QUALITY ANALYSIS OF FRESH CHEESE PREPARED USING PARTIALLY PURIFIED MILK CLOTTING PROTEASE FROM GINGER RHIZOME

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

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Approval Letter

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This *dissertation* entitled *Quality Analysis of Fresh Cheese Prepared using Partially Purified Milk Clotting Protease from Ginger Rhizome* presented by **Trilochan Pandey** has been accepted as partial fulfillment of the requirement for the **B. Tech. degree in Food Technology**

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(Trilochan Pandey)

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Abstract

The aim of the present study was to extract, partially purify and utilize the proteolytic enzyme from ginger (*Zingiber officinale)* rhizome for making fresh cheese. The pulp of ginger rhizome was blended with 20mM sodium phosphate buffer (pH 7.0) in the ratio of 1:1(w/v) under cool condition and the extract was centrifuged at 5000 rpm for 10 min at 4°C. The partial purification of the crude extract was obtained by ammonium sulphate precipitation at the concentration of 30-80%. The optimum saturation was selected on the basis of milk clotting activity (MCA). The optimum temperature and pH of milk in the cheesemaking, for maximum MCA and minimum time of coagulation (TOC), were determined by response surface methodology using "Design Expert" software. The physicochemical and microbiological properties of prepared cheeses were compared with rennet cheese. Storage stability of the protease was also determined.

The highest milk clotting activity was observed in the 50% ammonium sulfate precipitation. Numerical optimization study revealed that the optimum condition for milk clotting in cheesemaking was found to be at 55°C and pH 6.5. The optimized TOC and MCA were 28 s and 857.14 unit respectively. The sensory analysis showed that there were significant differences (P<0.05) in texture, spreadability, flavor and aftertaste except overall acceptance among rennet cheese and ginger protease coagulated cheese. The analysis of both cheeses revealed significant differences (P<0.05) in physico-chemical composition, and cheese yield. There was no significant difference (P>0.05) in yeast and mold count between the two cheeses, but there was a significantly higher level of total plate count in ginger protease coagulated cheese. Enzyme activity of protease was decreased gradually during 7 days storage at frozen condition.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CDFT	Central Department of Food Technology
CFU	Colony forming unit
DDC	Dairy Development Corporation
FAO	Food and Agriculture Organization
FDB	Fat on dry basis
FDM	Fat in dry matter
GDL	Glucono delta lactone
GP	Ginger protease
HOSCN	Hypothiocyanous acid
HTST	High temperature short time
IDF	International Dairy Federation

Abbreviation	Full form
LP	Lactoperoxidase
LP-s	Lactoperoxidase system
LTLT	Low temperature long time
MCA	Milk clotting activity
MCE	Milk clotting enzyme
MFFS	Moisture in fat free substances
NSLAB	Non starter lactic acid bacteria
OSCN	Hypothiocyanite
PA	Proteolytic activity
PCA	Plate count agar
PDA	Potato Dextrose Agar
RSM	Response surface methodology
SCN	Thiocyanite
SNF	Solid not fat
TCA	Tri-chloro acetic acid
TG	Triglycerides

Abbreviation	Full form
TOC	Time of coagulation
TPC	Total plate count
VRBA	Violet Red Bile Agar
WFFS	Water in fat free substances
WHO	World Health Organization

Part I

Introduction

1.1　General introduction

Cheese is the generic name for a group of fermented milk-based food products, produced throughout the world in a great diversity of flavours, textures and forms (Fox *et al.*, 2017a). Cheese is a concentrated protein gel, which occludes fat and moisture. Its manufacture essentially involves gelation of cheese milk, dehydration of the gel to form a curd and treatment of the curd (e.g., dry stirring, cheddaring, texturization, salting, moulding, and pressing). The moulded curd may be consumed fresh (shortly after manufacture) (Law and Tamime, 2011). The manufacture of cheese is a form of milk preservation as milk is highly perishable. The method used to clot milk for cheesemaking influences the overall structure, characteristics and firmness of the cheese (Farkye, 2004).

In the past, calf rennet is used in cheese manufacture. Rennet is a well-known enzyme that has been utilized in dairy processing since 6000 BCE. Traditionally, animal rennet is utilized as a milk-coagulant in the dairy manufacturing industry to produce high-quality cheeses. The global demand for cheese production has increased, while the availability of calf rennet has decreased. This led to the search for alternative sources of rennet (Abada, 2019). The selection of an appropriate plant coagulant is important due to rising global cheese demand and a limited supply of calf rennet (Ben Amira *et al.*, 2017). Many extracellular proteases of microbial origin act similar as chymosin and are, partially, suitable for cheese production. Such coagulants can be easily produced by fermentation and are, therefore, almost unlimited available. As the enzymes are not derived from ruminant tissue there are no constraints as regards bovine spongiform encephalopathy or scrapie, and cheeses made with microbial clotting enzymes are accepted by lacto-vegetarians. The enzymes show, however, higher proteolytic activity during cheesemaking, which may lead to a loss of protein degradation products into the whey and thus negatively affect cheese yield (Jacob *et al.*, 2011).

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). 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.*, 2013), artichoke (Cardosin) (Llorente *et al.*, 2014) and fig (ficin) (Liburdi *et al.*, 2019), sunflower seed (Egito *et al.*, 2007), pineapple (bromelain) (Gul *et al.*, 2021) lettuce (lettucine) (Lo Piero *et al.*, 2002), *Moringa oleifera* flowers (Zhao *et al.*, 2022) and thistle flower (Folgado and Abranches, 2020). Increased study and application of plant protease in cheesemaking is a result of political, religious, or cultural issues on the consumption of components obtained from animals (Mazorra-Manzano *et al.*, 2018).

Ginger protease is a cysteine protease characterized by a cysteine residue at the active center of the enzyme. Majority of previous studies focused on the proteolytic activity (PA), purification, structural analysis, and meat tenderization properties of ginger protease (Adhikari *et al.*, 2021). In ancient times, ginger was used as medicine in India, China and Europe. Today, ginger is one of the most important and widely used spices in the world. Ginger is also used in pharmacy due to the presence of the phenolic substances gingerol and shagaol in the rhizome which are reported to have anti-cancer and antioxidant activities (Nafi *et al.*, 2014). Ginger rhizome (*Zingiber officinale*) has often been used as a peptic drug or in other drugs in Chinese medicine and also in various food dishes from ancient times. It has also been applied as a meat tenderizing agent (Hashim *et al.*, 2011a). Also, ginger protease can be used in the proteolysis of animal and vegetal proteins, or as dough adjuster in baking, or milk solidification factor, or addictive in cosmetic (Qiao *et al.*, 2008).

A crucial step in selecting the best rennet substitute is to examine the enzymatic activities (namely milk clotting activity and proteolytic activity). The sensory and rheological qualities of the cheese produced are related to the ratio of milk clotting to proteolytic activities. The high value of this ratio suggests the best product, with desirable firmness and no bitter taste (Mazorra-Manzano *et al.*, 2013). As a result, the research into the use of ginger protease in cheese manufacturing promotes the greater acceptability by the vegetarians and may improve their nutritional intake.

1.2   Statement of the problem

Calf rennet is the most widely used milk clotting enzyme in cheesemaking. However, due to a rise in cheese consumption, calf rennet has been unable to meet the increasing market demand. It is vital to find calf rennet alternatives. Rennet is often limited in its use due to

issues such as the growth cycle, economic concerns, and humanism morality (Liu *et al.*, 2021). Rennet contains chymosin that causes coagulation of milk by cleavage of the bond between Phe_{105}-Met_{106} linkage of κ-casein (Kumar *et al.*, 2010). Extraction and subsequent purification of calf rennet from the tissues of animal stomach require various steps, which makes this enzyme very expensive. Moreover, the reduced supply of calf rennet and calf diseases, like Bovine Spongiform Encephalopathy (BSE), have led to an increase in the demand for alternatives sources of milk coagulants (Adhikari *et al.*, 2021).

Plant proteases are used instead of calf rennet due to its limited availability and high price, religious concerns, vegan, or a restriction on recombinant calf rennet in some countries. However, increased cheese production and a decreasing supply of animal rennet are driving up demand for alternative milk-coagulating sources (Chazarra *et al.*, 2007).

Fresh ginger are seasonal, perishable spices in nature and available in large quantities during the peak season in the local market. After harvesting it cannot be kept for longer period due to higher water activity (a_w) and during storage its suffer from weight loss, shrinkage, rotting and sprouting. Due to lack of processing and value addition practice in ginger, during harvesting season, a huge quantity of fresh produce becomes unmarketable (Alam *et al.*, 2018). The milk coagulating activity of ginger protease is the most important factor in cheese production, indicating its potential as a source of a novel rennet-like enzyme available for dairy production.

So using ginger protease as a novel protease and utilization in the manufacturing of fresh cheese help to fulfill the future need of the protease.

1.3 Objectives

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

1.3.1 General objective

To prepare fresh cheese using partially purified milk clotting protease from ginger rhizome.

1.3.2 Specific objectives

- To extract and partially purify the protease from ginger rhizome.

- To determine milk coagulating activity (MCA), proteolytic activity (PA), and protein content of the ginger rhizome protease.

- To optimize the temperature and pH of milk for maximum milk clotting activity (MCA) and minimum time of coagulation (TOC) in cheesemaking.

- To determine optimum enzyme: substrate ratio for cheesemaking.

- To prepare fresh cheese in the optimized condition of pH and temperature of milk.

- To study physicochemical, microbiological as well as sensory quality of prepared cheese.

- To study the storage stability of the extracted ginger protease.

1.4   Significance of the study

The coagulation of milk by enzymes is an important step in cheese making. The most common method is to use calf rennet to coagulate milk. Plant coagulants are more proteolytic and have broader specificity than chymosin (Esteves *et al.*, 2003).

One of the potential plant that possesses proteolytic enzyme is ginger. The traditional use of ginger protease in producing milk curd has also been reported (Su *et al.*, 2009). Ginger protease is, therefore, a rennet like enzyme that exhibits a strong coagulating activity and hydrolyzes α, β, and κ-casein, and have potential in preparing cheese and oriental dairy foods (Adhikari *et al.*, 2021). Ginger milk curd is a kind of snack in the south China area, produced by mixing the hot fresh ginger juice with milk to form a tofu pudding-like sweet snack. The milk-clotting activity of ginger protease is a key point in making this delicious snack. In the past, studies were focused on the protease activity of ginger proteases (Su *et al.*, 2009). Good milk clotting activity is also attributed to ginger protease and therefore, it is used in the preparation of ginger milk curd in the south of China (Nafi *et al.*, 2014). The use of these ginger proteases as milk coagulants is very interesting since they are natural enzymes that can be used for producing cheeses aimed at lacto-vegetarian consumers and ecological markets (Murtala *et al.*, 2017). Hashim *et al.* (2011b), reported the isolation and characterization of milk coagulating cysteine protease from the ginger rhizomes of Asian cultivars which showed higher milk clotting activity in comparison to both calf rennet and papain.

Plant protease is a rising topic of interest in the cheese industry due to its low cost and simple purification processes (Ben Amira *et al.*, 2017). A cheap and easily available salt, ammonium sulfate is used to purify. Therefore, the ginger protease can be used in cheesemaking at low cost.

1.5 Limitations of the study

The following are some of the difficulties encountered during the thesis:

- Molecular weight of the enzyme was not carried out.
- Casein hydrolysis pattern was not carried out.

Part II

Literature review

2.1   Introduction to cheese technology

Cheese is one of the most popular fermented dairy products, and demand from consumers is rising. It is a great food source of vitamins, minerals, and high-quality protein, including dietary calcium that is easily absorbed (Rana *et al.*, 2017). It is a pleasant and healthy milk product made by coagulating casein with fat under the influence of a proteolytic enzyme. The best approach to store and enhance the consumption of milk is to make cheese (Khan and Masud, 2013). Casein and calcium are important milk components for the transformation of milk into curd (Moatsou, 2019).

Cheesemaking is mainly a dehydration and acidification process in which the fat and protein (casein) in milk are concentrated 6- to 12-fold and the pH is decreased from 6.6 in milk to 4.6 to 5.4 in freshly formed curd. While the manufacturing process varies by variety, the basic steps are coagulation, acidification, dehydration (e.g., cutting the gel, syneresis/whey expulsion of resultant curd pieces by stirring and cooking in the expressed whey, drainage of whey, pressing of curd mass, continued acidification), moulding/shaping (by placing pieces of curd mass in forms and pressing), and salting. Fresh cheeses (such as Mozzarella, Quark, and Cottage cheese) can be eaten instantly, or they can be preserved (matured, ripened) for periods ranging from one week to two years, depending on the variety (e.g., Cheddar, Gouda, Parmesan) (Guinee and O'Kennedy, 2007). Compounds arising from the catabolism of free amino acids contribute directly to cheese taste and aroma (Singh *et al.*, 2003).

Cheese is a centuries-old cuisine with origins dating back before written history. There is no convincing evidence that cheese making began in Europe, Central Asia, or the Middle East, but the practice had spread throughout Europe before Roman times and had matured into a sophisticated process by the time the Roman Empire was created (Civitello, 2011).

2.2   Cheese production and consumption

In 2021, global cheese production amounted to about 21.86 million metric tons. The European Union was by far the top producer of cheese worldwide, with a production volume

of around 10.35 million metric tons of cheese (Shahbandeh, 2021). Cheese output in the world is $\sim 19 \times 10^6$ tonnes per year (35 percent of total milk production) and has expanded at a 4% annual rate over the last 30 years. Within the dairy industry, cheese is the most rapidly growing product. The rising intake of cheese can be attributed to a variety of factors, including a positive dietary image, ease and flexibility of use, and a wide range of flavors and textures (Fox *et al.*, 2017a).

2.2.1 Cheese production- a Nepalese scenario

All the milk produced by smallholder farmers in the Himalayan region of Nepal is converted into cheese. The Dairy Development Corporation (DDC) as well as the private sector manufacture yak cheese. All of the cheese operations are located in the mountainous regions of the country, where cheese is naturally refrigerated. Cheese is made with Swiss technology and is seasonal, meaning that it is only made for seven months of the year and the factories are closed for the five months between December and April. The production of yak cheese in the private sector is rising and has overtaken that of the DDC. In four areas, there are approximately 21 private yak cheese manufacturers (Joshi and Bahadur, 2015). In Nepal, yak cheese is a popular dairy product. The Dairy Development Corporation produces four types of cheese in the public sector: yak, kanchan (cow), mozzarella and processed cheese. Sherpas make a very hard acidic cheese called "Churpi" in the private sector (Neupaney *et al.*, 1997).

Cheese production began in Nepal during 1953/54, when a public-sector cheese plant was built over the Langtang mountain range. Gauri Prasad Sharma and Warner Schulthess, a Swiss national and senior FAO specialist, were the first to create a cheese industry in Nepal (Thapa, 2006). Nepal is rapidly increasing its cheese output. Jamunapari and Saanen goat milk have recently been employed in the country to make soft and hard cheeses, following in the footsteps of France. For the start-up of goat milk production in Nepal, France provided technology and other assistance. The spicy and peppery strong flavor of ripened goat cheeses is distinctive (Bhattarai, 2012).

Six yak cheese factories are operating in four districts (Ramechhap, Dolakha, Solukhumbu, and Rasuwa), four Kanchan cheese factories are operating in two districts (Illam and Panchthar), and one buffalo-milk cheese factory is operating in Nagarkot (Kavrepalanchowk). Dairy Development Corporation (DDC) is responsible for all of these

factories (Thapa, 2006). In comparison to the European and American regions, Nepal has less cheese variations. Due to increased awareness of the health benefits of cheese and the expansion of cattle husbandry, cheese supply and consumption are increasing in Nepal. Cheese production could be an appealing venture to overcome the milk holiday during the surplus season. There may be a lot of potential for exporting cheese if it meets the quality criteria set by national and international agencies (Khanal *et al.*, 2019).

2.3   Varieties and classification of cheese

Cheesemaking is a major industry worldwide, and much of it is still done on a small scale, which accounts for the wide varieties of cheeses available (Little *et al.*, 2008). The diversity of cheeses makes classification difficult, but the most widely accepted approach is one based on moisture content, with further subdivision based on milk type and the role of microorganisms in cheese ripening. The 'softness' or 'hardness' of the cheese is thus directly related to its moisture content, with higher moisture cheeses being softer than low moisture cheeses (Beresford and Williams, 2004).

Cheese is a broad category of foods (perhaps as many as 1500 varieties). Several cheese classifications schemes have been suggested and used to aid customers, retailers, and cheese technologists. Coagulating agent (rennet or acid); texture/moisture content (very hard, hard, semi-hard, semi-soft, soft); matured or fresh; microflora (internal bacterial, surface/smear bacterial, internal or surface mold, propionic acid bacteria) are among the classification criteria (Fox *et al.*, 2017b). According to Khanal *et al.* (2019), there are three types of cheese classification schemes: (i) texture, (ii) coagulation method, and (iii) ripening indices.

