EXTRACTION, PURIFICATION AND UTILIZATION OF PROTEOLYTIC ENZYME FROM THE LATEX OF *Calotropis gigantea* IN FRESH CHEESE MAKING

by

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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

This ***dissertation*** entitled ***Extraction, Purification and Utilization of Proteolytic Enzymes from the Latex of Calotropis gigantea for Making Fresh Cheese*** presented by **Peshal Raj Karki** has been accepted as the partial fulfillment of the requirement for **B. Tech. degree in Food Technology.**

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(Peshal Raj Karki)

Abstract

The main objective of the present study was extraction, purification and utilization of proteolytic enzyme from the latex of *Calotropis gigante* in fresh cheese making. The crude extract was subjected to TPP in varying optimization parameters of ammonium salt concentration (30-80%), ratio of *t*-butanol to crude extract (1;0.5-1:2) and the extraction pH (4-9). Both intermediate phase and aqueous phase were analyzed using milk clotting activity, protein content and caseinolytic activity for maximum activity recovery (%) and purification fold. Furthermore, the optimum temperature and pH of milk in the cheesemaking, for maximum and minimum time of coagulation (TOC), were determined by response surface methodology. The physicochemical properties of prepared cheese were compared with rennet cheese.

For the highest activity recovery and purification fold, the optimum condition parameters for TPP were found to be 60% ammonium sulphate precipitation, 1:1.25 ratio of crude extract to *t*-butanol (v/v) and extraction pH 6. Numerical optimization study revealed that the optimum temperature and pH for milk clotting in cheesemaking was found to be 55°C and pH 6 respectively. The optimized TOC and MCA were 12.91 s and 2779.65 units respectively. The analysis of rennet cheese and aank protease coagulated cheese revealed no significant difference ($P>0.05$) in fat, protein and ash content. There was also no significant difference in yield of both cheeses.

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List of Abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
BSE	Bovine spongiform Encephalopathy
CBS	Customized brewing solutions
CCP	Colloidal calcium phosphate
CE	Crude extract
DANIDA	Danish international development agency
DDC	Dairy development corporation
DNA	*Deoxyribonucleic acid*
FAO	Food and Agriculture Organization
FDB	Fat on Dry Basis
HTST	High temperature short time
IDF	International dairy federation
LSD	Least significant difference
LTLT	Low temperature long time
MCA	Milk clotting activity
MFFB	Moisture on Fat Free Basis
MFFS	Moisture in Fat Free Substance
MT	Metric tonnes
NDDB	Nepal dairy development board
NSLAB	Non-starter lactic acid bacteria
PPM	Parts per million
RCT	Rennet coagulation time
RO	Reverse osmosis
SCN	Thiocyanate

TOC	Time of Coagulation
TPP	Three phase partitioning
UF	Ultrafiltration
WFFS	Water in Fat Free Substance
WHO	World health organization

Part I

Introduction

1.1 General introduction

Cheese is a milk solid curd that has been stabilized through casein coagulation and milk fat being entrapped inside the coagulum. By separating the whey from the curd and removing it, the water content in the mixture is significantly decreased as compared to milk. The curd is textured, salted, molded, and pressed into molds before storage, ripening and curing (Fernandes, 2009). FAO/WHO defines cheese as a fresh or ripened solid or semi-solid product made by coagulating milk, skim milk, partly skim milk, cream, whey cream, or buttermilk with rennet or other acceptable coagulating agents, then draining the resulting whey. Cheese binds a lot of minerals and vitamins and is abundant in vital amino acids (Upadhyay, 2003).

In cheese manufacturing process, milk coagulation by enzyme incorporation is a crucial step where milk protein, casein, forms a gel network in which the fat gets entrapped (Duarte, et al., 2009) (Mazorra-Manzano, et al., 2013). The majority of the enzyme used to make cheese is taken from the fourth stomach of calves. The stomach of ruminants, particularly that of the calf, is where rennet enzyme is found. Its primary enzyme is chymosin (EC 3.4.23.4) (Khan & Masud, 2013) which is responsible for the particular cleavage of the k-casein Phe_{105}-Met_{106} bond (Nawaz *et al.,* 2011), which causes the breakdown of casein micelles and milk coagulation. However, very early on, coagulants from microorganisms and plants were also utilized (Freitas *et al.,* 2016).

Several proteolytic enzymes, including those from various animal species (such as pig, cow, and chicken pepsins), microbial proteases (such as *Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and proteases isolated from plants, can cause milk to coagulate. *Helianthus annus* (Egito *et al.,* 2007); *Ficus racemose* (Devaraj *et al.,* 2008); *Solanum dubium* (Ahmed *et al.,* 2009); *Bromelia hieronymi* (Bruno *et al.,* 2010); *Ficus religiosa* (Kumari *et al.,* 2010); *Euphorbia neriifolia* (Yadav *et al.,* 2011); *Moringa oleifera* (Pontual *et al.,* 2012); *Zingiber officinale* (Gagaoua *et al.,* 2015; Hashim *et al.,* 2011); *Withania coagulans* (Pezeshki *et al.,* 2011; Salehi *et al.,* 2017); *Carica papaya* (Maskey and

Shrestha, 2020) and *Actinidia deliciosa* (Maskey and Karki, 2022) are some plants found to have proteases that give strong milk clotting activity.

