimum time of coagulation (TOC) and milk clotting activity (MCA) for crude kiwifruit and ginger protease were found to be in pH 6.5 at 61℃ and 5.5 at 55℃ respectively.
2. The protease activities of the prepared crude kiwi fruit and ginger protease were found to be 0.7727 units/ml enzyme and 0.0508 units/ml enzyme respectively.
3. The protein concentration of prepared crude kiwi and ginger protease were found to be 15.018 mg/ml and 56.3 mg/ml respectively.
4. The physico-chemical analysis showed a significant difference ($P<0.05$) in moisture, fat, ash, calcium, acidity, pH and cheese yield, while protein content was non-significant ($P>0.05$) among the cheese made using rennet and crude plant proteases.
5. Coliforms were not detected in all samples. Microbial analysis showed a significant difference ($P<0.05$) in the total plate count (TPC) while non-significant difference ($P>0.05$) was found in the count of yeast and mold of all three samples.

5.2 Recommendations

Further researches can be done as follows:

- Purification of crude kiwifruit and ginger enzyme to enhance milk clotting activity (MCA) of milk.
- Use of other plant extracts such as bromelain, ficin, etc as rennet substitute to prepare cheese.

Part VI

Summary

Cheese is a ripened or unripened product made by coagulation of the proteins in milk through the action of rennet or another coagulant. Calf rennet has been widely employed as a milk coagulating agent from antiquity. However, the increase in cheese production, coupled with a diminishing supply of natural animal rennet, is responsible for increases in the demand for alternative milk-coagulating sources.

The main emphasis of this study was to utilize kiwifruit and ginger as a source of protease for the preparation of soft-unripened cheese using the crude protease as milk coagulant. The cheese thus obtained was compared with rennet cheese for physico-chemical and microbiological quality.

The impact of processing parameters namely pH of milk and temperature of milk was investigated on time of coagulation (TOC) and milk clotting activity (MCA) by response surface methodology. An empirical quadratic model was applied to experimental data pertaining to the average enzymatic activity and equation describing the optimal conditions was obtained. For crude kiwifruit protease, the optimized time of coagulation (120.513 s) and milk clotting activity (492.630 units) was obtained at pH of milk 6.5, temperature of milk 61.43℃ at enzyme concentration 2 g/1000 ml milk. Similarly, for crude ginger protease, the optimized time of coagulation (1591.667 s) and milk clotting activity (241.988 units) was obtained at pH of milk 5.5, temperature of milk 55℃ at enzyme concentration 2 g/1000 ml milk. Protein concentration of the crude proteases were also determined and were found to be 15.018 mg/ml and 56.3 mg/ml enzyme for crude kiwifruit and ginger protease respectively. Similarly, the protease activity of the enzyme was also determined using optimized condition which justified the use of those conditions. These optimized values were applied in the preparation of soft cheese.

The physicochemical parameters like protein, fat and acidity showed no significant ($p>0.05$) difference but significantly ($p<0.05$) higher levels of moisture, calcium and ash, and lower levels of fat were observed in the cheese produced by crude kiwi fruit and ginger protease compared to rennet cheese. Microbiological analysis showed significant

difference ($P<0.05$) in the TPC of all three samples while not-significant difference ($P>0.05$) was found in the count of yeast and mold.

Therefore, it was concluded that crude protease from kiwifruit and ginger showed good results in the production of soft cheese. The quality of the cheese made from this easily available source can further be improved by purification of the proteases used.

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Appendices

Appendix A

Table A.1 Product responses by independent variables crude kiwi enzyme

Std. no.	Factor 1 A: pH	Factor 2 B: Temperature ℃	Response 1 Time of Coagulation s	Response 2 Milk Clotting Activity (MCA) U/ml
1	5.5	45	30	40.00
2	6.5	35	2115	0.57
3	6	35	745	1.61
4	5.5	75	1	1200.00
5	6.5	75	190	6.32
6	6.5	65	225	5.33
7	5.5	35	390	3.08
8	6	65	1	1200.00
9	6.5	55	728	1.65
10	6.5	75	1	1200.00
11	6	45	295	4.07
12	6.5	45	1180	1.02
13	5.5	55	45	26.67
14	5.5	65	1	1200.00
15	6	55	374	3.21