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

- Country of origin.
- Raw milk: cow, buffalo, sheep, goat.
- Type of cheese hard, semi-hard, soft, fresh, acid coagulated or whey cheese.
- Internal characters: close or open texture, large medium or small eyes/holes, slit openings in curd blue or white mold ripened, color of curds.
- External characters: rind hard, soft, smooth or rough, smear or mold ripened spices or herbal addition type of coating (plastic, ash).

- Weight of cheese: shape and size.
- Fat in dry matter (FDM)/Fat-on dry basis (FDB): Percentage minimum/maximum.
- Water in fat free substances (WFFS)/ Moisture in fat free substance (MFFS).

Table 2.1 Classification of cheese on the basis of composition, firmness and maturation agents.

Types of cheese	Examples
1. Soft cheese (50-80% moisture)	
a. Unripened low fat	Cottage, Quark, Baker's
b. Unripened high fat	Cream, Neufchatels
c. Unripened stretched curd or pasta filata	Mozzarella, Scamorze
d. Ripened by external mold growth	Camembert, Brie
e. Ripened by bacterial fermentation	Kochkgse, Handkgse, Caciotta
f. Salt cultured or pickled	Feta- Greek; Domiati- Egyptian
2. Semi-soft Cheese (39-50% moisture)	
a. Ripened by internal mold growth	Blue, Gorgonzola, Roquefort
b. Surface-ripened by bacteria and yeast (surface smear)	Limburger, Brick, Trappist, Port du Salut, St. Paulin Oka
c. Ripened primarily by internal bacterial fermentation but may also have some surface growth	Miinster, Bel Paese, Tilsiter
d. Ripened internally by bacterial fermentation	Pasta Filata, Provolone, Low-moisture mozzarella

3.	Hard cheese (39% moisture maximum)	
a.	Internally ripened by bacterial fermentation	Cheddar, Colby, Caciocavallo
b.	Internally ripened by bacterial fermentation (CO_2 production resulting in holes or "eyes")	Swiss (Emmental), Gruyere, Gouda, Edam, Samsoe
c.	Internally ripened by mold growth	Stilton
4.	Very hard cheese (34% moisture maximum)	Asiago Old, Parmesan, Parmigiano, Grana, Romano, Sardo
5.	Whey Cheese	
a.	Heat and acid denaturation of whey protein	Ricotta (60 % moisture)
b.	Condensing of whey by heat and water evaporation	Gjetost (goat milk whey; 13% moisture), Myost, Primost (13-18 % moisture)
6.	Spiced Cheese	Noekkelost - cumin, cloves

Source: Bamforth and Ward (2018)

Table 2.2 Classification of cheese groups on the basis of mode of coagulation

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

Source: Fox *et al.* (2004)

2.4 Soft cheese

Soft cheeses are defined by Robinson (2002) as having more than 61% water in the fat free cheese matter and 10-50% fat in the dry matter (FDM). The FAO/WHO defines soft cheese as cheese that contains more than 67% water in the fat free cheese matter. Data on the composition of various soft cheeses in the literature show moisture contents ranging from 50 to 80%. Because most soft cheeses are consumed fresh, and to reduce the risk of food poisoning, it is critical that the milk or other dairy ingredients used in soft cheese production be adequately pasteurized.

Soft cheeses vary widely in nutritional composition, depending on whether they belong to the ripened or unripened varieties. Fresh, unripened cheeses, such as cottage cheese, are low in fat, relatively low in calcium, high in moisture and contain unfermented lactose (Buttriss, 2003).

2.5 The quality of milk for cheese manufacture

Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, five days after and 15 days before parturition (Parajuli *et al.*, 2018). The properties of milk for cheese can be defined as those that meet the needs of its users-both direct (the cheese manufacturer) and indirect (the cheese user, consumer) (Peri, 2006). According to Guinee and O'Brien (2010), the acceptability of milk for cheese production is defined as its ability to be transformed into cheese and produce cheese of the necessary quality and yield. He emphasized the following quality requirements:

- safety, which refers to the lack of any associated risk (e.g., pathogenic microorganisms, 'toxic' residues) in milk from eating the cheese from which it is manufactured;

- compositional/nutritional, which indicate conformity to minimum levels of particular components (fat, protein, casein, calcium) that make it suitable for cheese manufacture, for example, enable the milk to form a gel suitable for cutting within a certain time after addition of rennet; to give desired manufacturing efficiency (percentage recovery of fat and casein; product yield); and sensory characteristics;

- microbiological, ensuring that total bacterial count does not exceed a maximum value in order to reduce the risk of milk quality (level of intact casein, absence of rancidity associated with hydrolysis of milk fat) being compromised in terms of cheesemaking capacity (rennet coagulability, altered pH levels at various stages of manufacture), cheese yield efficiency (fat and casein recoveries, cheese yield) and cheese quality (flavour and physical properties);

- sensory and functional, implying that it has the desired hedonic (absence of taints) and physico-chemical characteristics (coagulability by rennet under defined conditions), allowing it to be satisfactorily made into cheese with the desired hedonic (taste, smell), usage (techno-functional), and nutritional properties; and

- ethical in terms of purity (unadulterated) and adherence to production standards, such as those governing animal breeding, animal welfare, and agricultural/husbandry systems.

Microbial (both pathogenic and non-pathogenic-bacteria), chemical, compositional, physicochemical, enzymatic, and adulteration concerns are some of the criteria that can be used to characterize milk quality (Sheehan, 2013). Microorganisms (ideally 10^4 CFU/ml milk) and somatic cells (100,000/ml) should be low in good quality milk for cheesemaking. To achieve this, the milk must come from healthy cows and be as fresh as possible, with as little time in the bulk tank as possible. Cold storage can lead to the growth of psychrotrophic bacteria and calcium phosphate leaking from micelles. If the milk needs to be stored, it should be thermized first to prevent the formation of microorganisms. The milk should have a high protein concentration, which is beneficial to cheesemaking. The milk should have a

good quality flavor with no off-flavors. The milk should be free of pathogens and clostridia, however helpful lactic acid bacteria may be present. Antimicrobial agents should not be present in the milk (Skeie, 2010).

2.5.1 Milk composition and constituents

Milk is a heterogeneous mixture that can be defined as a complex chemical substance in which fat is emulsified as globules (2,000–6,000 nm), major milk protein (casein, 50–300 nm), and some mineral matters are colloidal, and lactose is in true solution with some minerals and soluble whey proteins (4–6 nm). The diameter of lactose, salt, and other minor soluble constituents is 0.5 nm. In one ml of milk, there are 10^{10} milk fat globules, 10^{14} casein micelles, 10^{77} whey protein and 10^{19} lactose molecules respectively. Multiple constituents in various states and phases coexist in a delicate balance that ensures milk stability under normal conditions. Normal milk has a pH range of 6.5 to 6.7 (Mehta, 2015).

The composition of milk is influenced by several factors, including individuality of animal, stages of milking, intervals of milking, completeness of milking, frequency of milking, irregularity of milking, portion of milking, lactation period of milk, milk yield, season, feed, health status, age, weather, exercise and excitement (Chandan, 2007). Cheese made of buffalo milk has higher fat, protein, ash and total solids than cheese made of cow milk (Mijan *et al.*, 2010). Cheese composition and ultimately the interaction between casein molecules and adjacent micelles determines the firmness, melt, and chewiness of a cheese (Johnson *et al.*, 2001).

2.5.1.1 Milk protein

Milk proteins are divided into two major groups: casein and whey proteins, which differ fundamentally in their properties, particularly their solubility when the pH of milk is adjusted to 4.6 (isoelectric point) (Mehta, 2015). Casein is a mixture of α_{s1}-, α_{s2}-, β-, and κ- casein. The κ-casein protects the α_s- and β- caseins from calcium ion precipitation. However, κ-casein is easily attacked by the rennet enzyme, which splits off a portion of the κ- casein molecules, rendering it ineffective as a protective agent. As a result, in the presence of Ca ions, casein precipitates, which is the foundation of cheese production (Walstra *et al.*, 2006). Casein is responsible for the solid structure and texture of cheese, as well as the retention of fat and moisture in the cheese, whereas whey proteins are soluble in milk liquid and are largely removed from the final cheese product during draining (Provost *et al.*, 2016).

2.5.1.2   Milk fat

Milk fat plays a central role in dairy products. Many of the physical properties, manufacturing qualities, and organoleptic characteristics of dairy products are dependent on fat (Harvatine *et al.*, 2009). Milk fat is the most variable of all the milk constituents. It is mainly a mixture of triglycerides (TG). The other 1-2% of milk fat is composed of phospholipids, steroids, carotenoids, and fat soluble vitamins A, D, E, and K (Chandan, 2008). The milk fat in cheese contributes to its flavor, texture, color, and functional behavior. Fat has a variety of effects in cheese, including indirect effects on the metabolic functioning of the microbiota during cheese maturation (Waldron *et al.*, 2020).

Fat is a major component of the composition of most cheese varieties, and significant changes in its level result in aggressive forms in moisture, protein, and cheese yield. The protein-to-fat ratio in cheese milk is considered as the most important element in determining fat content. This is because it regulates the relative quantities of two of the three key compositional components of cheese, namely protein and fat. The third major component of cheese is moisture content (Guinee and McSweeney, 2006).

2.5.1.3   Milk salt

Milk salts are primarily sodium, potassium, calcium, and magnesium phosphates, citrates, chlorides, sulphates, carbonates, and bicarbonates. A variety of factors influence the composition of milk salts, including breed, individuality of the cow, stage of lactation, feed, mastitis infection, and season of the year (Fox *et al.*, 2015). Milk salts are important in the formation and stability of casein micelles. Many important functional properties of milk products are influenced by milk salts, including gelation, protein stability, emulsification, foaming, and cheese texture (Lucey and Horne, 2009). The calcium content of milk influences rennet coagulation. Cheeses with higher calcium content (higher cheese pH) display higher levels of protein aggregation than cheeses with lower calcium content (lower cheese pH) (Pastorino *et al.*, 2003). Due to calcium-dependent interactions between casein proteins, calcium influences the rheological and functional properties of cheese. Rennet coagulation is the first manufacturing step in the production of most cheese varieties, and calcium has a significant impact on this process (Papademas and Bintsis, 2017).

2.5.2 Abnormal milk

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

2.5.2.1 Mastitis milk

Mastitis is regarded as the most common and risky production disease in developed-country dairy herds (Seegers *et al.*, 2003). Mastitis is an inflammation of the mammary gland that is usually caused by a bacterial infection. An influx of white blood cells causes an increase in the number of somatic cells in the milk during the inflammatory response (Mazal *et al.*, 2007). Mastitis has an impact on total milk output, as well as milk composition and technological usability (Ogola *et al.*, 2007). Mastitis significantly reduces the lactose and casein: protein ratio while increasing proteolysis. Mastitis increases the moisture content of cheese and causes fat and protein losses (Martí-De Olives *et al.*, 2020).

2.5.2.2 Colostrum milk

Colostrum is the first milk produced after birth and contains a high concentration of immunoglobulins, antimicrobial peptides (such as lactoferrin and lactoperoxidase), and other bioactive molecules such as growth factors (Playford *et al.*, 2000). In comparison to milk, colostrum contains less lactose and more other nutrients such as fat, protein, ash, vitamins, hormones, and immunoglobulins. After three days, the lactose content increases while the percentage of the other components gradually decreases (Simon *et al.*, 2022).

2.5.2.3 Late lactation milk

The stage of lactation influences milk fat, protein, and calcium content, but not casein: protein ratio or phosphorus content. Late lactation produces milk with a higher pH and urea content. Late lactation milk is higher in calcium content (Coulon *et al.*, 1998). Due to its large sodium and potassium content, late lactated milk is poor for cheese making, resulting in more protein hydrated than normal milk (De, 2000).

2.5.3 Microbiological quality of raw and pasteurized milk

Milk is an excellent growth medium for many microorganisms. Because it is impossible to avoid contamination of milk with microorganisms due to the specific production, the microbial content of milk is a major feature in determining its quality (Torkar and Teger, 2008). Bacterial contamination in raw milk can occur from a number of sources, including the air, milking equipment, feed, soil, faeces, and grass (Coorevits *et al.*, 2008). In general, milk from healthy cows is sterile inside the mammary gland. Despite this, the number and type of bacteria found in milk immediately after milking are linked to direct contact with contaminated sources in a dairy farm environment, such as air, soil, worker hygiene, fecal matter, grass, and excretion from an infected udder of animal (Angulo *et al.*, 2009).

Pasteurization is the process of heating milk for a specific amount of time at a specific temperature. Pasteurization is used to kill microorganisms, increase safety, and extend the shelf life of milk and dairy products. Despite the fact that pasteurization kills most vegetative bacterial cells, some species, such as thermoduric microorganisms and, on rare occasions, Gram-negative rods, can survive and propagate in the final product, compromising the safety and quality of dairy products. As a result, careful microbiological monitoring of milk is required both before and after pasteurization. It aids in the evaluation of the effectiveness of milk heat treatment as well as the detection of potential post-contamination (Nada *et al.*, 2012).

The milk used to make cheese must be of high microbiological quality. There should be no pathogenic bacteria. Heat-resistant lipases and proteases produced by psychotropic bacteria may reduce yield but also cause undesirable flavors in ripened cheese. Clostridia spores cause late blowing in cheeses with eyes, but they also cause inedible flavors in cheeses with closed textures. Antimicrobial agents such as antibiotics, detergents, and so on must be avoided because lactic acid bacteria acidification will be greatly influenced (Skeie, 2007).

2.6 Pre-treatments of milk for cheesemaking

For centuries, milk for cheese-making was not pre-treated before curdling, and many cheese variations around the world are still made from raw milk, particularly but not only artisanal cheeses. However, most cheese-making today involves the treatment of milk by one or more processing steps prior to the addition of coagulant and starter culture, primarily for reasons

of safety, but also for consistency of quality and manipulation of product characteristics (Kelly *et al.*, 2008). Pretreatments have a significant impact on the cheese manufacturing schedule, cheesemaking efficiency, physicochemical, microbiological, and organoleptic properties of cheese, as well as shelf life (Walstra *et al.*, 2006).

The various treatments used are as follows:

- Chilling and cold storage
- Lactoperoxidase/thiocyanate/H_2O_2 treatment
- Thermization
- Bactofugation
- Microfiltration
- Standardization
- Pasteurization
- Homogenization
- CO_2 treatment

2.6.1 Chilling and cold storage

Due to the perishable nature of milk, immediate chilling of raw milk on the farm is suggested; however, this is practically impossible in underdeveloped nations for a variety of factors. Chilling raw milk below 4 °C is recommended and widely used around the world to extend its shelf life (Ajmal *et al.*, 2018). Chilled milk can undergo several biochemical changes. Proteases and lipases, which can hydrolyze milk proteins and fat, can be produced by several species of the genera Bacillus and *Pseudomonas fluorescens*. When the population of psychrophilic bacteria reaches 10^7 to 10^8/ml during raw milk storage at lower temperatures, the milk's microflora changes (Datta and Deeth, 2001).

2.6.2 Lactoperoxidase treatment

Lactoperoxidase (LP) is a member of the peroxidase family, a group of natural enzymes found in plants and animals, including humans. LP is a prominent enzyme found in bovine milk that catalyzes the inactivation of a variety of microorganisms in the lactoperoxidase system (LP-s) (Kussendrager and Van Hooijdonk, 2000). Lactoperoxidase system is made up of three components: the lactoperoxidase enzyme, thiocyanate, and hydrogen peroxide (H_2O_2). Lactoperoxidase oxidation of thiocyanate (SCN) produces intermediate

antimicrobial materials such as hypothiocyanite (OSCN) and hypothiocyanous acid (HOSCN). These materials have the potential to inhibit microorganism growth by oxidizing sulfhydryl (–SH) groups in their enzyme systems (Saravani *et al.*, 2019).

The lactoperoxidase method can be used to preserve raw milk instead of chilling and to solve the problem of psychrotrophs in stored milk. The ability of starter organisms to produce H_2O_2 results in the reactivation of the lactoperoxidase system unless lactoperoxidase is inactivated by appropriate heat treatment (e.g., 78°C/15 s). However, because such extreme heat treatment is not commonly used for cheese milk, lactoperoxidase is likely to be present in milk treated with lactoperoxidase. As a result, there is a possibility of reactivation of the lactoperoxidase system, which could cause problems in cheese production due to suppression of starter activity (FAO, 1999).

2.6.3 Thermization

Thermization is a heat treatment performed at sub-pasteurization temperature (57-65℃ for 10-20s) that could be used to treat raw milk and improve the safety of raw milk cheese without affecting the organoleptic properties (Centorotola *et al.*, 2021). Thermization of raw milk for cheese is an empirically preferred preventive measure over pasteurization due to its milder effects on raw milk flora and the functionality of milk caseins and salts. These milk factors are important for improving the texture of the milk curd and the sensorial qualities of the finished cheese (Samelis *et al.*, 2009). Thermization has little effect on the renneting properties of cheese milk (Panthi *et al.*, 2017).