A number of proteolytic enzymes are obtained from the latex of the aank plant, *Calotropis. gigantea*. Among these are the calotropins FI and FII (Abraham and Joshi, 1979) and the Calotropins DI and DII (Sengupta *et al.*, 1984). All these enzymes contain a free sulfhydryl group on which the activity depends, and therefore they are classified as plant sulfhydryl protease (Heinemann *et al.*, 1982). Crude proteases from latex of aank plant has promising milk clotting activity and protease activity and bears the potentiality to replace calf rennet as milk coagulant in cheesemaking process. The crude protease showed optimum activity at 37°C and pH of 5.5 (Rajagopalan *et al.*, 2019).

To industrialize useful and affordable products, the search for fresh plant proteases from various plant sections continues. With very few exceptions, the majority of plant rennet developed to date has been unsuitable for manufacturing commercial cheese due to a high proteolytic activity. As a result of the high degree of proteolytic activity, short peptides are produced, which influence the flavor and texture of cheese and give it an overly acidic and bitter flavor (Beigomi *et al*., 2014). When selecting a prospective plant rennet substitute, it is crucial to understand how casein degrades because it affects the yield, consistency, and flavor of the cheese. In order to produce commercial cheese, it is imperative to ensure the proper ratio of protein to peptides and to minimize general non-specific proteolysis (Singh *et al.*, 2014).

Three phase partitioning (TPP) is an effective and environmentally friendly bio separation technology. This technique, which is also used to isolate enzymes, entails the successive mixing of a crude extract or suspension with an organic solvent (most often *t*-butanol) and an adequate quantity of an aqueous anti-chaotropic salt, usually ammonium sulphate. The mixture separates into three distinct phases during agitation and centrifugation: an upper *t*-butanol phase, a lower watery phase, and an intermediate phase that is protein-enriched. While certain polar chemicals, such saccharides, are concentrated in the lower aqueous phase, the crude extracts often contain pigments, lipids, enzyme inhibitors, and other non-polar compounds that are enriched in the upper *t*-butanol phase. A protein-rich intermediate phase (in the form of a precipitate) is required to create the third phase, and this intermediate phase often exhibits an increase in enzymatic activity. TPP can be thought of

as a unique fusion of alcoholic precipitation and salting out techniques, with several benefits (Yan *et al.*, 2018).

1.2 Statement of problem

The demand for alternative milk-coagulating sources has increased due to a rise in cheese production around the world and a shortage of natural animal rennet (Ben Amira *et al.,* 2017). Systematic studies on the feasibility and acceptability of their replacement by alternative enzymes of plant origin have been prompted by the rising costs of calf rennet as well as ethical considerations related to the manufacture of such enzymes for general cheesemaking (Jacob *et al.,* 2011). The cost of rennet, religious objections, dietary restrictions, or the prohibition of recombinant calf rennet are all plausible explanations for the widespread usage of microbial coagulants and coagulants derived from plants. The aforementioned concerns are the causes of the growing interest in studies looking into alternative milk clotting enzymes to calf rennet (Mistry et al, 2017).

In addition to these, plant proteases outperform enzymes originating from microorganisms mostly due to safety issues like pathogenicity or other unfavorable consequences like increased proteolysis and lower production. Plant sources are now a good and accessible alternative to microbial and animal proteases (Sharma and Vaidya, 2018). A suitable, affordable non-rennet coagulating agent must be found in order to solve the growing problem and assist cheese producers as well as consumers.

1.3 Objectives

The objective of the research was divided into two parts:

1.3.1 General objective

The general objective of the dissertation work is to extract, purify and utilize proteolytic enzyme from the latex of *aank* (*Calotropis gigantea*) in fresh cheese making.

1.3.2 Specific objectives

The specific objectives are as follows:

- To extract and purify the proteolytic enzyme from the latex of *aank* by three phase partitioning method.

- To determine the milk coagulating activity (MCA), caseinolytic activity (CA), as well as the protein content of the purified protease.
- To optimize the pH and temperature of milk by response surface methodology (RSM) for making fresh cheese.
- To study physicochemical quality of prepared cheese.

1.4 Significance of the study

Chymosin (EC 3.4.23.4), the primary enzyme component of calf rennet, has been used extensively as a milk clotting enzyme (Ahmed *et al.,* 2009). Both plant coagulants and chymosin can break the phenylalanine-to-methionine peptide link in k-casein, although plant coagulants are more proteolytic and have a wider specificity than chymosin (Esteves *et al.*, 2003).

In the previous few decades, a lot of purification techniques have been proposed, but none of them are applicable on a broad scale. Most of these separation processes are costly, time-consuming, involve several stages, and are challenging to scale up (Ramos and Malcata, 2016). Three phase partitioning (TPP) is a practical and affordable method for the extraction, concentration, and recovery/purification of macromolecules, including enzymes, from various biological sources (Sharma et al, 2001).