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

Std. no.	Factor 1 A: pH	Factor 2 B: Temperature ℃	Response 1 Time of Coagulation s	Response 2 Milk Clotting Activity (MCA) U/ml
1	5.5	35	5100	2.35
2	6	35	10800	1.11
3	5.5	45	104	115.28
4	5.5	55	40	300.00
5	6	45	3465	3.46
6	6.5	35	10800	1.11
7	6.5	45	5258	2.28
8	6	55	1426	8.42
9	6.5	55	2754	4.36

Appendix B

Table B.1 Model summary statistics for Time of Coagulation for crude kiwi enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	336.20	0.7148	0.6673	0.4758	2.493E+06	
2FI	225.06	0.8828	0.8509	0.6677	1.580E+06	
Quadratic	**156.15**	**0.9539**	**0.9282**	**0.8280**	**8.178E+05**	**Suggested**
Cubic	128.36	0.9792	0.9515	0.8126	0.8912E+05	Aliased

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

Table B.2 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Time of Coagulation for crude kiwi enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.536E+06	5	9.072E+05	37.21	< 0.0001	significant
A-pH	1.577E+06	1	1.577E+06	64.67	< 0.0001	
B-Temperature	1.822E+06	1	1.822E+06	74.74	< 0.0001	
AB	7.992E+05	1	7.992E+05	32.78	0.0003	
A^2	1.432E+05	1	1.432E+05	5.88	0.0384	
B^2	1.945E+05	1	1.945E+05	7.98	0.0199	
Residual	2.194E+05	9	24381.82			
Cor Total	4.756E+06	14				

Table B.3 Model summary statistics for Milk Clotting Activity for crude kiwi enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	393.00	0.5551	0.4810	0.2998	2.917E+06	
2FI	**334.05**	**0.7054**	**0.6250**	**0.4986**	**2.089E+06**	**Suggested**
Quadratic	319.55	0.7794	0.6568	0.3585	2.673E+06	
Cubic	282.22	0.8853	0.7323	0.2286	3.214E+06	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 for crude kiwi enzyme

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.939E+06	3	9.795E+05	8.78	0.0029	significant
A-pH	6.026E+05	1	6.026E+05	5.40	0.0403	
B-Temperature	1.710E+06	1	1.710E+06	15.32	0.0024	
AB	6.259E+05	1	6.259E+05	5.61	0.0373	
Residual	1.227E+06	11	1.116E+05			
Cor Total	4.166E+06	14				

Table B.5 Model summary statistics for Time of Coagulation for crude ginger enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**1738.31**	**0.8637**	**0.8183**	**0.7086**	**3.877E+07**	**Suggested**
2FI	1783.33	0.8805	0.8088	0.6928	4.087E+07	
Quadratic	1023.49	0.9764	0.9370	0.7188	3.740E+07	
Cubic	437.00	0.9986	0.9885	0.7384	3.480E+07	Aliased

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

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

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.149E+08	2	5.745E+07	19.01	0.0025	significant
A-pH	3.068E+07	1	3.068E+07	10.15	0.0189	
B-Temperature	8.423E+07	1	8.423E+07	27.87	0.0019	
Residual	1.813E+07	6	3.022E+06			
Cor Total	1.330E+08	8				

Table B.7 Model summary statistics for Milk Clotting Activity for crude ginger enzyme

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	79.81	0.5342	0.3789	-0.3165	1.080E+05	
2FI	**57.54**	**0.7983**	**0.6772**	**0.0285**	**79713.54**	**Suggested**
Quadratic	49.58	0.9101	0.7603	-0.0885	89312.71	
Cubic	11.25	0.9985	0.9877	0.7189	23064.08	Aliased

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

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

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	65499.48	3	21833.16	6.60	0.0344	significant
A-pH	28000.58	1	28000.58	8.46	0.0335	
B-Temperature	15830.93	1	15830.93	4.78	0.0804	
AB	21667.97	1	21667.97	6.55	0.0507	
Residual	16552.68	5	3310.54			
Cor Total	82052.16	8				

Appendix C

Table C.1 Solutions of optimization result for crude kiwi enzyme

Number	pH	Temperature	Time of coagulation	MCA	Desirability	
1	**6.500**	**61.430**	**492.630**	**120.513**	**0.896**	**Selected**
2	6.500	61.926	474.979	123.584	0.816	