2.6.4 Bactofugation

Cheese can be affected by a wide variety of microorganisms, including *Clostridium*, coliforms and yeasts. *Bacillus* spores and *Pseudomonas* species can produce proteolytic and lipolytic enzymes affecting cheese quality. Milk with high levels of somatic cells is known to adversely affect cheese making (coagulation time, yield, and quality of cheese, especially flavour and texture) (Te Giffel and Van Der Horst, 2004). The presence of enzymes (catalases, proteases, and lipases) in somatic cells also has putative effects on cheese quality and yield (Maubois, 2002). Bactofugation of milk is the process of removing from milk the spores of organisms that undergo late acid fermentation ('late blowing' defect) - a condition known as a cheese-making defect. Temperature of bactofugation is 50-68℃. Bactofuges are currently classified into two types. The first type is a one-phase bactofuge, which functions

similarly to a standard clarifier. It only has one outlet at the top. The second type is a two-phase bactofuge, which is similar to a cream separator. It has two top outlets, one for bacteria-reduced milk and one for continuous discharge of bactofugate (about 3% of total liquid flow) via a special top disc. In the cheese industry, double bactofugation is used to effectively reduce the load of bacterial spores (spore removal up to 99 %) (Gésan-Guiziou, 2010).

2.6.5   Microfiltration

The microfiltration process separates particles with molecular weights greater than 200,000 Da. Milk materials removed by microfiltration include somatic cells, fat globules, bacteria, casein micelles, aggregated whey components, β-casein, and β-lactoglobulin, depending on the membrane pore size (Mistry and Maubois, 2017). Microfiltration of skim milk prior to cheesemaking has the advantage of allowing milk serum proteins to permeate the membrane and thus not be retained in the cheese, whereas caseins are retained (Papadatos *et al.*, 2003).

2.6.6   Standardization

To produce a cheese with the desired solids composition, milk was generally standardized to the desired casein to fat ratio or at least to a desired fat level (Johnson and Lucey, 2006). Standardization of milk protein/casein levels, either by concentration of milk solids or by producing a protein rich fraction that may be subsequently added to milk prior to cheese manufacture, may be used to reduce some negative defects associated with a seasonal milk supply, such as variable protein/casein contents, which result in poor curd-forming properties and variations in yield, composition, and consistency of resultant cheeses. Reduced losses of fat and casein particles in whey and better retention of whey proteins in the aqueous phase of cheese result in increased yield (Kelly *et al.*, 2008).

2.6.7   Pasteurization

The production of cheese from raw milk obviously increases the risk of pathogenic bacteria survival and incorporation into cheese. Pasteurization is a process used to make milk safe for human consumption by inactivating the most heat-resistant vegetative pathogenic bacteria found in raw milk (*Mycobacterium tuberculosis* and *Coxiella burnettii*) (McSweeney, 2007). Milk pasteurization was first done in vats or kettles at temperatures around 63–65°C for 30 min i.e., Low Temperature Long Time (LTLT pasteurization), and

more recently in continuous-flow plate heat exchangers at 72–74℃ for 15–30 s i.e., High Temperature Short Time (HTST pasteurization). Pasteurization is the only treatment applied to cheese-milk for a large proportion of cheese varieties. Pasteurization also inactivates some enzymes, corrects shifts in milk mineral balance caused by cold storage, and influences the microflora of non-starter lactic acid bacteria (NSLAB) in the final cheese (Kelly *et al.*, 2008). While pasteurization reduces the risk of producing poor quality cheese due to the growth of undesirable bacteria and destroys food-poisoning microorganisms, it may harm the cheesemaking properties of milk if the heat treatment is too severe (due to heat denaturation of the whey proteins and their interaction with κ-casein) (Fox *et al.*, 2017a).

2.6.8 Homogenization

Homogenization of cheese-milk can be useful in cheese-making for preventing creaming of fat globules, reducing fat losses in the whey, or controlling the development of free fat in the cheese (Kelly *et al.*, 2008). Homogenization is typically done at 60°C, and the milk is processed to break down milk fat globules into fine lipid droplets, preventing cream separation and increasing the stability and shelf life of the milk emulsion. A two-stage homogenization process is commonly used, in which the first stage reduces the size of fat globules and the second stage disrupts any clusters that form. Although homogenization of whole milk reduces curd forming properties and curd syneresis, it improves rennet action and increases cheese yield due to improved fat recovery (Zamora *et al.*, 2007).

2.6.9 CO$_2$ treatment

CO$_2$ can be added to raw milk as an anti-microbial agent (Hendricks and Hotchkiss, 1997; Martin *et al.*, 2003). The addition of 20-30 mmol/L at refrigeration temperatures has been recommended for the extension of raw milk storage, while keeping in mind the pressure and temperature conditions of the treatment, as they can promote protein precipitation (Rajagopal *et al.*, 2005). The effect of CO$_2$ on various species and strains is variable. In general, the lag phase of aerobic plate counts increases substantially and more dramatically than the increase in psychrotrophs lag phase. Inhibition of coliforms by at least 1 log CFU/ml is also possible. This treatment has a more pronounced effect on Gram-negative bacteria than on Gram-positive bacteria and spores (Martin *et al.*, 2003; Singh *et al.*, 2012). However, the reduction in pH caused by CO$_2$ may cause dissociation of the casein

micelles, which can be detrimental for the efficiency of heat exchangers due to fouling (Loss and Hotchkiss, 2003).

2.7   Additives in cheese milk

A variety of additives are used in the production of cheese milks and cheese curds. The additives commonly used in cheese processing are listed below.

- Salt is used to restore the calcium balance in milk.
- Salt is used to inhibit to undesirable organisms.
- Acid is used alone or as supplements to lactic acid.
- Colours and bleaching agents.
- Added flavours in spices and herbs (Robinson and Wilbey, 1998).

Starters are bacterial cultures used in cheese production to produce lactic acid through controlled lactose fermentation, resulting in a pH decrease. Starter cultures can also contribute to an open texture or eye formation by producing CO_2 and to microbial safety by lowering pH, reducing redox potential, and inhibiting pathogens competitively. Starters also affect cheese flavor and aroma via citrate metabolism or the activities of peptidases, esterases, lipases, and other enzymes released during ripening (McSweeney, 2007). Starter cultures are commonly classified as mesophilic (optimum temperature of ~30°C) or thermophilic (optimum temperature of ~42°C). Mesophilic cultures are mostly *Lactococcus lactis* subsp. *cremoris*, with a few *Lactococcus lactis* subsp. *lactis* and/or *Leuconostoc* sp. *Streptococcus thermophilus* and either *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *lactis*, or *Lactobacillus helveticus* are present in thermophilic cultures (Fox *et al.*, 2017a).

2.8   Rennet

Rennet is a clotting agent that is used in the production of many cheeses. Its main purpose is to coagulate milk protein like casein. The proteolytic activity of rennet added to milk facilitates the coagulation of milk proteins to 'curds' in the cheese production process (Bezie and Regasa, 2019).

The most commonly used milk clotting enzyme in the cheese industry is calf rennet, which is derived primarily from the fourth stomach of suckling calves (Ben Amira *et al.*,

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2017; Kethireddipalli and Hill, 2015; Mamo and Balasubramanian, 2018) . Calf rennet has a high milk clotting activity/proteolytic activity (MCA/PA) ratio and is heat sensitive, resulting in high cheese yield (Moschopoulou, 2011). Calf rennet was the first milk clotting enzyme used in cheese production, and it is also well known for its high specificity for cleaving caseinomacropeptide from κ-casein (Liu *et al.*, 2021). The Phe_{105}–Met_{106} site of κ-casein in casein is hydrolyzed by calf rennet. The hydrophilic macropeptide and para-κ-casein diffuse into the whey. More than half of the κ-casein is hydrolyzed at pH 5.2–6.6 and temperature 35℃. The para-κ-casein approach each other to form the three-dimensional network micelle structure (Ghorbel *et al.*, 2003). The calcium ions then neutralize the negative charge on the micelle surface by acting as a "bridge" between the micelles, causing them to aggregate and appear curd (Liu *et al.*, 2021). Calf rennet is particularly expensive since it must be extracted from animal stomach tissues and then purified in a number of procedures (Adhikari *et al.*, 2021). Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactorily replace calf rennet produced by genetically engineered bacteria have proven suitable substitutes for animal rennet, but increasing attention has been directed towards natural rennet extract from plants (Kheir *et al.*, 2011).

2.9 Plant protease as rennet substitute

Proteases are enzymes that can hydrolyze peptide bonds. They can act at the ends of polypeptide chains (exopeptidases) or within them (endopeptidases) (Palma *et al.*, 2002). Cysteine proteases, serine proteases, metalloproteases, and aspartic proteases are the four catalytic classes of proteases (Van der Hoorn, 2008). Rennet substitutes have the following features:

- high clotting to general proteolysis ratio;
- proper specificity on κ-casein;
- good activity in milk;
- easily denatured during whey processing (so that products made from whey do not contain active coagulant) (McSweeney, 2007).

Plant-derived proteases have a high potential as processing aids in the production of cheese, food (e.g., the production of novel dairy products, meat tenderizers, and protein

hydrolyzates), and medicine (e.g., digestive and anti-inflammatory agents) (Huang *et al.*, 2011; Katsaros *et al.*, 2010). According to Roseiro *et al.* (2003), the flavor of cheese made with plant protease is slightly more acidic and bitter than cheese made with rennet. As milk clotting agents, several plant proteases are used. Some have a high level of milk clotting activity, while others have a low level.

2.9.1   Production of plant proteases

Plant proteases are important in a variety of biological processes that occur throughout the life cycle of plant. Plant germination is one of the most important stages in which proteases play a key role, as it involves the hydrolysis and mobilization of other proteins accumulated in seeds and cereal grains (Martinez *et al.*, 2019). Plant proteases can be obtained in vivo (via a biological vector expressing a specific vegetable protein), in vitro (via cell, tissue, and organ cultures), or directly from vegetable biomass (latex, flowers, fruits, and roots) and can be extracted from natural sources (Feijoo-Siota and Villa, 2011).

2.9.1.1   Production from natural sources

Aqueous maceration of various plant organs such as flowers, seeds, roots, and leaves can be used to extract plant proteases from natural sources (Shah *et al.*, 2014). Depending on the degree of purification, the crude extract can be further purified to obtain partially purified enzyme or pure enzyme. Precipitation with ammonium sulfate is an efficient method for producing large amounts of active proteases (Barros *et al.*, 2001).

2.9.1.2   In vitro production

To increase protease volumetric productivity, in vitro tissue culture can be performed using genetically modified plants. In vitro methodologies are classified into two types: (i) micropropagation and somatic embryogenesis, and (ii) callus and cell suspension cultures (Troncoso *et al.*, 2022). *Mirabilis jalapa* culture (Tamer and Mavituna, 1997), *Centaurea calcitrapa* (Raposo and Domingos, 2008; Reis *et al.*, 2000) cell suspension culture, and *Silybum marianum* (Cimino *et al.*, 2006) and *Cynara cardunculus* (Oliveira *et al.*, 2010) callus culture were used to produce proteases. Pérez *et al.* (2013) used micropropagation to create enzymes from *Hohenbergia penduliflora*. Lufrano *et al.* (2012) investigated the molecular cloning, expression, and characterization of procirsin from the flowers of *Cirsium vulgare*.

2.9.1.3   In vivo production

Protease production through in vivo is a complex process that can include post-translational modifications (proper folding, glycosylation, and phosphorylation) of the proteins to improve their proteolytic activity and stability (Streatfield, 2007). Aspartic, cysteine, and serine proteases have been successfully produced in invivo from a wide range of tissues, including seed (*Arabidopsis thaliana*, rice, and barley), leaves (tomato and potato), and flowers (cardoon). Cardosin A (*Cynara cardunculus*) is an aspartic protease whose accumulation in pistils tissue has been increased through in vivo production. As a result, the proteases have increased milk-clotting activity and their specificity to the substrate target (κ-casein) (Castanheira *et al.*, 2005).

2.9.1.4   Plant protease applications

Apart from calf rennet, proteolytic enzymes from different animal species, microbial proteases, and proteases derived from fruits and plants can all be used to coagulate milk (Hashim *et al.*, 2011a). Plant proteases are becoming more common in industrial applications. For decades, vegetable proteases have been used in manufacturing processes such as cheese-making, dairy industry, meat tenderization, brew industry, leather tanning, and peptide production (Troncoso *et al.*, 2022). Plant proteases can coagulate milk proteins and have thus been used as milk clotting enzymes in cheesemaking for centuries. These proteases are used in either crude or purified form as a substitute for calf rennet (Shah and Mir, 2019). Exogenously added proteolytic enzymes from plants, such as papain, bromelain, and ficin, are commonly used in commercial meat tenderization (Abdel-Naeem and Mohamed, 2016). Garg and Mendiratta (2006) discovered that proteases derived from ginger rhizome and *Cucumis trigonus* Roxb plant fruits were effective in meat tenderization.

2.10   Ginger (*Zingiber officinale*)

Ginger is herbaceous plant in the Zingiberaceae family. Perennial herbs with creeping horizontal or tuberous rhizomes, lobed thick structures, branched with tuberous joints that grow horizontally, and a thin skin with a yellow or brown color. Ginger (*Zingiber officinale*), a monocotyledonous perennial plant native to Asia, is widely used as a food and dietary supplement, as well as in traditional medicine in several countries. Fresh ginger is mostly made up of water (80.9%), carbohydrates (12.3%), fiber (2.4%), proteins (2.3%), minerals

(1.2%), and lipids (0.9%). Ginger contains potassium, calcium, phosphorous, sodium, and iron, as well as thiamine, riboflavin, niacin, and vitamin C (Beristain-Bauza *et al.*, 2019).

2.10.1 Ginger protease (Zingibain)

Ginger protease is a cysteine protease that belongs to the peptidase family C1 (papain family) and is distinguished by a cysteine residual at the active center of enzyme (Huang *et al.*, 2011). Ginger protease (GP) or zingibain, which was discovered as a new source of protease in 1973, has remarkable proteolytic activity. GP is an extremely effective meat tenderizer against collagen and other connective tissue proteins. Because of its milk clotting activity, GP is used in the preparation of ginger milk curd in the south of China (Nafi *et al.*, 2014). Zingibain from ginger received a considerable commercial importance due to their activity properties over a wide range of temperature and pH (Gagaoua *et al.*, 2015).

2.10.2 Characteristics of ginger protease

Ginger proteases are found in ginger rhizomes and are separated into two fractions (GP I and GP II) with a molecular mass of approximately 22.5 kDa. The complete amino acid sequence for GP II is 221 amino acids long, while approximately 98% of the amino acid sequence for GP I is known. The protease activity of ginger proteases has a pH optimum of 5.0–5.6 when combined, but the protease activity of GP I and GP II has a pH optimum of 6.5–7.0 when separated (Su *et al.*, 2009). Ginger proteases are also capable of hydrolyzing native collagen, showing preferences for the peptide bonds where Pro is in the P2 position (Huang *et al.*, 2011). Gagaoua *et al.* (2015) separated GP into three fractions by isoelectrofocusing, following which the 3-D structure of GP-II was elucidated by X-ray crystallography. They were shown to have high activity against protein substrates such as gelatin, casein, bovine serum albumin and collagen. They are inactivated by sulfhydryl reagents (Gagaoua *et al.*, 2015). 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). The protease extracted from the *Taffin Giwa* ginger rhizome cultivar that was obtained from northwestern Nigeria showed optimum activity at temperatures near 60 °C and at a broad range of pH values of 5.0 to 8.0. The enzyme protein was completely denatured at 100 °C and pH value of 12 (Murtala *et al.*, 2017). Su *et al.* (2009) also reported that ginger proteases could be a choice for cheese making as well as a milk-clotting enzyme source to improve on the bitterness of milk products caused by papain, ficin and bromelain.

2.10.3   Effect of substrate on zingibain activity

There are two isoforms of ginger protease, and both isoforms have unique substrate specificity with preferential peptide cleavage with Pro at the P_2 position. Ginger protease to develop a novel gelatin (heat-denatured collagen) hydrolysate because collagen is a Pro-rich protein (approximately 20%) (Taga *et al.*, 2017). Ginger protease has high protease activity toward various proteins, such as gelatin and casein, and is the only reported plant collagenase with the ability to hydrolyze native collagen (Taga *et al.*, 2016).

2.10.4   Milk clotting activity of zingibain

Ginger protease possessed high milk clotting activity (MCA) and proteolytic activity (PA), and exhibited a higher ratio of MCA to caseinolytic activity at temperatures above 60°C, proving its potential use as a rennet substitute and accelerating agent in the dairy industry; for example, in cheese maturation and manufacture of cheese (Huang *et al.*, 2011). The extracted enzyme displayed optimum milk clotting activity at pH 5, temperature 35°C and enzyme concentration 15µl/ml of milk (Adhikari *et al.*, 2021).

Table 2.3 Purification of milk coagulating protease from ginger rhizome

Purification step	Total activity (Unit)[a]	Total protein (mg)	Specific activity (Unit/mg) [b]	Purification (fold)	Recovery (%)
Crude extract	34,210	1612	21.22	1.0	100
$(NH_4)_2SO_4$ (20-65%)	29,500	523	56.40	2.66	86.23

Source: Hashim *et al.* (2011b)

[a] One protease activity unit is defined as the amount of enzyme needed to raise the absorbance of 1.0 in 1 h at 440 nm.