Several target proteins from plant sources were purified using the three-phase partitioning (TPP) method (Gagaoua *et al.*, 2014, Rajagopalan *et al.*, 2018). Hence the use of TPP in purification of the *aank* protease will be a significant approach for their utilization in broad spectrum. In addition, the optimization of the TPP purification process of these enzymes leads to preferably cheaper extraction comparable to the chromatographical purification process.

1.5 Limitations of the study

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

- Sensory evaluation of the product was not carried.
- Texture evaluation of the product was not carried.

Part II

Literature review

2.1 Introduction to cheese technology

The term "cheese" refers to a category of fermented dairy products that come in a variety of flavors, shapes, and textures (Singh *et al.,* 2003). The definition of cheese is a dairy product made from milk by coagulating the casein with rennet or comparable enzymes in the presence of lactic acid produced by additional or accidental microorganisms, from which part of the moisture has been removed by cutting, cooking, or pressing, which has then been shaped in a mold, and which has then been allowed to ripen at the proper temperature and humidity level (Mijan *et al*., 2010).

Mankind has known about cheese for thousands of years. The cheese dates before the time of the Bible. From 6000 to 7000 BC, cheese first appeared. Over 12 million tons are produced annually globally, and the pace of growth is around 4%. Numerous cheese variants have developed over the years. Local conditions, climate, kind of milk, and other economic and geographical considerations all have a significant impact on the creation of cheese (Walstra *et al.,* 2006). The transformation of milk from a liquid into a solid (the curd), which retains the casein and fat of the milk but often removes most of the water, the whey proteins, and some of the lactose content, is a crucial step in the cheese-making process (Law and Tamime, 2010). The formation of a wide range of flavor sensations in cheese as it aged was caused by the natural contamination of milk or cheese by bacteria, yeasts, and molds (Johnson *et al*., 2001).

The goal of creating cheese is to create a product that is appealing and durable and contains concentrated amounts important components of milk, however making cheese is a difficult process that requires numerous process steps and biochemical changes (Walstra *et al*., 2005). In order to make cheese, milk fat and protein (casein) must be concentrated six to twelve times, and the pH must be lowered from roughly 6.6 in milk to between 4.6 and 5.4 in freshly created curd. The essential production procedures similar to all variations include coagulation, acidification, dehydration, forming (shaping), and salting, despite the fact that the production method varies widely from variety to variety (Kheir *et al.,* 2011).

The cheese pH, microflora, moisture content, and salt content regulate and control the biochemical changes that take place during age, which in turn affect the flavor, aroma, and texture of the final product. The manufacturing processes play a big role in the finished cheese's quality. However, during the ripening process, the distinctive flavor and texture of various cheese varieties develop (Guinee *et al.*, 2004).

2.2 Cheese production

Over the past 20 years, the output of cheese has increased globally (approximately 4% annually): from just under 6 million metric tons in 1961 to 7.6 million in 1970–1971, 12.3 million in 1984, and 14.65 million in 1994 (Farkye, 2004). In 2015, 2016, 2017, 2018, 2019, 2020, and 2021, the total amount of cheese produced worldwide was 19.52, 19.81, 20.44, 20.72, 20.98, 21.22, and 21.69 million metric tons, respectively. With a production output of over 10.35 million metric tons of cheese in 2020, the European Union was the world's leading cheese producer (Shahbandeh, 2021).

2.2.1 Cheese production- a Nepalese scenario

The first cheese business in Nepal was established in 1953, when the government began making yak cheese in the Langtang and Rasuwa districts with assistance from the FAO. Under the Department of Agriculture (DOA), a dairy development section was created, and a small-scale milk processing facility was launched in the Kavre district's Tusal hamlet. Dairy Development Corporation was established in 1955 (Khanal *et al*., 2019). Along with government assistance, private dairy plants with medium-sized facilities (that can process 10,000–30,000 L/day) have grown recently (NDDB, 2021).

According to the Department of Food Technology and Quality Control (DFTQC) and other offices under this department, 53 small and medium-sized cheese industries have been registered in Nepal to date (DFTQC, 2020). There is currently no precise information on cheese exports from Nepal. The Himalayan Dog Chew company has just begun exporting dry cheese, or "dog chew" as it is known in Nepali, to the United States, Britain, Canada, and Japan (Khanal *et al.*, 2019).

The majority of Nepalese do not particularly enjoy eating cheese. Many people are unfamiliar with the taste and flavor of cheese. In the food market of Nepal, it is a dairy item that is relatively expensive. Demand for pasteurized milk and milk products, including

cheese generally, has increased as awareness of health requirements and hygienic products has grown. Future growth of this demand is inevitable (Pradhan, 2000).