Table C.2 Solutions of optimization result for crude ginger enzyme

Number	pH	Temperature	Time of coagulation	MCA	Desirability	
1	**5.500**	**55.000**	**1591.667**	**241.988**	**0.898**	**Selected**
2	5.500	54.913	1558.903	240.895	0.896	
3	5.504	55.000	1572.554	240.789	0.895	
4	5.548	55.000	1374.442	228.356	0.872	
5	5.500	53.500	1029.660	223.243	0.862	
6	6.470	35.000	10288.157	2.310	0.014	

Appendix D

Calibration curve for protease activity

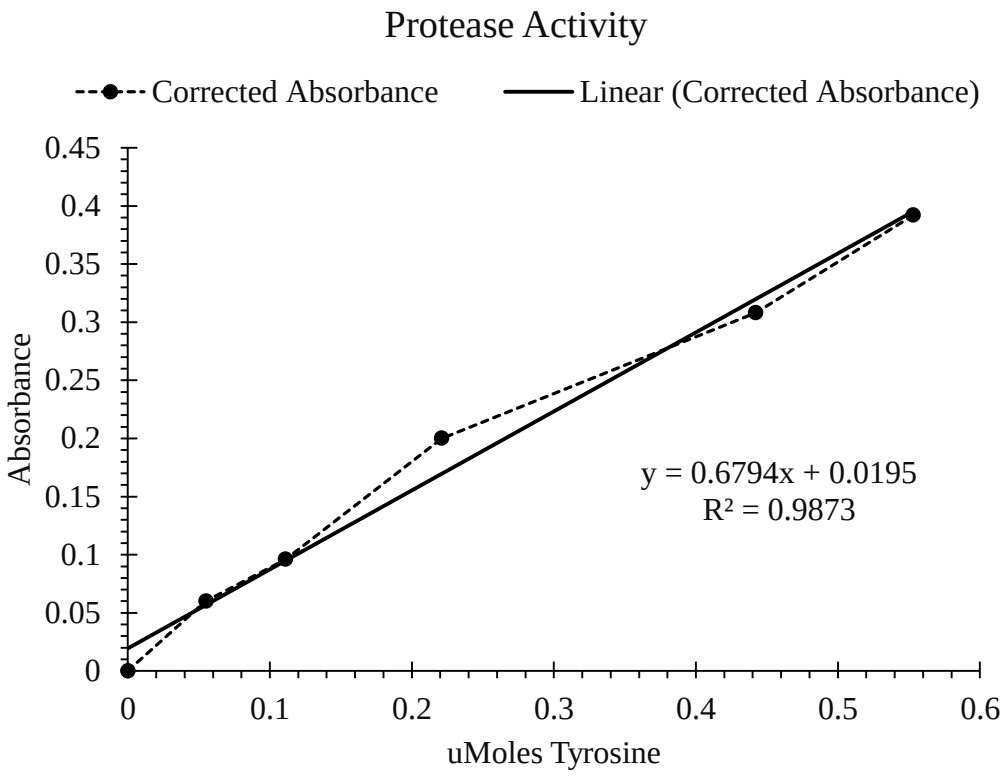

Fig. D.1 Standard curve of L-Tyrosine for protease activity

Calibration curve for protein determination

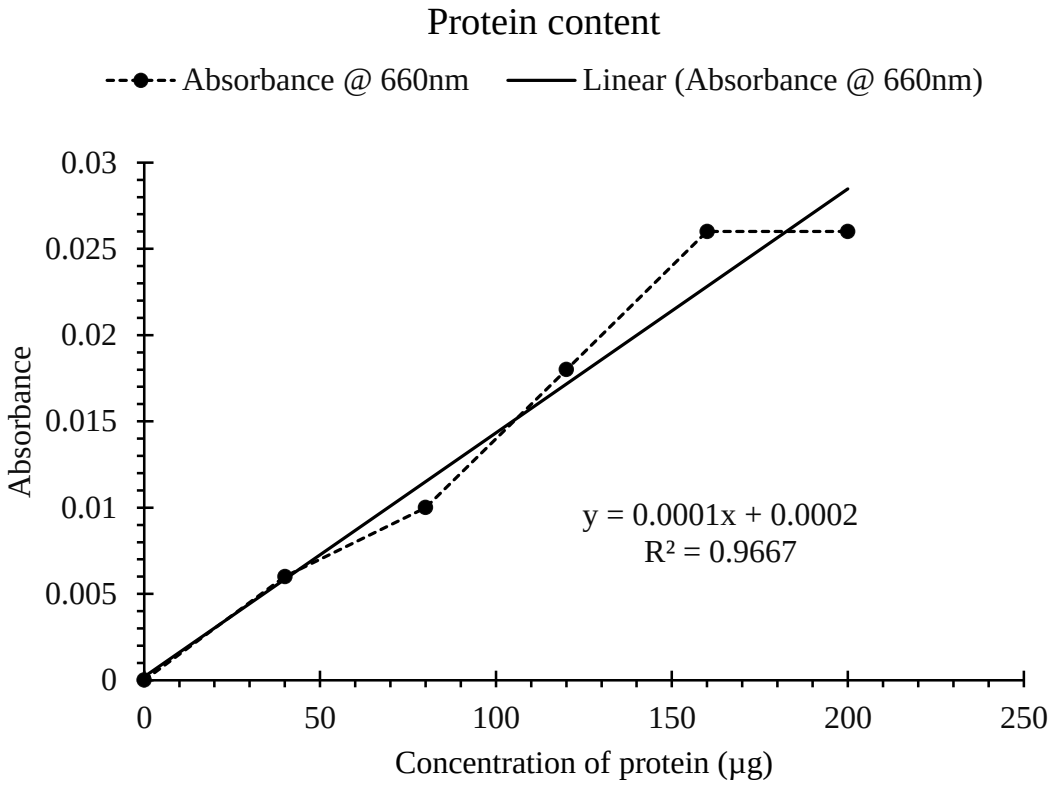

Fig. D.2 Standard curve of protein concentration for protein determination

Appendix E

Table E.1 Theoretical and Actual yields of soft cheese

Source of Variation	Actual Yield	Theoretical Yield
A	$11.84^{b} \pm 0.005$	$14^{a} \pm 0.015$
B	$12.91^{c} \pm 0.085$	$14.88^{b} \pm 0.01$
C	$10.44^{a} \pm 0.075$	$15^{c} \pm 0.011$

Table E.2 Microbiological analysis of soft cheese

Sample	Coliform (cfu/g)	TPC (cfu/g)	Yeast & Mold (cfu/g)
A	ND	$218.67^{a} \pm 6.51$	$26.33^{a} \pm 3.05$
B	ND	$262.67^{b} \pm 8.62$	$27^{a} \pm 4.35$
C	ND	$222.33^{a} \pm 6.81$	$25^{a} \pm 6.24$

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

Appendix F

Statistical analysis (ANOVA Tables)

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

Variate: Moisture Content

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	21.106	23.1641	4871	2.33e-10
Residual	6	0.013	0.002		
Total	8	21.119			

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

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

Variate: Fat

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	3.1489	1.5744	10.19	0.0118
Residual	6	0.9267	0.1544		
Total	8	4.0756			

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

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

Variate: Protein

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	0.6148	0.3074	1.851	0.237
Residual	6	0.1661	0.1661		
Total	8	0.7809			

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