[b] Specific activity is the number of protease activity unit/mg protein.

Table 2.4 Partial purification of ginger protease extracted from *Taffin Giwa* ginger cultivar from northwestern Nigeria

Purification step	Proteolytic activity (Unit)[a]	Total protein (mg)	Specific activity (Unit/mg)	Purification (fold)	Recovery (%)
Crude extract	300.0 ± 07	674.81	2.25	-	-
$(NH_4)_2SO_4$ (15%-30%) saturation	71.1 ± 13	92.02	1.95	0.57	57%

Source: Murtala *et al.* (2017)

[a] One Unit of enzyme activity is the amount in micromoles of tyrosine equivalents released from casein per minute.

2.10.5   Application of ginger protease in cheesemaking

Ginger protease may have potential application for the manufacture of cheese. The influence of ginger protease in comparison to calf rennet on the physicochemical, microbiological, and sensory characteristics of Peshawari cheese manufactured from cow's milk was examined. Importantly, no bitterness was noted in the Peshawari cheese made with ginger protease (Hashim *et al.*, 2011a). Adhikari *et al.* (2021) explained that the potential use of ginger protease as a coagulant in the preparation of mozzarella cheese. Yonas *et al.* (2014) explained that the possibility of making soft unripened cheese from camel milk by coagulating it using locally available ginger crude extract.

2.11   Protein precipitation

Precipitation is useful as it can be used to denature the protein, destroying its drug binding ability depending on the binding mechanism (Polson *et al.*, 2003). Proteins must be brought into solution by breaking down tissue or cells or extracted from various sources, depending

on the source. To extract proteins from solid samples, they must first be disintegrated. There are several methods for accomplishing this task, including repeated freezing and thawing, sonication, homogenization by high pressure, filtration and permeabilization by organic solvents. In the case of tissues and cells, each method consists of three steps: sample disintegration, protein extraction, and precipitation (Novák and Havlíček, 2016). Different methods for protein precipitation are ammonium sulfate precipitation, acetone precipitation, acetone-Trichloroacetic acid precipitation, Trichloroacetic acid (TCA) precipitation and chloroform/methanol (Jiang *et al.*, 2004; Xue-xue *et al.*, 2011).

2.11.1   Ammonium sulfate precipitation

Protein purification using selective ammonium sulfate precipitation is a common method (Wood, 1976). Because of its high solubility, ammonium sulfate, $(NH_4)_2SO_4$, is frequently used for salting out due to its low price and availability of pure material (Duong-Ly and Gabelli, 2014). Ammonium sulfate precipitation is a quick and low-cost method of concentrating large starting volumes. Polypeptides "salt out" at high salt concentrations because the salt competes with the polar side chains of the protein for ion pairing with the water molecules and reduces the effective volume of solvent. The amount of ammonium sulfate needed to precipitate a given protein is determined primarily by the surface charge, surface distribution of polar side chains, and polypeptide size, as well as the pH and temperature of the solution (Harlow and Lane, 1988).

Proteins in solution can also be fractionated out since they will precipitate out as a function of salt concentration. The high salt content inhibits any microbial growth or protease activity. This is because salt precipitation only changes the solubility of proteins, not their nature, as they become salt-solubility changes (Mukherjee, 2019). Dialysis of the protein solution against large volumes of the desired buffer easily removes ammonium sulfate. Although dialysis is still a popular method of salt removal, it is time-consuming (Kent, 1999).

2.11.2   Trichloroacetic acid (TCA) precipitation

TCA (Trichloroacetic acid) is a very effective protein precipitant. TCA is added to the extract at a final concentration of 10–20%, and the proteins are allowed to precipitate for 30 min on ice. In this method, tissue can also be homogenized directly in a 10–20 % TCA solution. Proteolysis and other protein changes are limited using this method. The pellet should next

be centrifuged and washed with acetone or ethanol to eliminate any remaining TCA (Link and LaBaer, 2011).

TCA is an analog of acetic acid in which the three hydrogen atoms of the methyl carbon have been replaced with chlorine atoms. TCA is a relatively weak acid so it cannot hydrolyze the peptide bonds of proteins, but it does maintain an acidic pH in water. Addition of TCA to proteins in an aqueous solution disrupts the hydrogen-bonded water molecules surrounding a protein. These protein molecules no longer remain soluble and can be recovered by centrifugation. However, because TCA disrupts hydrogen bonding, the proteins will also lose their secondary structure and become denatured (Koontz, 2014).

2.11.3   Acetone precipitation

Acetone precipitation is a common method for precipitation and concentration of proteins (Simpson and Beynon, 2010). For acetone precipitation, the combination of specific salts, temperature control, solvent composition, and precipitation time is critical (Nickerson *et al.*, 2022). Proteins are insoluble in acetone (particularly at low temperatures) whilst many small molecules which could interfere with downstream protein work are soluble. By precipitating proteins in this solvent it can remove buffer contaminant and concentrate protein into a pellet which can be re-dissolved by other solvents (IGEM, 2011). Cold acetone (-20°C) is slowly added into the crude extract and the mixture was gently agitated to allow precipitation. This was followed by centrifugation using a centrifuge at $10,000 \times g$ for 15 min. The precipitate was then dissolved in phosphate buffer (50 mM), pH 7.2 and dialyzed (Coorevits *et al.*, 2008).

2.11.4   Acetone-TCA precipitation

TCA and acetone are often employed to precipitate proteins during 2-D electrophoresis sample preparation, and the combination is more successful than either TCA or acetone alone (Niu *et al.*, 2018).

Cold acetone (-20°C) is slowly added into the crude extract with the immediate addition of 10% TCA and the mixture was gently agitated to allow precipitation for 12hr. This was followed by centrifugation using a centrifuge at $10,000 \times g$ for 15 min. The precipitate was then dissolved in phosphate buffer (50 mM), pH 7.2. After that, it was collected and stored at 4°c until further use (Lenga, 1988).

2.12   Enzymatic activities of plant proteases

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

Mazorra-Manzano *et al.* (2013) compared the MCA of three crude extracts obtained from kiwi (*Actinidia deliciosa*), ginger (*Zingiber officinale*), and melon (*Cucumis melo*). There is considerable variation due to different types of proteases (actinidin, cucumisin, and zingibain) detected in kiwi, melon, and ginger extracts. The MCA/PA ratio evaluation showed that the chymosin was 67, 95 and 500 times higher than that obtained for the three extracts, respectively, when casein was used as a substrate. To overcome reduced MCA/PA ratios found in plant extracts, (Amira *et al.*, 2017) varied the pH of *Cynara cardunculus* extract from 3 to 6 and evaluated enzymatic activities of the crude extracts. They found that the MCA/PA ratio increased with pH drop and peaked at 28.71 for extract at pH 3, which was higher than 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. Furthermore, the high amount of phenolic compounds causes their rapid oxidation to create pigments, which may bind to native enzymes and inactivate them (Barros *et al.*, 2001).

2.13   Basic principle of cheesemaking

According to Nielsen (2004), the basic principle of cheesemaking are as follows:

- Concentration
  The first step in making cheese is coagulating the milk. Milk can be coagulated using rennet enzyme or by acidification. The coagulum has a natural tendency to syneresis-contraction and expelling of whey (i.e., water and milk-soluble compounds). Larger particles, such as fat globules and bacteria, will be retained in the shrinking coagulum,

30

or cheese curd. The syneresis process can be accelerated by cutting up the gel and heating it. The resulting firm curd can be pressed and formed into a variety of cheese shapes.

- Preservation
Because fresh curd contains valuable nutrients, it can quickly spoil due to bacteria, yeasts, and molds if not preserved. Low moisture content, acidification by adventitious or added lactic acid bacteria, and salting are the main factors that contribute to preservation. Spoilage of the cheese by molds on the surface can be avoided by drying and cleaning the surface, excluding oxygen by immersing the cheese in brine, or coating the cheese with wax or synthetic film material.

- Ripening
Cheese can be consumed as fresh curd or it can be ripened using enzymes from milk, rennet, and microorganisms in the cheese or on the surface. During ripening, which can take months, the organic solids of the cheese are gradually hydrolyzed and metabolized, resulting in the development of flavor, consistency, and texture of cheese.

2.14 Manufacturing steps involved in cheesemaking

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

- Selection, standardization and, in most cases, pasteurization of the milk.
- Acidification, usually via the in-situ production of lactic acid by selected bacteria.
- Coagulation of the milk.
- Dehydration of the coagulum to yield cheese curd, by a range of techniques, some of which are variety specific.
- Forming the curds into characteristic shapes.
- For most varieties, ripening (maturation) of the curd during which the characteristic flavour and texture of the cheese develop.

2.14.1 Selection of milk

The composition of cheese is heavily influenced by the composition of the cheesemilk, particularly the content of fat, protein, calcium, and pH. Milk constituents are influenced by several factors, including species, breed, individuality, nutritional status, health, and stage of lactation of the producing animals. Milk from cows in the very early or late stages of

lactation, as well as those suffering from mastitis, should be avoided due to significant compositional abnormalities. The number of somatic cells (leucocytes) is a good indicator of quality. Contaminating bacteria will be concentrated in the cheese curd and may cause defects or public health problems if the milk is of poor microbiological quality (Fox *et al.*, 2017a).

2.14.2 Pre-treatments of milk

The various pre-treatments of milk are:

- Standardization

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

- Pasteurization

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

- Filtration/centrifugation

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

2.14.3 Conversion of milk to cheese curd

After the milk has been standardized and pasteurized or otherwise treated, its temperature is adjusted to a value in the range 30–35°C, depending on the variety, and transferred to

vats (or kettles), which vary in shape (hemispherical, rectangular or vertical or horizontal cylinders), may be open or closed and may range in size from a few hundred litres to 30,000 L or more (Bennett and Johnston, 2004), where it is converted to cheese curd by a process which involves three basic operations: acidification, coagulation and dehydration.

2.14.3.1 Acidification

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

Direct acidification method involves addition of lactic acid/phosphoric acid to cold milk (2°C–12°C) to achieve a pH of 5.2 followed by addition of glucono-δ-lactone, which is slowly hydrolyzed to gluconic acid, resulting in a gradual reduction in pH to 4.6-4.8 (Makhal and Kanawjia, 2008). Direct acidification is more controllable than biological acidification, and, unlike starters, it is not susceptible to phage infection. In addition to acidification, the starter bacteria helps in cheese ripening, and hence chemical acidification is used mainly for cheese varieties for which texture is more important than flavor (Fox *et al.*, 2000).

2.14.3.2 Coagulation

The property of the protein, casein, which permits its coagulation by acid or rennet, is a key essential to cheese-making. Rennet is added to the acidified milk when it reaches pH 5.4-5.6 at 31-35ºC. After the coagulum is completely formed, the curds are stirred and cooked with whey to a temperature of about 60-70ºC (Phadungath, 2005). The vast majority of cheese varieties (representing about 75% of total production) are produced by rennet coagulation,

but some acid coagulated varieties, such as Quarg and Cottage cheese, are of major importance. The acid heat coagulated cheeses are of relatively minor importance (Fox *et al.*, 2000).

2.14.4   Curd treatment

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

2.14.4.1   Cutting the coagulum

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

2.14.4.2   Cooking the curd

The combination of heat and the developed acidity (decreasing pH) causes syneresis with the consequent expulsion of moisture, lactose, acids, soluble minerals and salts, and whey proteins (Goff, 2003). Cooking temperature in cheese vat influences moisture removal from curd during cheese making and differences in moisture content could be a significant impact on cheese. Thus differences in cooking temperature may affect chemical composition and sensory characteristics of cheese (Abdalla and Mohamed, 2009).

2.14.4.3   Drainage

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

2.14.5 Salting

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

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

2.14.6 Ripening or aging

Ripening is the natural process of microbial and biochemical reactions that occurs in a cheese after its manufacture and during storage. Ripening gives different cheeses their unique flavor, texture, and appearance. Except for some soft cheeses (e.g., cottage cheese, cheese, quark, etc.) almost all cheeses are held under controlled conditions to develop distinct attributes. Ripening essentially results from the action of microorganisms present within the curd mass and on its surface (Gunasekaran and Ak, 2002).

Further moisture loss occurs during ripening, and a complex combination of microbial and enzymic reactions occur, involving milk enzymes, the coagulant, and proteases and peptidases from the starter culture and non-starter organisms, which remain viable despite their growth being inhibited. Ripening conditions differ depending on the type of cheese. Soft, high-moisture cheeses ripen quickly, whereas hard, strongly flavored cheeses can ripen for more than a year (Fernandes, 2009).

2.15   Fresh cheese manufacturing method

The methodology for fresh cheese manufacturing given by Zheng *et al.* (2021) has been shown in Fig. 2.1.

Standardized raw milk (9.5-10% TS)

↓

Pasteurization (71-75°C/15s)

↓

Cooling to 30-32°C

↓

Addition of starter culture (1-2%)

↓

Incubation at pH 5.4-5.6

↓

Addition of coagulant

↓

Clot and cut

↓

Stirring to 25-50 min

↓

Heat up (35-40°C)

↓

Discharge whey (35-50%)

Mould and squeeze (5-6 h)

Salting (0.5-3%)
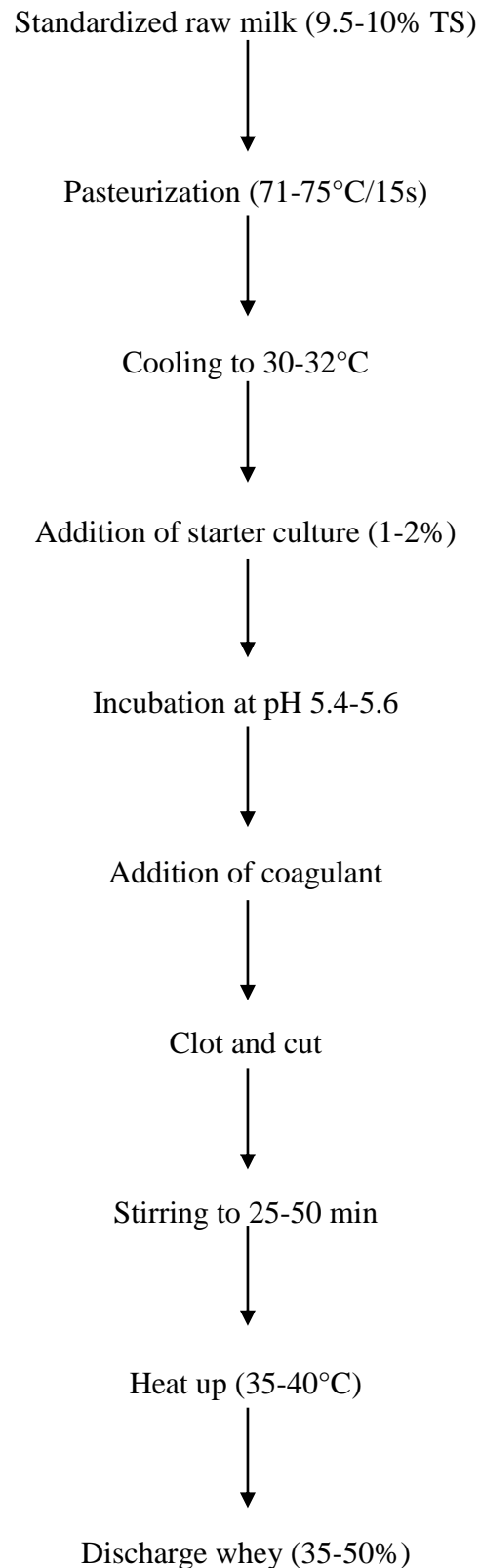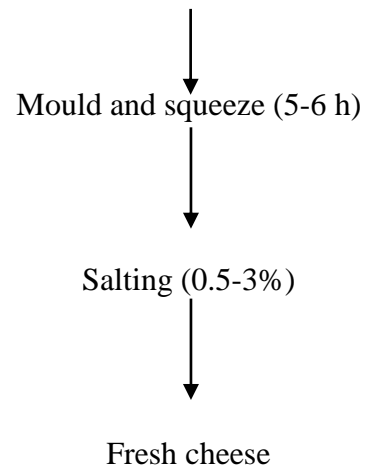
Fresh cheese

Fig. 2.1 Processing steps for fresh cheesemaking

Source: Zheng *et al.* (2021)

Part III

Materials and methods

3.1   Materials

3.1.1   Milk

Raw cow milk of local breed was collected from local market of Dharan.

3.1.2   Ginger rhizome

The ginger (*Zingiber officinale*) rhizome of the *Zingiber officinale var. roscoe* variety was purchased from the local market of Dharan.

3.1.3   Rennet

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

3.1.4   Glucono delta lactone

The food grade glucono delta lactone (GDL) (Thermo Fisher Scientific India Pvt. Ltd.) was used from laboratory of Central Department of Food Technology (CDFT).