2.3 Varieties and classification

Cheese comes in more than 2,000 different varieties. There are likely about 18 different types of natural cheese; these differ in many steps including setting the milk, cutting the curd, stirring, heating, draining, pressing, and salting of the curds or ripening of the cheese. They can be categorized based on their origin, composition, firmness, the maturation agents used in their production, and the processes employed in their manufacture and maturation (Bamforth and Ward, 2018). Roquefort and Gorgonzola are examples of certain hard cheeses that are matured by molds. Additionally, microorganisms ripen a number of semi-hard cheeses, including brick. Unripened, acid curds like cottage cheese, Camembert that has been mold-ripened, and Limberger that has been bacteria-ripened make up soft cheeses (Mijan *et al.,* 2010). Some of the earliest cheese kinds are from the Middle East and the Mediterranean and are known as brined cheeses (Dimitreli *et al.*, 2017).

Khanal *et al.* (2019) stated that there are three alternative approaches for classifying cheese:

- Based on texture,
- Based on coagulation process and
- Based on ripening indices.

However, the following headings are where the qualities of cheese are included in the International Dairy Federation study (Upadhyay, 2003).

- Nation of origin.
- Raw milk: from cows, buffalos, sheep, goats, etc.
- Type of cheese hard, semi-hard, soft, fresh, acid coagulated or whey cheese.
- Internal characteristics: close or open texture, big, medium, or small eyes/holes, and slit apertures in curds that are ripened with blue or white mold.
- External characteristics: type of coating, ripening spices or herbal additions, rind hard, soft, smooth or rough, smear or mold (plastic, ash, etc.).
- Weight of cheese: shape and size.
- Fat in dry matter (FDM)/Fat on a dry basis (FDB): Minimum/Maximum percentages.

- Moisture in fat-free substances (MFFS)/Water in Fat-Free Substances (WFFS).

According to Codex Alimentarius, FAO/WHO, and Standard A6, cheese is categorized according to its texture in Table 2.1

Table 2.1 Classification of cheese according to Codex Alimentarius

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

Source: Scott (1986)

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

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

Cheese was categorized by Bamforth and Ward (2018) based on (i) Composition, (ii) Firmness, and (iii) Maturation agents, as indicated in Table 2.2.

Table 2.2 Classification of cheese on the basis of composition, firmness and maturation agents.

Type 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, Portdu 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)
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)

Almost all matured cheeses and nearly 75% of all cheese produced are rennet-coagulated cheeses. About 25% of all cheese production is made up of acid-curd cheeses, which are typically eaten raw. Coagulation employing a combination of heat and acid is employed for a limited number of kinds, including Ricotta and Manouri. Rennet-coagulated varieties were subdivided into relatively homogeneous groups based on the characteristic ripening agents or manufacturing technology.

The classification of cheese on the basis of method of coagulation suggested by (Fox *et al*., 2004) has been illustrated in the Table 2.3.

Table 2.3 Classification of cheese on the basis of method 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)

Both yak cheese and kanchan cheese are hard cheeses (MFFB, 55-56%, Full fat (FDB min. 45%), close textured, bacteria ripened, and rennet cheeses that are manufactured in Nepal. Swiss cheeses are similar to yak and kanchan cheese.

2.4 Fresh cheese

Fresh cheese represents a diverse group of varieties produced by the coagulation of milk, cream or whey via acidification, acidification with a small quantity of rennet, or a combination of acid and heat and which are ready for consumption once manufacturing is complete. 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 flavor and texture of the cheese develop.

2.4.1 Selection of milk

The composition of cheese is heavily influenced by the composition of the cheese milk, 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*., 2017).

2.4.1.1 Raw milk quality

Numerous mammals' milks, including those of goats, sheep, buffalo, reindeer, camels, zebras, and yaks, can be used to make cheese. The finest milk for creating cheese is from ruminants since it has high quantities of casein, a milk protein needed to create a sufficient coagulum (Goff, 2003). Cheese was traditionally manufactured from raw milk, a technique that persisted until the 1940s. In Europe, a sizable portion of cheese is still produced with raw milk (Fox *et al*. 2000). The use of raw milk in cheese making is undesirable because of the following reasons:

- It may be contaminated with a number of potential pathogenic bacteria that can make people sick.
- The undesirable microorganisms may also deteriorate the flavor and texture of cheese.

The use of milk as the primary ingredient in cheese production has a significant impact on the production process, yield, and cheese quality. Changes in the quality of raw milk may have an impact on how cheese is made, necessitating adjustment of the current production method, disruption of regular manufacturing operations, or altered product output, composition, and quality. Therefore, using high-quality milk to make cheese is necessary (Upadhyay, 2003). Factors include milk composition, anomalous milk, microbiological quality of milk, and many other factors might affect the milk quality used to make cheese (Nassar *et al*., 2015).

2.4.1.2 Chemical composition of milk

Animal-related factors (breed, milking, lactation, gestational stage, age, parity, and nutritional/health status), husbandry-related factors (milking intervals, feeding, herd size, and physical activity), as well as environmental factors (season, temperature, and weather) have an impact on milk composition (Dominguez-Salas *et al.*, 2019). Compared to cheese prepared from cow milk, buffalo milk cheese has higher levels of fat, protein, ash, and total solid (Mijan *et* al., 2010). The yield and characteristics of the resulting cheese are highly related to the content of the milk. The casein-containing milk proteins, milk fat, and calcium-rich mineral salts are the milk components that are most crucial for creating cheese (Scott, 1986). The firmness, melt, and chewiness of a cheese are ultimately determined by the casein molecule's interaction with nearby micelles in the cheese's composition (Johnson *et al.,* 2001).