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

Variate: Ash

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	1.3016	0.6508	43.94	0.000261
Residual	6	0.0889	0.0148		
Total	8	1.3905			

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

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

Variate: Acidity

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	0.0126	0.006300	5.4	0.0456
Residual	6	0.0070	0.001167		
Total	8	0.0196			

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

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

Variate: pH

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	1.6422	0.8211	367.7	5.3e-07
Residual	6	0.0134	0.0022		
Total	8	1.6556			

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

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

Variate: Calcium

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	2123.22467	1061.6	193.8	3.54e-06
Residual	6	32.9	5.5		
Total	8	2156.12467			

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

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

Variate: Theoretical Yield

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	1.6373	0.8187	4723	6.44e-09
Residual	5	0.0009	0.0002		
Total	7	1.6382			

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

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

Variate: Actual Yield

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	7.115	3.557	1047	2.77e-07
Residual	5	0.017	0.003		
Total	7	7.132			

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

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

Variate: TPC

Source of variation	Degree of freedom	Sum of squares	Mean of squares	v.r.	F pr.
Sample	2	3641	1820.5	38.27	0.000931
Residual	5	238	47.6		
Total	7	3879			

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

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

Variate: Yeast and Molds

Source of variation	**Degree of freedom**	**Sum of squares**	**Mean of squares**	**v.r.**	**F pr.**
Sample	2	26.71	13.35	0.687	0.545
Residual	5	97.17	19.43		
Total	7	123.88			

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