3.2   Methods

3.2.1   Preparation of ginger rhizome protease

3.2.1.1   Extraction of crude protease from ginger rhizome

The ginger rhizome (*Zingiber officinale*) was peeled and the pieces of ginger rhizome was blended in a grinder (Model: Havel's max grind 14000) with cold sodium phosphate buffer 20mM (pH 7.0) in the ratio of 1:1 (w/v). Then the extract was filtered using muslin cloth. The filtrate thus obtained was centrifuged at 5000 rpm for 10 min in refrigerated centrifuge at 4°C (Model: DLAB company D3024R) (Setiasih *et al.*, 2018). The supernatant was labelled as "Crude enzyme".

3.2.1.2 Partial purification of ginger rhizome enzyme

Ammonium sulfate saturation to a concentration of 30-80% saturation (w/v) was performed to precipitate the protease. This was followed by centrifugation at 15000 rpm for 10 min at 4°C. The precipitate was then dissolved in 20mM sodium phosphate buffer (pH 7.0). It was then dialyzed overnight under refrigeration with three times buffer exchanges to remove the remaining salt (Andevari *et al.*, 2019; Sharma and Vaidya, 2018).

3.2.1.3 Milk clotting activity

The milk clotting activity (MCA) was determined following the procedure described by IDF (1992). 60 g of skimmed milk powder (HiMedia) was reconstituted in 500 ml of 0.01 mol/L $CaCl_2$ (pH 6.5) and the mixture was stored at 4°C. The extract was added at a proportion of 0.1 ml per 1.0 ml of milk (v/v). The clotting point was estimated during the manual shaking of the test tube, at very short time intervals (5-10 s). The coagulation time was noted. One milk clotting unit was defined as the amount of enzyme that clots 10 ml of substrate within 40 min (2400 s) at 37°C (Berridge, 1952).

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

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

3.2.1.4 Protease activity

Protease enzymatic activity was determined using protocol given by Cupp-Enyard and Aldrich (2008). 5ml of 0.65% substrate and 0.5ml of enzyme was used in this assay. The absorbance of the sample was measured by a double beam spectrophotometer (Agilent Cary 60 UV-vis) using a wavelength of 660 nm. The protease activity was determined by measuring the amount of tyrosine released during the reaction using the tyrosine standard curve. One unit of protease activity is defined as the amount of tyrosine (µmole) released per min per ml under the assay condition.

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

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

3.2.1.5 Protein estimation

Protein content of the ginger rhizome extract was determined using the procedure given by Bradford (1976). The absorbance were measured in a double beam spectrophotometer (Agilent Cary 60 UV-vis) at 595 nm and a standard curve was plotted by taking concentration of BSA (0.1 to 1.6 mg/ml) along X-axis and absorbance at 595 nm along Y-axis.

3.2.1.6 Specific activity

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

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

3.2.1.7 Purification fold

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

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

3.2.1.8 Activity recovery percent

Activity recovery percent was calculated by dividing the total enzyme activity of partially purified enzyme with total activity of crude enzyme (Blaber *et al.*, 2004).

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

3.2.1.9 Experimental design

The effect of two independent variables, namely temperature and pH of milk on MCA and TOC (time of coagulation) of extracted protease from ginger rhizome was investigated using response surface methodology (RSM). The independent variables and their levels were selected on the basis of literature and preliminary researches. The two-factor central composite rotatable design was employed. The response variables were TOC and MCA of the ginger rhizome protease. The experimental design, data analysis and model building were performed using "Design Expert" software (Version 13.0, Stat-Ease Inc., USA).

Table 3.1 Different constraints of optimization for ginger rhizome protease

Name	Goal	Range
Temperature of milk	To be in range	31-59°C
pH of milk	Target is 6.5	5.7-6.6
Time of coagulation (TOC)	To be minimized	To be determined
Milk clotting activity (MCA)	To be maximized	To be determined

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

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

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

3.2.1.10 Optimization of concentration of enzyme to substrate for milk clotting activity

The optimization of enzyme at different concentrations (5%, 2.5%, 1% and 0.5%) was carried out at 6.5 pH and 55°C temperature of milk as shown in Table 3.2.

Table 3.2 Optimization of concentration of enzyme to substrate for milk clotting activity

Enzyme concentration (%)	Temperature of milk (°C)	pH of milk	Time of coagulation (s)
5	55	6.5	63.03±0.05
2.5	55	6.5	88±0.02
1	55	6.5	240±0.04
0.5	55	6.5	612±0.01

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

3.2.2 Storage stability

Storage stability at refrigerated condition (around 4°C) and at frozen condition (-10°C) of the partially purified enzyme from 0 to 7 days was assayed and the results were expressed as milk clotting activity (MCA) (U/ml).

3.2.3 Preparation of fresh cheese

A general process for the preparation of fresh cheese is shown in Fig. 3.1. The cheese prepared using rennet by direct acidification was labelled as cheese A. Similarly, cheese prepared using ginger rhizome protease was labelled as cheese B. Milk was heated until it reached the temperature of 80-85°C. For cheese A, the rennet was added at the rate of 2.5 g/100 L after the milk attained pH 5.6 by food grade 2% glucono delta lactone (GDL) addition at temperature of 37°C. For cheese B, pH 6.5 and temperature 55°C of the milk were adjusted. The milk was stirred gently and allowed to coagulation for 40 min. The curd was then cut by a stainless-steel knife and it was further cooked at 45-50°C for 1 h to separate the whey. The whey and the curd were separated and the curds were drained using cheese cloth. The pressing was performed for 5-6 h and the curds were mixed with 1.5% common salt. The cheese was stored in refrigerator at below 5°C (Zheng *et al.*, 2021).

Milk

↓

Pasteurization (80-85°C for 15 s)

Cooling to 37°C   Cooling to 55°C

↓   ↓

Adjustment of pH 5.6 by 2% GDL   Adjustment of pH 6.5 by 2% GDL

↓   ↓

Addition of rennet (2.5 g/100 L) milk   Addition of 0.5% ginger protease

↓

Coagulation

↓

Cutting by a stainless-steel knife

↓

Cooking (45-50ºC for 1 hr)

↓

Discharge of whey

↓

Pressing (5-6 h)

↓

Salting (1.5%)

Fresh cheese A   Fresh cheese B
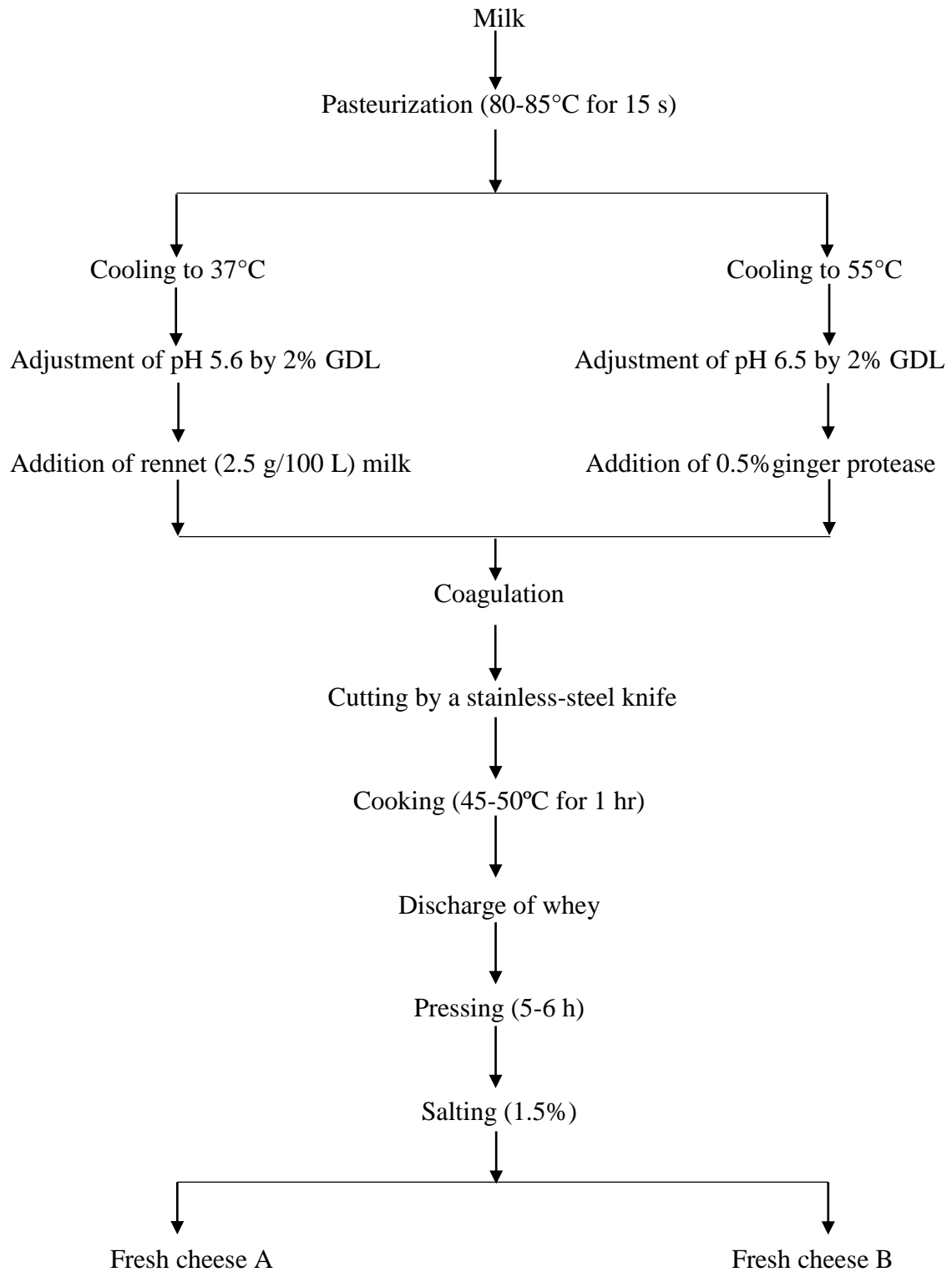
Fig 3.1 Preparation steps of fresh cheese

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 were 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 were 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 Ranganna (1986).

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 Emmons and Modler (2010):

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

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

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

Actual yield was calculated by weighing the curd as described by Nasr *et al.* (2016). 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 Sensory evaluation of cheese

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

3.2.6 Microbiological analysis of cheese

3.2.6.1 Coliform test

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

3.2.6.2 TPC of cheese

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

3.2.6.3 Yeast and molds count

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

3.2.7 Statistical analysis

Data were statistically processed using paired t-test in Microsoft Excel 2013 at 5% level of significance. The independent process variables were correlated using a second order quadratic model. Multiple regression analysis with Design Expert® software was used to

determine the second order polynomial coefficient for each term of the equation. The statistical significance of the terms was investigated using analysis of variance (ANOVA) for the response after the data was fitted to the selected model. The adequacy of the model was tested considering R^2 (coefficient of determination of the amount of variation around the mean explained by the model), adjusted R^2 (a measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model), predicted R^2 (a measure of how good the model predicts a response value) and Fischer's F test.

PART IV

Results and discussion

In this research work, protease was extracted and partially purified from ginger rhizome. The impact of pH and temperature of milk on time of coagulation (TOC) and milk clotting activity (MCA) were analyzed by response surface methodology (RSM). The cheeses prepared from rennet (A) and partially purified ginger protease (B) were analyzed for their physico-chemical (moisture, fat, protein, ash, pH, acidity and calcium) as well as microbiological (total plate count, yeast and molds count and coliform count) properties. Various sensory attributes (texture, spreadability, flavor, aftertaste and overall acceptability) of prepared cheeses were also analyzed. Storage stability of protease was also determined.

4.1 Partial purification of ginger rhizome protease

The crude enzyme from ginger rhizome was subjected to ammonium sulphate precipitation, at different concentrations ranging from 30-80%. The protease precipitation at 50% saturation, gave maximum milk clotting activity (857.14 U/ml) as shown in Table 4.1.

Table 4.1 Activity of ginger rhizome protease at different salt concentrations

Ammonium sulphate precipitation (%)	Time of coagulation (s)	Milk clotting activity (U/ml)
30	41.08±0.01	584.27±0.08
40	37.04±0.03	647.95±0.46
50	28±0.01	857.14±0.31
60	40.06±0.02	599.15±0.35
70	53.01±0.03	452.74±0.23
80	72.07±0.11	333.03±0.5

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

4.2 Specific activity, purification fold and activity recovery (%) of ginger rhizome protease

Crude and partially purified proteases were subjected to protease activity (PA) and protein determination. The results for PA were calculated using equation deduced from standard curve (Fig. B.1) and the results for protein concentration were calculated using equation deduced from standard curve (Fig. B.2). The results of protein concentration, protease activity, specific activity, purification fold and percentage yield are tabulated in Table 4.2. With 50% ammonium sulphate precipitation, a 89% of activity recovery (%), 1.03 purification fold and 0.20 U/mg of protein specific activity were found in ginger rhizome protease.Hashim *et al.* (2011b) reported that the enzyme obtained from the crude extract of the ginger rhizome, using 20–65% $(NH_4)_2SO_4$ saturation with 2.66-fold purification and 86.23% recovery.

Table 4.2 Protein concentration, protease activity, specific activity, purification fold and activity recovery (%) of ginger protease

Purification step	Protein (mg/ml)	Proteolytic activity (U/ml)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Crude enzyme	2.24	0.4357	0.1945	1	100
50% Ammonium sulphate precipitation	1.93	0.3902	0.2021	1.03	89

4.3 Storage stability of ginger rhizome protease

Partially purified enzyme of ginger rhizome was stored at refrigerated temperature (around 4°C) and at frozen condition (-10°C) for 7 days. The milk clotting activity of the stored enzyme was assayed using milk as substrate as shown in Fig. 4.1.
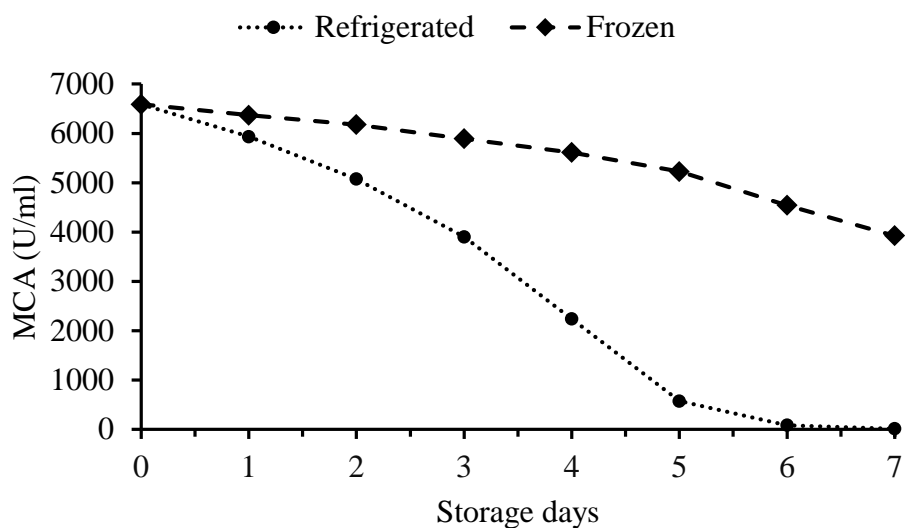
Fig. 4.1 Storage stability of partially purified ginger protease

Fig. 4.1 showed MCA reduced gradually at frozen temperature and drastically at refrigerated temperature. This indicates that the enzyme retains its milk clotting activity at lower temperature. A loss in activity with increasing time might be due to protein-protein interaction, degradation, or molecular rearrangements (Gagaoua *et al.*, 2015). Also, the reduction of the enzyme activity during storage could be attributed to the degradation by other contaminating proteolytic enzymes since the enzyme is not fully purified yet. Complete purification will remove contaminating proteases while freeze drying will terminate autodigestion (Tajalsir *et al.*, 2021). The lower storage stability of the partially purified enzyme from ginger rhizome could be overcome by either complete purification or by the freeze drying of the enzyme.

4.3 Numerical optimization of pH and temperature for time of coagulation and milk clotting activity

The measured values of the time of coagulation (TOC) and milk clotting activity (MCA) for partially purified ginger rhizome protease varied from 20-187 s and 128.342-1200 units respectively as shown in Table 4.3.

Table 4.3 Product responses by independent variables

Std. no.	Factor 1	Factor 2	Response 1	Response 2
	A: Temperature (°C)	B: pH	Time of Coagulation (s)	Milk Clotting Activity (MCA) U/ml
1	45	6.2	75	320
2	45	6.2	80	300
3	59	6.2	20	1200
4	45	6.2	75	320
5	55	6.5	28	857.14
6	35	5.8	92	260.86
7	45	6.6	100	240
8	45	5.7	56	428.57
9	45	6.2	80	300
10	55	5.8	25	960
11	31	6.2	187	128.3422
12	35	6.5	109	220.18

Table A.1 and A.2 show the coefficients of the model and other statistical attributes of TOC whereas Table A.3 and A.4 show that of MCA.