2.4.1.2.1 Milk protein (casein)

Numerous protein types can be found in milk, albeit most only exist in trace amounts (Haenlein and Park, 2013). Whey protein (also known as serum proteins) and caseins are the two main forms of milk proteins. Over 80% of the protein in milk is made up of casein, while the ratio of whey protein to casein fluctuates depending on the stage of lactation (Chandan, 2008). 20% of milk protein is made up of whey protein, sometimes referred to as serum protein, which is composed of lactoglobulin (8.5%), lactalbumin (5.1%), immunoglobulins (1.7%), and serum albumins. From a cheesemaking perspective, milk's proteins are its most crucial components (Fox *et al*., 2004).

Milk that is high in casein and low in lactose and serum protein is ideal for making cheese. The majority of the latter two ingredients are lost in whey during the cheese-making process. In addition, milk with a higher whey protein content takes longer to coagulate with

rennet and yields a weaker coagulum. In general, milk with a high casein level has desirable cheesemaking qualities. It has been discovered that milk with a higher casein concentration produces curd with greater strength, a quicker rate of curd firming, a longer syneresis time, and cheese with a firmer body and texture (Kosikowski, 1982).

2.4.1.2.2 Milk fat

Major variations in fat content have a direct impact on moisture and protein levels as well as the yield of cheese in the majority of cheese varieties. It is a crucial factor in determining the distinctive body, composition, biochemistry, microstructure, yield, rheological and textural properties, cooking properties, cheese texture, and flavor (Abd El-Gawad *et al.,* 2007; Guinee and McSweeney, 2007). Due to moisture retention issues, cheese manufactured from nonfat or low-fat milk develops a hard, dry body (Pradhan, 2000).

Since fat and casein account for >90% of the total cheese solids, milk fat content and cheese output are also closely connected. Because high fat milk typically produces less fatty casein than milk rich in fat, the cheese output per kilogram of utilized fat dropped as milk fat content increased (Upadhyay, 2003). Due to increased cross-linking between the curd, cheese with a high fat content is less firm and elastic, whereas cheese with a low-fat content is harder and less smooth (Nassar., 2015).

2.4.1.2.3 Milk salt

Sodium, potassium, calcium, and magnesium phosphates, citrates, chlorides, sulphates, carbonates, and bicarbonates make up the majority of milk salts. Breed, cow individuality, lactation stage, nutrition, mastitis infection, and season of the year are only a few of the variables that affect how milk salts are composed (Fox *et al*., 2015). The creation and stability of casein micelles depend on milk salts. Milk salts have an impact on many crucial functional aspects of milk products, such as cheese texture, emulsification, protein stability, gelation, and protein stability (Lucey and Horne, 2009). Rennet coagulation is influenced by the calcium level of milk. Protein aggregation is more pronounced in cheeses with higher calcium contents (higher cheese pH) than to cheeses with lower calcium contents (lower cheese pH) (Pastorino *et al.*, 2003). The rheological and functional characteristics of cheese are influenced by calcium due to calcium-dependent interactions between casein proteins. The majority of cheese kinds are produced via the rennet coagulation method, and calcium has a big influence on this procedure (Papademas and Bintsis, 2017).

2.4.1.3 Abnormal milk

Any type of milk that differs significantly from regular milk is referred to as abnormal milk in general. Mastitis milk, colostrum, and late lactation milk are typically included. Abnormal milk is not suitable for cheesemaking due to factors or circumstances that cause "slow starter," sluggish rennet coagulation, and weak curd formation. Atypical enzyme levels may interfere with fruit ripening and flavor development (De, 2000).

2.4.1.3.1 Mastitis Milk

In dairy herds in affluent countries, mastitis is thought to be the most prevalent and dangerous production illness (Seegers *et al.,* 2003). Mammary gland irritation known as mastitis is typically brought on by a bacterial infection. During the inflammatory reaction, an influx of white blood cells raises the quantity of somatic cells in the milk (Mazal *et al.,*2007). Mastitis affects the quantity of milk produced, the composition of the milk, and the technological utility of the milk (Ogola *et al.,* 2007). Mastitis dramatically lowers the casein:protein and lactose: protein ratios while elevatingproteolysis. Cheese's moisture content rises due to mastitis, which also results in protein and fat loss (Martí-De Olives *et al.,* 2020).

2.4.1.3.2 Colostrum milk

The first milk produced after birth is called colostrum, and it has high levels of immunoglobulins, antimicrobial peptides (including lactoferrin and lactoperoxidase), and other bioactive compounds like growth factors. Colostrum differs from milk in that it has less lactose and more fat, protein, ash, vitamins, hormones, and immunoglobulins. Three days later, the percentage of the other ingredients steadily declines while the lactose level rises (Tripathi, 2006).