The coded equations for ginger rhizome protease are:

$$TOC = 75.90 - 66.53A + 13.89B - 5.60AB + 12.13A^2 - 8.57B^2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.4.1$$

$$MCA = 317.96 + 502.15A - 68.72B - 24.87AB + 383.99A^2 + 40.45B^2 \ldots\ldots\ldots\ldots\ldots.4.2$$

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

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

Similarly, the quadratic equation 4.2 shows that temperature of milk (A) had significant ($P<0.05$) positive effect but pH of milk (B) had significant ($P<0.05$) negative effect on MCA at 95% level of confidence. The quadratic term of temperature of milk (A^2) had significant ($P<0.05$) positive effect and the quadratic term of pH of milk (B^2) had not significant ($P>0.05$) positive effect on MCA as given by Table B.4.
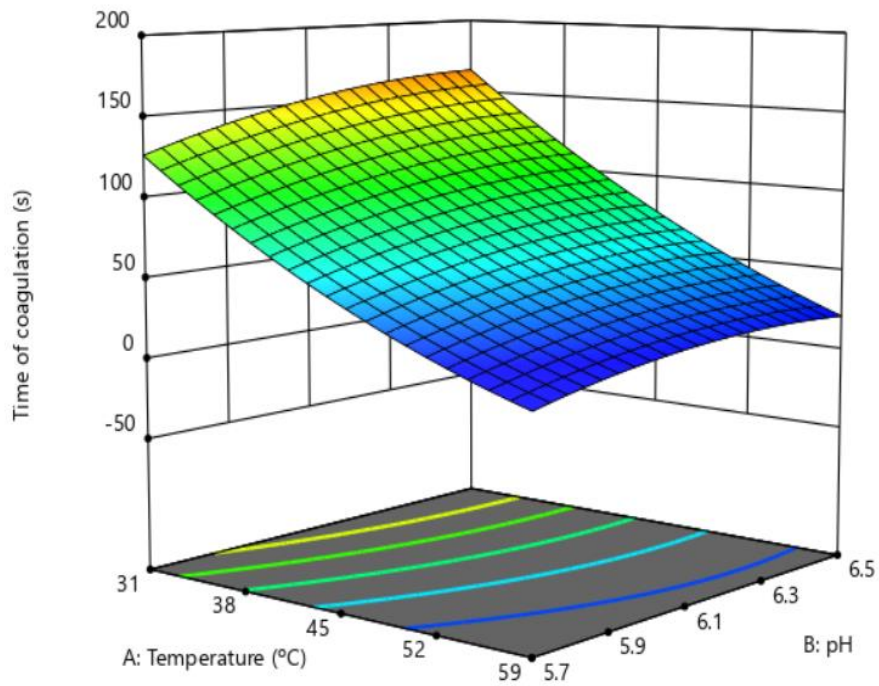
Fig. 4.2 Response surface plot of TOC of ginger rhizome protease as a function of pH and temperature of milk.

In Fig. 4.2, it was found that TOC decreased greatly with higher temperature while increased gradually with increase in pH. Shrestha (2022) also reported that the TOC of kiwi protease increased gradually with the increase in pH, while decrease in TOC was seen with the increase in temperature of milk.
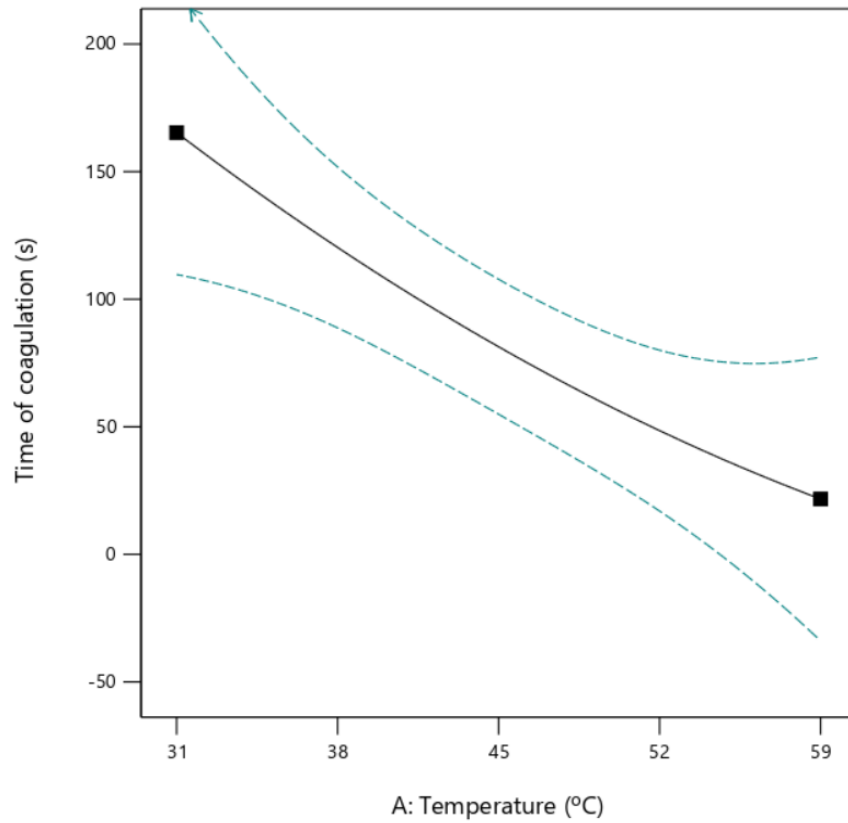
Fig. 4.3 Single factor graph of TOC of ginger rhizome protease for individual factor A: temperature

From Fig. 4.3, the increase in temperature led to decrease in TOC of purified ginger rhizome protease. This finding correlate to the findings of Shrestha (2021).
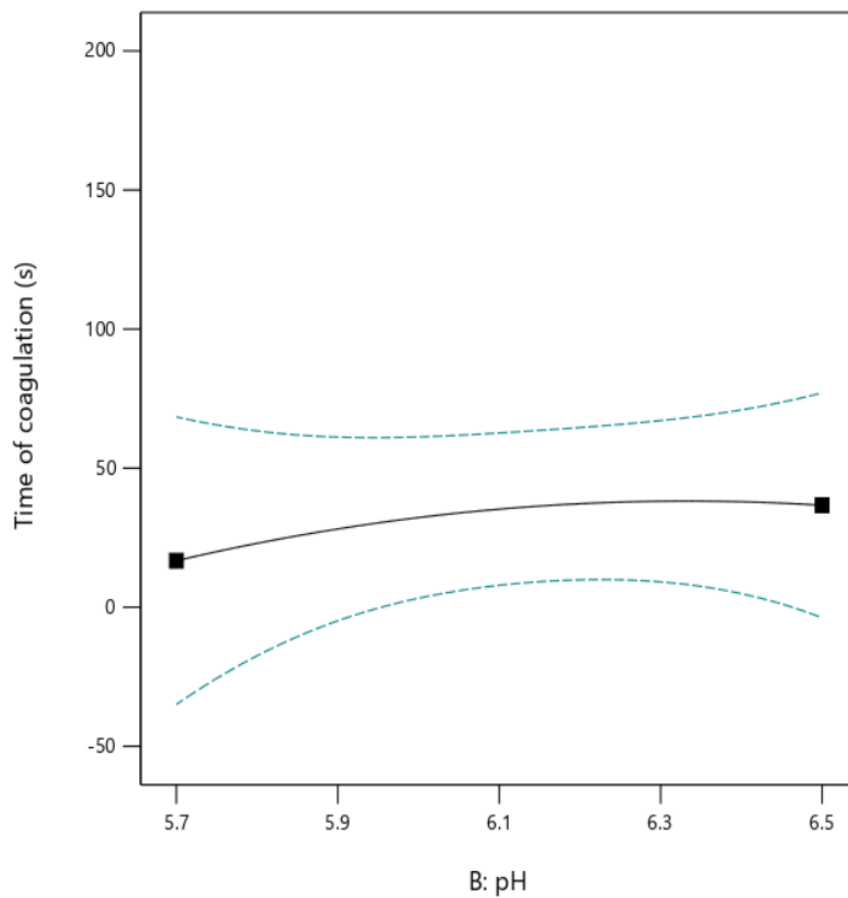
Fig. 4.4 Single factor graph of TOC of ginger rhizome protease for individual factor B: pH

From Fig. 4.4, the increase in pH led to gradual increase in TOC of purified ginger rhizome protease. Shrestha (2022) also reported similar findings.
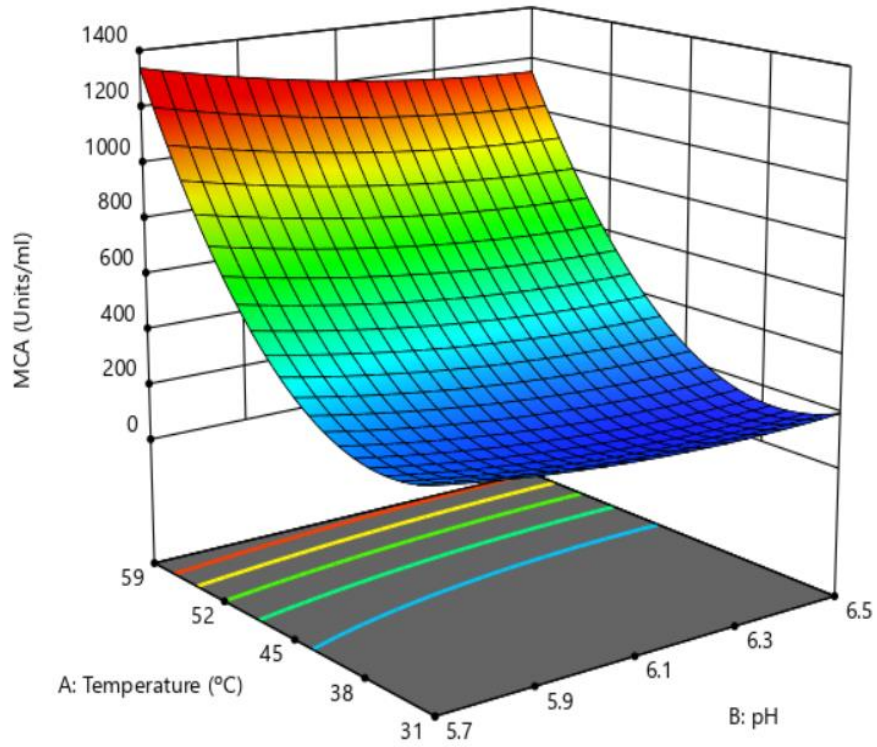
Fig. 4.5 Response surface plot of MCA of ginger rhizome protease as a function of pH and temperature of milk

In the Fig. 4.5, MCA increased with the increase in milk temperature and decreased with the increase in pH. Shrestha (2022) also expressed similar views.
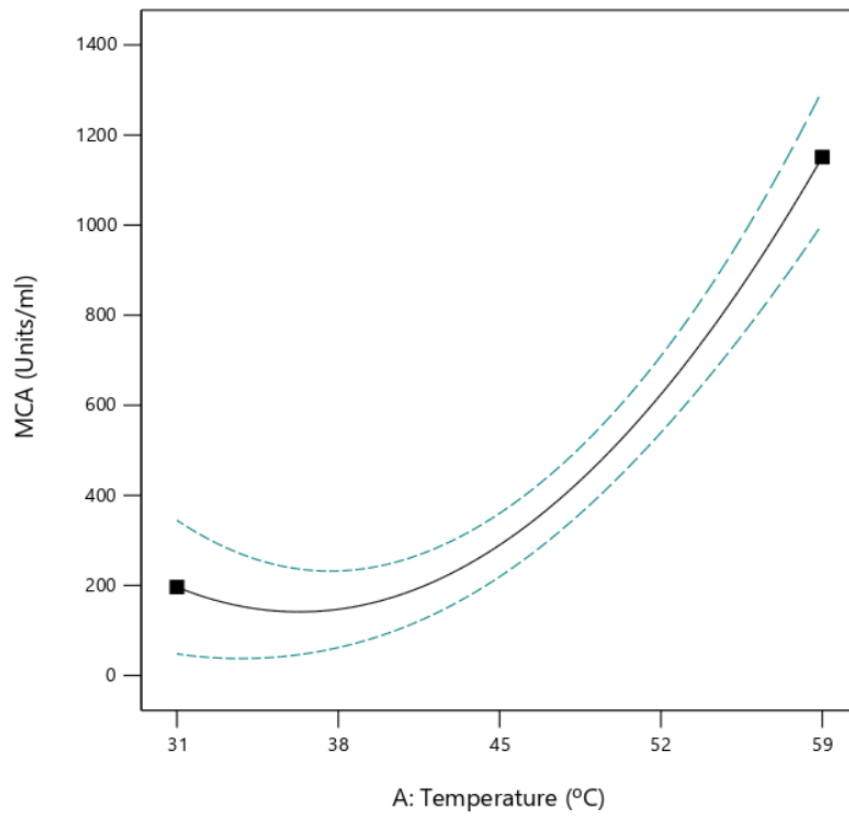
Fig. 4.6 Single factor graph of MCA of ginger protease for individual factor A:
temperature

Fig. 4.6 showed that MCA of purified ginger protease increased with increase in temperature. Shrestha (2022) also reported similar findings.
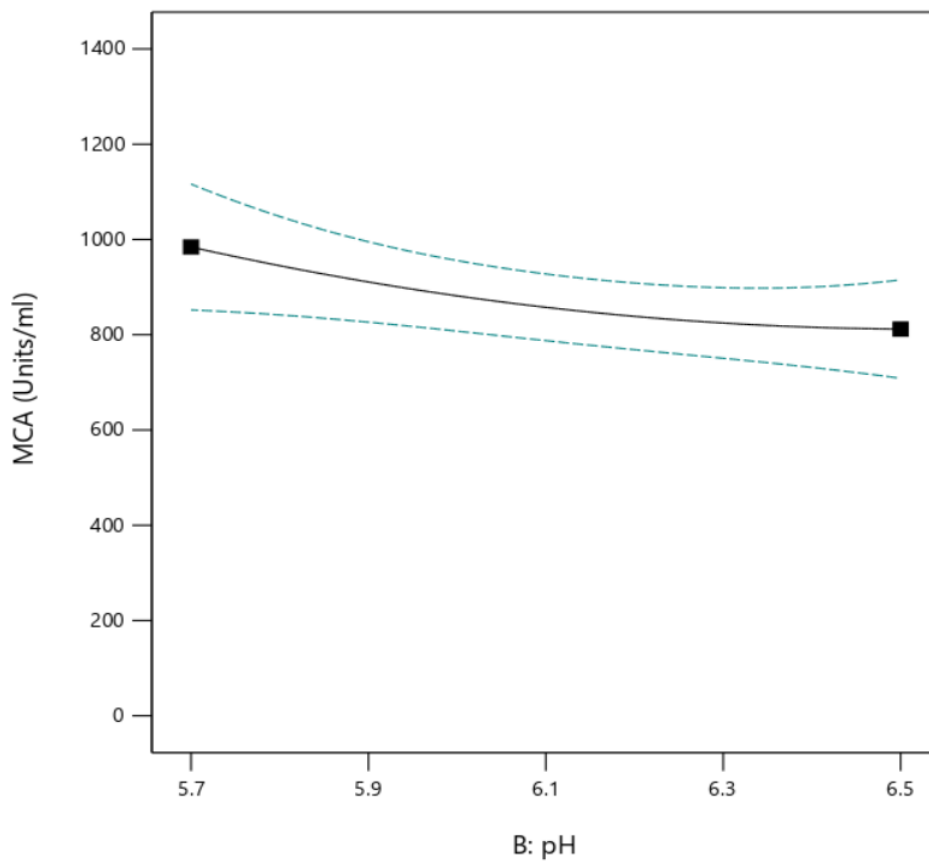
Fig. 4.7 Single factor graph of MCA of ginger rhizome protease for individual factor B: pH

Fig. 4.7 showed that MCA of purified ginger rhizome decreased gradually with the increase in pH. Shrestha (2022) reported that MCA of purified kiwi protease decreased with the increase in pH.

4.3.1 Optimization of partially purified 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 4.4.

Table 4.4 Different constraints for optimization of partially purified ginger protease

Name	Goal	Lower Limit	Upper Limit
Temperature of milk	is in range	31	59
pH of milk	is target 6.5	5.7	6.6
Time of coagulation	Minimize	20	187
Milk clotting activity	Maximize	128.3422	1200

Under the assumptions by Design Expert (version 13), the optimum operating conditions for minimum TOC and maximum MCA of enzyme were found to be 6.5 pH and 55°C temperature of milk. The responses for these optimum conditions reported MCA of 857.14 units at 28 s of coagulation time.