2.4.1.3.3 Late lactation milk

Due to its high salt and potassium levels, late lactated milk is not good for creating cheese since it causes more protein to be hydrated than in regular milk (De, 2000).

2.4.1.4 Changes in the milk after production

As a result, milk is highly perishable and is prone to changes in its physical, chemical, bacteriological, and organoleptic properties between the time it is produced and the start of the cheese-making process. After processing, milk may acquire taints such as those that are naturally present in feed, marijuana, and other substances. As a result, after manufacturing,

milk may exhibit oxidation, acid generation, lipolysis, proteolysis, alterations in casein micelles, and proliferation of off flavors (Devi, 2023).

2.4.1.5 Inhibitory substances

It is well known that raw milk has built-in inhibitory mechanisms. Additionally, leftover antibiotics, detergents, and sanitizers may be present in milk (Walstra *et al.*, 2006).

- Immunoglobulin, lactoferrin, lysozyme, and the peroxide system of lactoperoxidase-thiocyanate-hydrogen make up the natural inhibitory mechanism in raw milk. These mechanisms have been demonstrated to be effective against lactic streptococci proliferation and acid formation. Some lactic acid bacteria strains can produce a lot of hydrogen peroxide under aerobic circumstances, and self-inhibitory effects can be seen when manufacturing cheese (FAO, 1999).
- Residual antibiotics in milk are extremely unsatisfactory from the standpoint of public health as well as the fact that they can inhibit cheese starter, which can result in a number of flaws, including slow whey drainage, high moisture content in cheese, early and late blowing, weak and pasty body, and various types of taints, cracks, open texture, and sponginess (Farkye, 2004).
- Detergents and sanitizers are used for a wide range of applications at production farm, at a chilling center and at dairy factory. When applied by 'Good practice' they cause no residue problems in milk. However, their misuse possesses major problem (Walstra *et al.*, 2006).

2.4.1.6 Microbiological quality of milk

A complete food, raw milk has all the essential nutrients: protein, fat, sugar, vitamins, and minerals. Even though raw milk is sterile when it is secreted, handling, storing, and other pre-processing processes can contaminate milk with bacteria (de Silva *et al.*, 2016). Low count milk that is as free as possible from microorganisms that cause flaws in cheese, such as coliforms, yeast, clostridia, and specific species of lactobacilli, Propionibacterium, and Micrococci, is ideal for making cheese. These microorganisms can grow in milk's acid environment and cause flaws like gassiness and cheese discoloration. Milk must also be free of bacteria and organisms that create the toxins implicated in food poisoning for the sake of public health (Upadhyay, 2003).

2.4.2 Pre-treatments of milk for cheesemaking

Many types of cheese, particularly, but not solely, artisanal cheeses, are still manufactured from raw milk. For centuries, milk used to make cheese was not pre-treated before curdling. However, the majority of cheese manufacture today involves the treatment of milk through one or more processing steps prior to the inclusion of coagulant and starter culture, mostly for reasons of safety but also consistency of quality and modification of product features (Kelly *et al.*, 2008). They have a significant impact on the production schedule, production efficiency, physiochemical, 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
- CO_2 treatment
- Microfiltration
- Bactofugation
- Standardization
- Pasteurization
- Thermization
- Lactoperoxidase/thiocyanate/H_2O_2 treatment
- Filtration/centrifugation

2.4.2.1 Chilling and cold storage

Before being transformed into cheese, the majority of raw milk in developed dairy countries is chilled to 4°C and stored on the farm in refrigeration bulk tanks as well as in insulated or refrigerated storage tanks. This practice of retaining cooled raw and/or processed milk for an extended period of time alters the physiochemical condition of milk components, particularly casein and minerals, which raises the probability of psychotropic development (Upadhyay, 2003).

2.4.2.2 CO_2 treatment

Raw milk can be given CO_2 as an anti-microbial additive (Hendricks and Hotchkiss, 1997; Martin *et al.*, 2003). The prolongation of raw milk storage has been suggested, while taking into account the pressure and temperature conditions of the treatment since these can result

in protein precipitation, by adding 20 to 30 mmol/L at refrigeration temperatures (Rajagopal *et al.,* 2005). Different species and strains respond differently to CO_2. Generally speaking, the lag phase of aerobic plate counts rises significantly and more sharply than the lag phase of psychrotrophs. It is also feasible to inhibit coliforms by at least 1 log CFU/ml. Gram-negative bacteria and spores are more strongly affected by this therapy than are Gram-positive bacteria and spores (Martin *et al*., 2003; Singh et *al.,* 2012). However, the casein micelles may dissociate as a result of the pH decrease brought on by CO_2, which could reduce heat exchanger efficiency due to fouling (Loss and Hotchkiss, 2003).

2.4.2.3 Microfiltration

Particles with molecular weights larger than 200,000 Da are separated using microfiltration. Depending on the membrane pore size, milk solids removed by microfiltration include somatic cells, fat globules, bacteria, casein micelles, aggregated whey components, κ-casein, and β-lactoglobulin (Mistry and Maubois, 2017). The benefit of micro filtering skim milk before manufacturing cheese is that milk serum proteins can pass through the membrane and are not kept in the cheese, in contrast to caseins, which are retained (Papadatos *et al*., 2003).