4.3.2 Verification of model

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

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

Response	Conditions		Predicted value	Mean observed value
	Temperature of milk (°C)	pH of milk		
TOC (s)	55	6.5	35.8815	28
MCA (U/ml)	55	6.5	826.523	858.721

4.3.3 Optimization of enzyme concentration for cheesemaking

The optimization of enzyme at different concentrations (5%, 2.5%, 1% and 0.5%) was carried out at 6.5 pH and 55°C temperature of milk as shown in Fig 4.8.
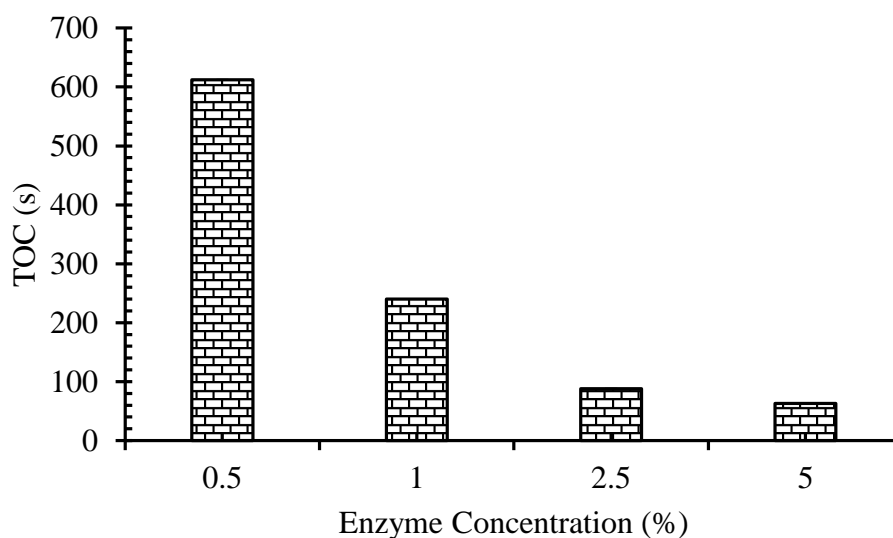
Fig. 4.8 Optimization of enzyme concentration for cheesemaking

Fig. 4.8 showed optimum enzyme concentration to prepare ginger protease coagulated cheese was found to be 0.5%. The increased enzyme concentration can cause an extensive cleavage of casein and thus affect the milk gelation and taste. Increase in enzyme concentration increases hydrophobic amino acid residues which is associated with the accumulation of bitter peptides (Pontual *et al.*, 2012).

4.4 Physicochemical properties of milk

4.4.1 Chemical composition of milk

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

Table 4.6 Proximate composition of milk

Parameters	Cow milk
Moisture (%)	87.44±0.12
Fat (%)	2.93±0.05
Protein (%)	3.72±0.06
SNF (%)	7.98±0.02
Acidity (% L.A.)	0.14±0.02
pH	6.7±0.06

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

The values for moisture, protein, acidity and pH are similar to those reported by Walstra *et al.* (2006). The values for fat and SNF are similar to those reported by Parajuli *et al.* (2018) but lower than that of Walstra *et al.* (2006). The variation could be due to difference in breed, feeding and management practices which have important effect on milk composition quality (Parajuli *et al.*, 2018).

4.4.2   Chemical composition of fresh cheese

The chemical composition of the fresh cheeses made from rennet and ginger rhizome protease has been shown in Table 4.7.

Table 4.7 Chemical composition of rennet and ginger protease coagulated fresh cheeses

Parameters	Cheese samples	
	Rennet	Ginger protease
Moisture (%)	$48.96^a \pm 0.1$	$52.06^b \pm 0.02$
Fat % (wb)	$26.66^b \pm 0.28$	$22.84^a \pm 0.98$
Protein % (wb)	$19.91^a \pm 0.05$	$20.35^b \pm 0.19$
Ash % (wb)	$2.66^a \pm 0.01$	$3.31^b \pm 0.03$
pH	$5.68^b \pm 0$	$4.82^a \pm 0.03$
Acidity (% lactic acid)	$0.21^a \pm 0.01$	$0.25^b \pm 0.01$
Calcium (mg/100 g)	$632.73^b \pm 0.35$	$622.66^a \pm 1.56$

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

Significant differences ($P<0.05$) were found for moisture, fat, protein, ash, pH, acidity and calcium in cheeses made with both types of coagulants. The moisture percentage of ginger protease coagulated cheese is in the line with the findings of Khan and Masud (2013). For rennet cheese, the result of moisture content was similar to Adhikari *et al.* (2021) who reported moisture content of cheese as 48.99% but higher than that of Rana *et al.* (2017). This may due to difference in processing techniques and activity of coagulant.

Significant differences ($P<0.05$) were observed between two samples regarding moisture content (Appendix E.1). Moisture content in fresh cheese made by using ginger protease was higher than that of rennet. Longer coagulation time for plant proteases results in more moisture retention in the final product which might be the reason for significant difference (Johnson *et al.*, 2001).

Analysis of variance (Appendix E.2) regarding fat content revealed significant difference (P<0.05) among rennet and ginger protease coagulated cheese. Cheese made using ginger protease showed comparatively lower fat content. For rennet cheese, the result of fat content was similar to that of Nawaz *et al.* (2011) but higher than that of Hashim *et al.* (2011a). The fat content of ginger protease coagulated cheese is similar to that of the Hashim *et al.* (2011a). Khan and Masud (2013) revealed that plant protease takes a longer time for coagulation compared to rennet and higher fat loss during whey drainage. This may be responsible for the retention of less fat in the final product.

Analysis of variance (Appendix E.3) regarding protein contents revealed that significant difference (P<0.05) was recorded between two samples. The protein in fresh cheese made using ginger protease was higher than that of rennet. The protein content of rennet cheese is in the line with the findings of Hashim *et al.* (2011a) but higher than that of Kheir *et al.* (2011). For ginger protease coagulated cheese, the result of protein content is in the line with the findings of Shrestha (2021). The result of protein content for cheese made from ginger protease was higher than that of Hashim *et al.* (2011a) who reported protein content of cheese as 19.53% but lower than that of Adhikari *et al.* (2021). The slight variation might be due to the cheesemaking techniques and the quality of milk used, as the milk quality changes with the lactating stage of animal, nutrition, breed and age of milking animal (Mijan *et al.*, 2010). Proteolytic enzymes are responsible for the formation of nitrogenous products of intermediate size, such as proteases, peptones, polypeptides, peptides and free amino acids. Enzymes act on these and other substances to form products like amino acids, amines, fatty acids, esters, aldehydes, alcohols and ketones (Fox *et al.*, 2004).

The ash content of rennet cheese was similar to the findings of Mijan *et al.* (2010) but lower than the findings of Rana *et al.* (2017) who found ash content to be 2.75%. The possible reason for the less ash contents may be the seasonal variation in the composition of milk. The ash content of fresh cheese made from rennet was lower than that of ginger protease. Analysis of variance (Appendix E.4) regarding ash contents revealed that significant difference (P<0.05) was found between two samples.

The pH of rennet cheese was similar to the findings of Shrestha (2022) while the pH of ginger protease cheese was similar to the findings of Shrestha (2021). Between two samples, the average pH was more in rennet cheese. The pH of milk and milk products is measured

to ensure the quality of foodstuff (Razzaq, 2003). The texture and firmness of fresh cheese are greatly influenced by the pH (Phadungath, 2005). The possible variation may be due to the processing techniques, effect of coagulants, incubation and coagulation time and temperature. Analysis of variance (Appendix E.5) regarding pH revealed that there were revealed that significant difference ($P<0.05$) was found between two samples.

The titratable acidity of fresh cheeses were similar to the findings of Shrestha (2021) but lower than Mijan *et al.* (2010). Due to longer coagulation time of plant protease, a higher acidity was reached in curd (Kheir *et al.*, 2011). Analysis of variance (Appendix E.6) regarding titratable acidity revealed that significant difference ($P<0.05$) was found between two samples.

The difference found in the calcium contents of the cheeses produced by two coagulants was significant ($P<0.05$). Among two samples, calcium content was more in rennet cheese followed by ginger protease coagulated cheese. The calcium content of fresh cheeses is similar to the findings of Shrestha (2021) but higher than the findings of Adhikari *et al.* (2021). Joshi *et al.* (2004) reported that caseins are more hydrated as the level of bound calcium decreases in milk. According to Adhikari *et al.* (2021), calcium content inversely correlates to the moisture content of the cheese.

4.4.3   Theoretical and actual yield

The theoretical and actual yield of fresh cheeses A and B is presented in Appendix D.1 and shown in Fig. 4.9.
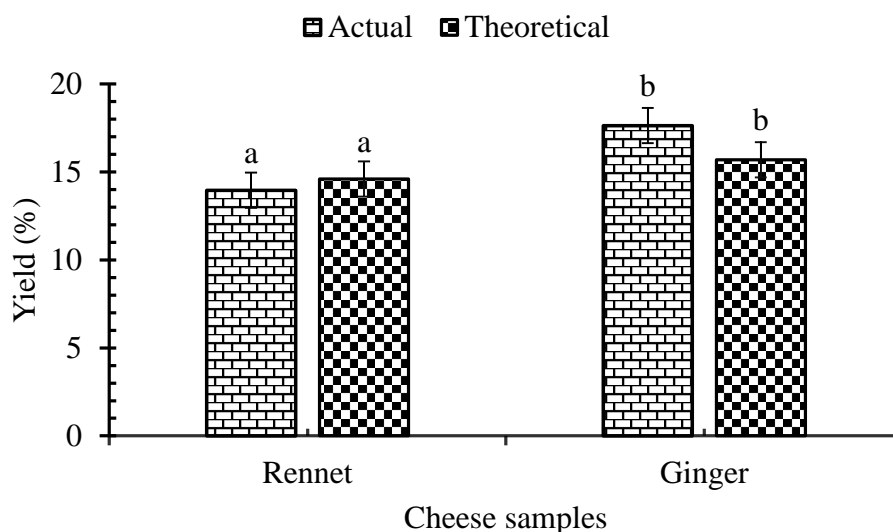
Fig. 4.9 Theoretical and actual yield of fresh cheeses

*Bars with different alphabets are significantly different at P<0.05

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

Actual yield was lower than the theoretical yield of rennet cheese. Cheese yield depend on the factors such as milk quality and composition, heat treatment of milk, type of cheese, and the processing methods used (Mazorra-Manzano *et al.*, 2013). Among both samples, the average actual yield was more in ginger protease coagulated cheese. The longer coagulation period, which led to more moisture content, may be responsible for the higher actual yield in cheese made with ginger protease coagulated. (Shrestha, 2022) also expressed similar views. Analysis of variance (Appendix E.9) regarding actual yield revealed that there was significant difference (P<0.05) among two samples.

4.5 Microbiological analysis

The result of microbiological analysis of fresh cheeses are presented in Fig. 4.10 and in (Appendix D.3). It showed the average value for TPC and yeasts and molds of samples.
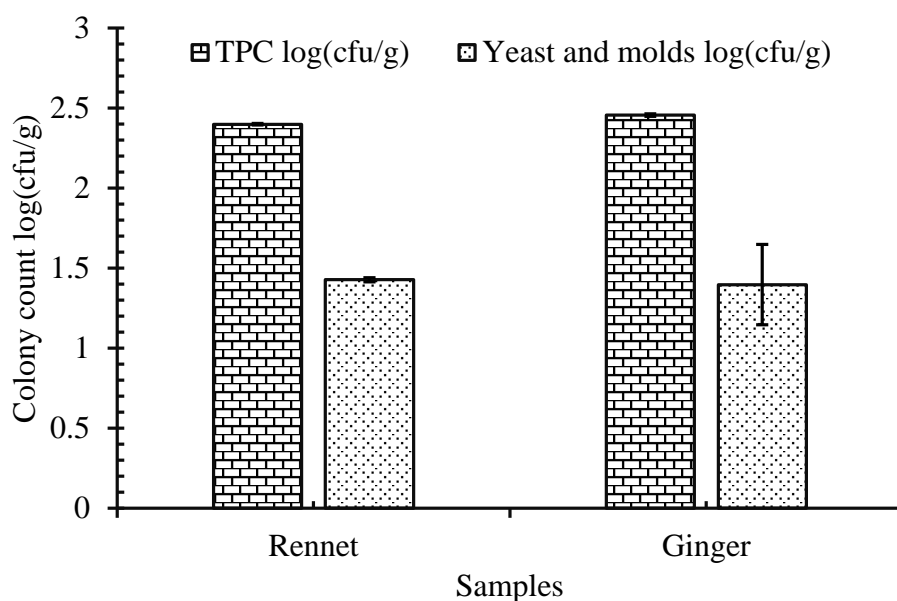
Fig. 4.10 Microbiology of fresh cheese

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

Significant difference (P<0.05) was found for TPC (Appendix E.10) but no significant difference (P>0.05) was found for yeast and molds count (Appendix E.11). Coliforms were not detected in both samples. This shows the effect of heating which completely eliminated coliforms. However, the presence of TPC and yeasts and molds were detected which might be due to handling contamination or oxygen trapped in the package (Maskey and Shrestha, 2020).

4.5 Sensory evaluation of cheese

The sensory scores (5-point hedonic scales) of the fresh cheeses are shown in (Appendix D.2). Graphical representation is given in Fig. 4.11.
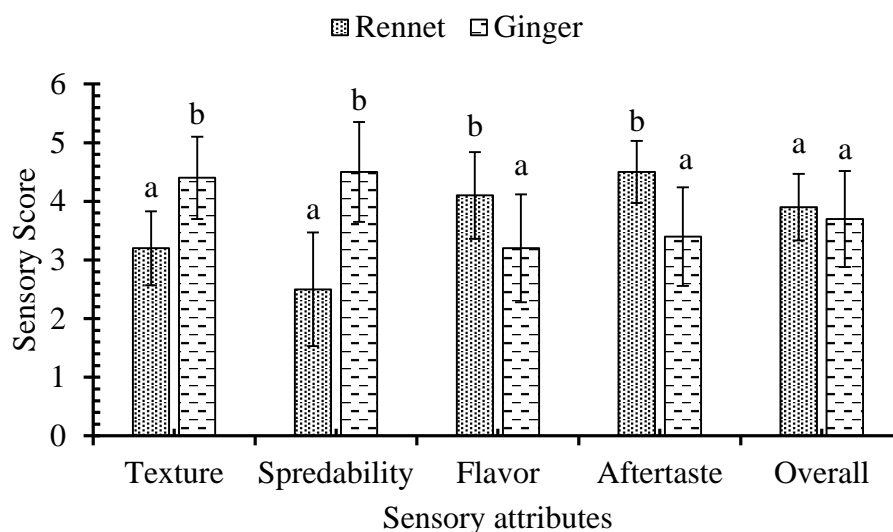
Fig. 4.11 Graphical view of mean sensory scores of fresh cheeses

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

The average texture score was comparatively higher for cheese made from ginger protease, which means that ginger protease coagulated cheese was found to be better in texture. As soft-fresh cheeses are meant for use as a spread, so its texture must naturally be smooth without any grainy feel. The lower primary proteolysis by chymosin might be the reason for slightly hard and rubbery texture in rennet cheese (Adhikari *et al.*, 2021). Similar findings have been reported for ewe's milk cheese made using vegetable coagulant from *Cynara cardunculus*, which were softer and creamier than those made using rennet. The intense level of proteolysis which occurred in cheeses made using vegetable protease hydrolyze the casein network, creating a more homogeneous structure, thus prompting greater creaminess and softening of the cheese (Tejada *et al.*, 2007). Analysis of variance (Appendix E.12) regarding texture revealed that significant difference (P<0.05) between two samples.

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

Cheese made using rennet had higher score for flavor as compared to ginger protease coagulated cheese. Rennet cheese was found to be slightly better which is in agreement with Kheir *et al.* (2011) who reported that the flavour of cheese made with rennet scored higher compared kiwi protease coagulated cheese. Analysis of variance (Appendix E.14) regarding flavor revealed that significant difference ($P<0.05$) was observed between two samples.

The aftertaste score was higher for rennet cheese (Table D.2) followed by cheese made from ginger protease. A mild bitter taste was observed in cheese made from ginger protease. This bitterness is associated with accumulation of the bitter peptides that contain more hydrophobic amino acid residues when coagulants from plant sources are used (Singh *et al.*, 2003). Analysis of variance (Appendix E.15) regarding aftertaste revealed that significant difference ($P<0.05$) was observed between two samples.

Despite the slightly lower overall sensory rating, cheese manufactured with ginger protease is comparable to cheese made with rennet. The data regarding aftertaste showed a significant difference ($P<0.05$) among the samples. Aftertaste is a very important attribute since it determines consumer preference. The results showed that ginger rhizome protease cheese was slightly bitter which is in agreement with Roseiro *et al.* (2003) who observed that plant protease coagulated cheese had a more bitter flavor than rennet cheese.

Part V

Conclusions and recommendations

5.1   Conclusions

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

1. Numerical optimization study revealed that the optimum condition for maximum milk clotting activity and minimum time of coagulation for partially purified ginger rhizome protease were 857.14 U/ml and 28 s at pH 6.5 and temperature 55°C.

2. The degree of the purity of partially purified ginger rhizome protease increased from 1 to 1.03-fold.

3. The optimum enzyme concentration in the milk to prepare fresh cheese was found to be 0.5%.

4. The physico-chemical parameters (moisture content, fat, protein, ash, calcium, acidity, pH and cheese yield) of cheese prepared from rennet and ginger rhizome protease was found to be significantly different.