2.4.2.4 Bactofugation

Bactofugation is a technique that is frequently used to eliminate spores from items that have undergone little pasteurization. This might entail removing Bacillus spp., Clostridium, and related species' spores from cheese milk. Despite the spores' small size, the density difference with plasma is bigger than it is for bacteria, and at separation temperatures of 60 to 65°C, a significant portion can be removed, often 90 to 95% (Walstra *et al.,* 2006). The sludge is then reintroduced back to the milk after being sterilized to remove the germs and spores (Fox *et al.,* 2004).

Bactofugation helps remove spores when 3% of the milk is lost. The shelf-life of pasteurized milk is increased using this pricey technique. For the elimination of 99% of spores, two separators must be used in a row (Haenlein and Park, 2013).

2.4.2.5 Standardization

Standardization can eliminate any significant imbalance in milk content, helping to preserve the cheese's homogenous quality (Phelan, 2007). Since fat and protein make up the majority of cheese, it makes sense to standardize the milk at a fat/protein ratio that ensures high cheese quality and consistent production throughout the year (Scott, 1986). It 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 30 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).

2.4.2.6 Pasteurization

Pasteurization of milk is done to eliminate any pathogenic and hazardous bacteria as well as to inactivate the phosphatase and xanthine oxidase enzymes that are already present in the milk. Pasteurization also boosts cheese production by insolubilizing a portion of the serum protein (Banks, 2011). Most counties use one of three pasteurization methods: flash heating (no holding) to a temperature of 75–95°C, HTST: 71–75°C/14–40s, and LTLT: 61–65°C/20–40 min (Scott, 1986). 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℃ for 15 s is commonly used for continuous pasteurization (Ong *et al*., 2017).

2.4.2.7 Thermization

The most popular method is applying a light heat treatment (Thermization), which involves heating to about 57 to 68°C for 15 to 20 seconds and then quickly cooling to 6°C. This significantly lowers the psychotropic population and can significantly increase the amount of time raw milk can be stored. Thermization is not a reliable risk control since it cannot get rid of vegetative pathogens. *Listeria monocytogenes*, for instance, can endure the procedure and proliferate during refrigerated storage. (Fernandes, 2009)

2.4.2.8 Lactoperoxidase treatment

Milk contains the enzyme lactoperoxidase. It doesn't have any inherent antibacterial properties, but when hydrogen peroxide, which is typically of microbial origin, is present, it oxidizes thiocyanate to generate inhibitors such hypothiocyanite. This is known as the lactoperoxidase (LPO) system, and it has some bacteriostatic activity against numerous infections as well as bactericidal activity against many Gram-negative spoilage organisms (Fernandes, 2009).

It catalyzes the conversion of SCN by H_2O_2 to OSCN and is the second most prevalent enzyme in cow's milk after xanthine oxidoreductase. SCN and OSCN are both safe for animals, however they stop the majority of bacteria from creating H_2O_2. The concentration of SCN and pH affect the activity of the LPO system in milk, which also produces H_2O_2 (mostly from bacterial metabolism) and thiocyanate anions from cyan glucosides in animal feeds. The preservation of raw milk during storage or the lengthening of milk shelf life, if performed prior to pasteurization, can be accomplished by activating the LPO system in milk through the addition of thiocyanate ions and H_2O_2 or activation of a H_2O_2 system (for example, glucose oxidase). This procedure is referred to as "cold pasteurization" when neither refrigeration nor heat treatment are used (Pruitt, 2003; Seifu *et al*., 2005).

2.4.2.9 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.,* 2006b).

2.4.3 Additives in cheese milk

The starter culture and the rennet are crucial ingredients in the cheese-making process. In some circumstances, it can also be essential to give other ingredients such calcium chloride ($CaCl_2$) and saltpeter (KNO_3 or $NaNO_3$) (Bylund, 1995).

2.4.3.1 Saltpeter ($NaNO_3$ or KNO_3)

Inhibitory salts (Saltpeter) are added to milk during the production of less acidic cheeses like Edam, Gouda, and Swiss to stop the growth of organisms that produce gas, such as the coliform/aerogenes group of bacteria that cause "early blowing" defects in cheese and the butyric acid bacteria that cause "late blowing" defects in cheese. Nitrite or nitrate concentrations of 10 to 100 ppm or 2 to 5 ppm, respectively, are adequate to prevent spore growth (Farkye, 2004).