5. Microbial profile for cheese made using rennet and ginger rhizome protease was similar, however, yeast and mold count of cheese made from ginger rhizome protease was less than that of cheese produced using rennet.

6. The sensory analysis were significantly different in texture, spreadability, aftertaste and flavor among cheeses made using rennet and ginger rhizome protease while overall acceptance was not different.

7. The storage stability of partially purified ginger protease revealed that milk clotting activity decreased gradually at frozen temperature but rapidly at refrigerator temperature during storage.

5.2   Recommendations

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

1. Complete purification of ginger rhizome protease using three phase partitioning system or ion-exchange chromatography can be used.

2. Use of other plant proteases such as actinidin, bromelain and calotropin to prepare fresh cheese can be studied.

3. Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis can be done to determine the molecular weight of the protease.

4. The cleavage pattern of the enzyme can be analyzed.

5. Texture evaluation of the product can be performed.

Part VI

Summary

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

The present work showed that the partial purification of crude extract using ammonium sulphate precipitation at 50% saturation give 89% protease yield of 1.03 purification fold and 0.20 U/mg of protein specific activity. The enzyme showed optimum temperature at 55°C and pH 6.5 which gives maximum milk clotting activity (857.14 U/ml) with minimum time of coagulation (28 s).

The physico-chemical analysis showed that there were significant differences (P<0.05) in moisture content, fat, protein, ash, calcium, acidity, pH and cheese yield among cheeses made using rennet and ginger rhizome protease. Microbiological analysis showed that the average TPC and yeast and molds count for ginger protease coagulated cheese was 2.44 log cfu/gm and 1.41 log cfu/gm but coliforms were not detected. Significant difference (P<0.05) in all sensory attributes (texture, flavor, spreadability and aftertaste) except overall acceptability were observed between ginger protease coagulated and rennet cheeses. Storage stability of partially purified ginger protease revealed that milk clotting activity decreased gradually at frozen temperature but during 7 days of storage.

This study indicate that partially purified ginger rhizome protease can be used to produce fresh cheese with a slightly bitter taste. The quality of the cheese prepared from ginger rhizome protease can further be improved by three purifying the enzyme. Therefore, ginger rhizome protease can be viewed as a possible substitute for calf rennet in the manufacturing of fresh cheese.

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Appendices

Appendix A

Table A.1 Model summary statistics for time of coagulation

Model Summary Statistics

Source	Std. Dev.	R²	Adjusted R²	Predicted R²	PRESS	
Linear	**19.45**	**0.8500**	**0.8167**	**0.6637**	**7633.24**	**Suggested**
2FI	20.48	0.8522	0.7967	0.4592	12274.13	
Quadratic	21.23	0.8808	0.7815	0.1585	19100.96	
Cubic	19.43	0.9335	0.8170	-3.1894	95092.44	Aliased

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

Table A.2 Analysis of variance (ANOVA) for response surface quadratic model of time of coagulation

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	19993.43	5	3998.69	8.87	0.0097	significant
A-Temperature	17358.01	1	17358.01	38.50	0.0008	
B-pH	1072.19	1	1072.19	2.38	0.1740	
AB	49.00	1	49.00	0.1087	0.7528	
A²	245.03	1	245.03	0.5435	0.4888	
B²	275.63	1	275.63	0.6114	0.4640	
Residual	2704.82	6	450.80			
Lack of Fit	2679.82	3	893.27	107.19	0.0015	significant

Table A.3 Model summary statistics for milk clotting activity

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	171.51	0.7967	0.7515	0.6174	4.982E+05	
2FI	181.58	0.7975	0.7215	0.4727	6.866E+05	
Quadratic	**54.26**	**0.9864**	**0.9751**	**0.9052**	**1.235E+05**	**Suggested**
Cubic	54.19	0.9910	0.9752	0.4418	7.269E+05	Aliased

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

Table A.4 Analysis of variance (ANOVA) for response surface quadratic model of milk clotting activity

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.285E+06	5	2.569E+05	87.25	0.0001	significant
A-Temperature	9.888E+05	1	9.888E+05	335.81	<0.0001	
B-pH	26244.40	1	26244.40	8.91	0.0245	
AB	966.31	1	966.31	0.3282	0.5876	
A²	2.457E+05	1	2.457E+05	83.42	<0.0001	
B²	6139.68	1	6139.68	2.09	0.1989	
Residual	17667.58	6	2944.60			
Lack of Fit	17267.58	3	5755.86	43.17	0.0057	significant

Appendix B

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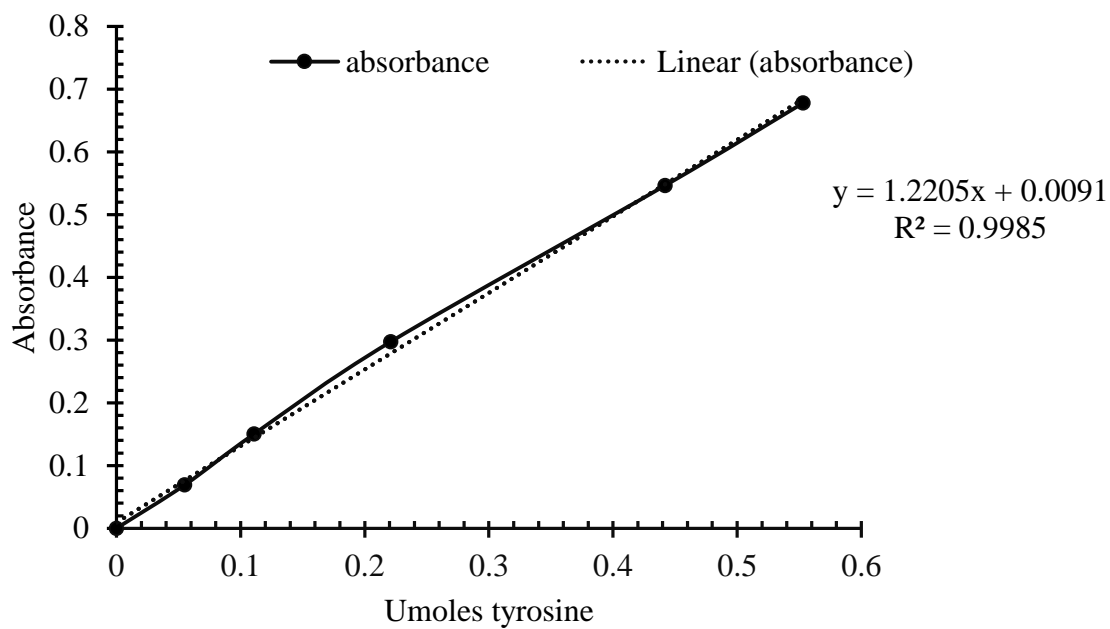
Fig. B.1 Standard curve of L-tyrosine for protease activity

Within the figure:
- Y-axis: Absorbance
- X-axis: Umoles tyrosine
- Legend: absorbance, Linear (absorbance)
- $y = 1.2205x + 0.0091$
- $R^2 = 0.9985$

Calibration curve for protein determination
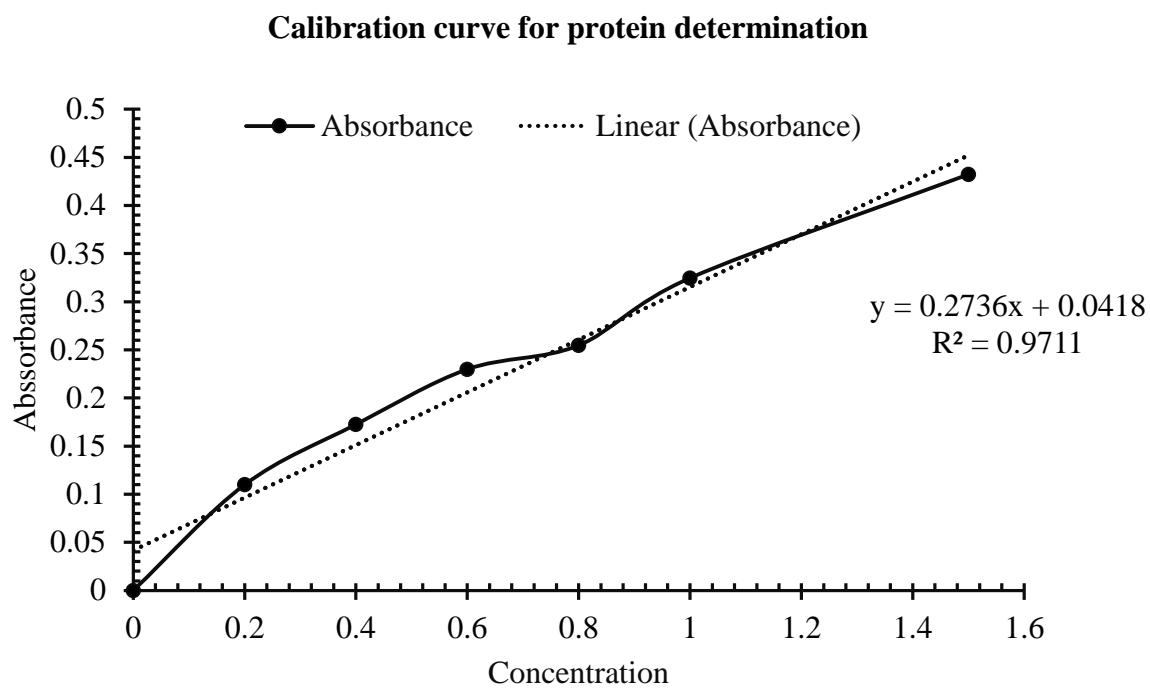
Fig. B.2 Standard curve of protein concentration for protein determination

Appendix C

Sensory Evaluation Card

Date: _____

Name: _____

Product: Fresh Cheese

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

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

Samples	Parameters				
	Texture	**Spreadability**	**Flavor**	**Aftertaste**	**Overall**
A					
B					

Comments (if any) …………………………………………………………………...
…………..………………………………

_____
Signature

Appendix D

Table D.1 Theoretical and actual yields of fresh cheese

Source of Variation	Actual Yield	Theoretical Yield
A	$13.96^a \pm 0.01$	$14.61^a \pm 0.07$
B	$17.64^b \pm 0.05$	$15.69^b \pm 0.02$

Table D.2 Mean scores of sensory attributes of fresh cheese

Source of variation	Texture	Spreadability	Flavor	Aftertaste	Overall
A	$3.2^a \pm 0.63$	$2.5^a \pm 0.97$	$4.1^b \pm 0.74$	$4.5^b \pm 0.53$	$3.9^a \pm 0.57$
B	$4.4^b \pm 0.7$	$4.5^b \pm 0.85$	$3.2^a \pm 0.92$	$3.4^a \pm 0.84$	$3.7^a \pm 0.82$

Table D.3 Microbiological analysis of fresh cheese

Sample	Coliform log(CFU/g)	TPC log(CFU/g)	Yeast & Mold log(CFU/g)
A	ND	$2.3987^a \pm 0.0043$	$1.4276^b \pm 0.0128$
B	ND	$2.4559^a \pm 0.0086$	$1.3972^a \pm 0.2504$

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

Appendix E

Statistical analysis (T- test Tables)

Table E.1 T-test: Two-sample assuming equal variances for moisture content

Variate: Moisture content

	Variable 1	*Variable 2*
Mean	48.95666667	52.05666667
Variance	0.010133333	0.000433333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-69.08368263	
P(T<=t) one-tail	1.31526E-07	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	2.63053E-07	
t Critical two-tail	2.776445105	

Table E.2 T-test: Two-sample assuming equal variances for fat

Variate: Fat

	Variable 1	*Variable 2*
Mean	26.65666667	22.83666667
Variance	0.080033333	0.968233333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	6.462314683	
P(T<=t) one-tail	0.001476506	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.002953012	
t Critical two-tail	2.776445105	

Table E.3 T-test: Two-sample assuming equal variances for protein

Variate: Protein

	Variable 1	*Variable 2*
Mean	19.91333333	20.35333333
Variance	0.002533333	0.034433333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.963767369	
P(T<=t) one-tail	0.008312566	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.016625132	
t Critical two-tail	2.776445105	

Table E.4 T-test: Two-sample assuming equal variances for ash

Variate: Ash

	Variable 1	*Variable 2*
Mean	2.663333333	3.31
Variance	0.000133333	0.0009
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-34.84342859	
P(T<=t) one-tail	2.02422E-06	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	4.04843E-06	
t Critical two-tail	2.776445105	

Table E.5 T-test: Two-sample assuming equal variances for pH

Variate: pH

	Variable 1	*Variable 2*
Mean	5.68	4.823333333
Variance	0	0.000933333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	48.56843478	
P(T<=t) one-tail	5.37624E-07	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	1.07525E-06	
t Critical two-tail	2.776445105	

Table E.6 T-test: Two-sample assuming equal variances for acidity

Variate: Acidity

	Variable 1	*Variable 2*
Mean	0.21	0.253333333
Variance	1E-04	3.33333E-05
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-6.5	
P(T<=t) one-tail	0.001445004	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.002890007	
t Critical two-tail	2.776445105	

Table E.7 T-test: Two-sample assuming equal variances for calcium

Variate: Calcium (mg/100g)

	Variable 1	*Variable 2*
Mean	632.7333333	622.6566667
Variance	0.124633333	2.447033333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	10.88354011	
P(T<=t) one-tail	0.000202295	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.00040459	
t Critical two-tail	2.776445105	

Table E.8 T-test: Two-sample assuming equal variances for theoretical yield

Variate: Theoretical yield

	Variable 1	*Variable 2*
Mean	14.61	15.685
Variance	0.005	0.00045
Observations	2	2
Hypothesized Mean Difference	0	
df	2	
t Stat	-20.59326513	
P(T<=t) one-tail	0.001174862	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.002349724	
t Critical two-tail	4.30265273	

Table E.9 T-test: Two-sample assuming equal variances for actual yield

Variate: Actual yield

	Variable 1	*Variable 2*
Mean	13.96	17.635
Variance	0.0002	0.00245
Observations	2	2
Hypothesized Mean Difference	0	
df	2	
t Stat	-100.9600145	
P(T<=t) one-tail	4.90464E-05	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	9.80928E-05	
t Critical two-tail	4.30265273	

Table E.10 T-test: Two-sample assuming equal variances for TPC

Variate: TPC

	Variable 1	*Variable 2*
Mean	2.398730264	2.455984411
Variance	3.03989E-06	2.3811E-06
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-42.59204303	
P(T<=t) one-tail	9.08266E-07	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	1.81653E-06	
t Critical two-tail	2.776445105	

Table E.11 T-test: Two-sample assuming equal variances for yeast and molds

Variate: Yeast and Molds

	Variable 1	*Variable 2*
Mean	1.42749	1.39652
Variance	0.00027	0.00093
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	1.54855	
P(T<=t) one-tail	0.09821	
t Critical one-tail	2.13185	
P(T<=t) two-tail	0.19641	
t Critical two-tail	2.77645	

Table E.12 T-test: Two-sample assuming equal variances for texture

Variate: Texture

	Variable 1	*Variable 2*
Mean	3.2	4.4
Variance	0.4	0.488888889
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	-4.024922359	
P(T<=t) one-tail	0.000397244	
t Critical one-tail	1.734063607	
P(T<=t) two-tail	0.000794488	
t Critical two-tail	2.10092204	

Table E.13 T-test: Two-sample assuming equal variances for spreadability

Variate: Spreadability

	Variable 1	*Variable 2*
Mean	2.5	4.5
Variance	0.944444444	0.722222222
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	-4.898979486	
P(T<=t) one-tail	5.7793E-05	
t Critical one-tail	1.734063607	
P(T<=t) two-tail	0.000115586	
t Critical two-tail	2.10092204	

Table E.14 T-test: Two-sample assuming equal variances for flavor

Variate: Flavor

	Variable 1	*Variable 2*
Mean	4.1	3.2
Variance	0.544444444	0.844444444
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	2.414953416	
P(T<=t) one-tail	0.013298532	
t Critical one-tail	1.734063607	
P(T<=t) two-tail	0.026597063	
t Critical two-tail	2.10092204	

Table E.15 T-test: Two-sample assuming equal variances for aftertaste

Variate: Aftertaste

	Variable 1	*Variable 2*
Mean	4.5	3.4
Variance	0.277777778	0.711111111
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	3.497993004	
P(T<=t) one-tail	0.001284073	
t Critical one-tail	1.734063607	
P(T<=t) two-tail	0.002568146	
t Critical two-tail	2.10092204	

Table E.16 T-test: Two-sample assuming equal variances for overall acceptance

Variate: Overall

	Variable 1	*Variable 2*
Mean	3.9	3.7
Variance	0.322222222	0.677777778
Observations	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	0.632455532	
P(T<=t) one-tail	0.267520708	
t Critical one-tail	1.734063607	
P(T<=t) two-tail	0.535041417	
t Critical two-tail	2.10092204	

Color plates

Plate 1 Clotting of milk in vials

Plate 2 Ginger protease
coagulated curd

Plate 3 Rennet coagulated curd

Plate 4 Protein content
determination of the extract

Plate 5 Sensory analysis of cheese