2.4.3.2 Starters

In cheese-making, starter is of prime importance. Two principal types of culture are used in cheesemaking (Fox *et al.*, 2000):

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

2.4.3.3 Calcium salt

When rennet is acting in its second phase, calcium is crucial. As a result, for the coagulation to be successful, calcium balance between the soluble, colloidal, and complexed is crucial (Lucey and Fox, 1993). Adding $CaCl_2$ to cheese milk enhances the milk's rennet coagulation and syneresis capabilities. It is a typical supplement used in cheesemaking (McSweeney, 2007). To precipitate the para-casein created by renneting, milk must include Ca++; the more Ca++, the stronger the rennet action, the firmer the coagulation, and the quicker the curdling. The right balance of calcium salts, sodium salts, and potassium salts must be present in the milk. The coagulum will be excessively solid if there is too much calcium present, and too loose and soft if there is not enough calcium. In order to increase the rennet ability that was decreased by cooling and/or homogenizing the milk, replace the calcium that is precipitated during pasteurization, or when the milk initially contains too few calcium ions, $CaCl_2$ is added (Acharya, 2010).

2.4.3.4 Cheese color

It is customary to add additional color to light-colored milk in order to give cheese an appealing and tasty appearance. Riboflavin and carotenoids, two vital colors, are present in milk but absent in whey. Commonly, 88 g/1000 kg of vegetable-derived annatto cheese is used to add color (Kosikowski, 1982).

2.4.3.5 Salt

In cheese, salt has three main purposes: it preserves cheese, enhances flavor, and provides dietary sodium. Salt has a significant impact on the casein matrix's ability to bind water, as well as on its rheological and textural qualities and cooking abilities, together with the required pH and calcium level (McSweeney, 2007).

2.4.3.6 Rennet

Numerous cheeses are produced using rennet, a clotting agent. Its primary function is to coagulate casein, a milk protein. In the making of cheese, the proteolytic activity of the

rennet that is added to the milk makes it easier for the milk proteins to coagulate into "curds" (Bezie and Regasa, 2019).

Calf rennet, which is predominantly obtained from the fourth stomach of suckling calves, is the most widely utilized milk clotting enzyme in the cheese industry (Ben Amira *et al.,* 2017; Kethireddipalli and Hill, 2015; Mamo *et al.,* 2018). High cheese yield is achieved using calf rennet, which has a high milk clotting activity/proteolytic activity ratio and is heat sensitive (Moschopoulou, 2011). Calf rennet is well known for its great specificity for cleaving caseinomacropeptide from κ-casein, and it was the first milk clotting enzyme employed in cheese making in the year 21. (Liu *et al*., 2021). Calf rennet hydrolyzes the Phe-Met site of κ-casein in casein. Para-casein and the hydrophilic macropeptide diffuse into the whey. At pH 5.2-6.6 and 35°C, more than half of the -casein is hydrolyzed. The three-dimensional network micelle structure is formed as the para-casein approaches one another (Ghorbel *et al.,* 2003). The calcium ions then operate as a "bridge" between the micelles, causing them to aggregate and take on the appearance of curd, neutralizing the negative charge on the micelle surface (Liu *et al*., 2021). Since it must be isolated from animal stomach tissues and then purified using various techniques, calf rennet is extremely expensive (Adhikari *et al.,* 2021). Finding a milk-clotting enzyme that would satisfactorily replace calf rennet has received a lot of research interest, but there is growing interest in natural rennet extract from plants. This is because genetically modified bacteria have generated suitable substitutes for animal rennet (Kheir *et al.,* 2011b).

2.4.3.6.1 Factors affecting the rennet activity

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

a) pH

The optimal pH for chymosin is 5.4, which is related to the primary phase of the breaking of -casein in milk. The activity of the rennet enzymes is significantly influenced by even slight changes in the acidity of the milk. When the pH is decreased from 6.7 to 6.4 in the instance of chymosin, the enzymes react twice as quickly (De, 2000).

b) Temperature

Rennet works best at 42°C, and both higher and lower temperatures result in less activity. The enzyme is destroyed around 55–60°C. Coagulation takes two to three times longer at

30°C than it does at 42°C, so variations in milk temperature have an impact on the process (Adhikari *et al.,* 2000).

c) Calcium ion concentration

Only if the milk contains free calcium ions will the para-casein, which is the casein that the rennet enzyme converts, precipitate. Changes in coagulation time, curd hardness, and whey exudation will result from variations in the amount of free calcium ions present in milk (Acharya, 2010).

2.4.4 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 liters to 30,000 L or more, where it is converted to cheese curd by a process which involves three basic operations: acidification, coagulation and dehydration (Bennett and Johnston, 2004).

2.4.5 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 31 micro flora of the milk. However, this process is difficult to control and tends to give a variable product that may suffer from taints and inconsistent flavors. As a result, most cheeses are now made using selected starter, that gives predictable and desirable results. Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus and Lactobacillus delbrueckii are the primary species of starter bacteria used in cheese manufacture (Fernandes, 2009).

Direct acidification method involves addition of lactic acid/phosphoric acid to cold milk (2°C–12°C) to achieve a pH of 5.2 followed by addition of glucono-δ-lactone, which is slowly hydrolyzed to gluconic acid, resulting in a gradual reduction in pH to 4.6-4.8 (Makhal and Kanawjia, 2008). Direct acidification is more controllable than biological acidification,

and, unlike starters, it is not susceptible to phage infection. In addition to acidification, the starter bacteria help 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).

2.4.6 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).

2.4.7 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).

2.4.8 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 32 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).

2.4.9 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).

2.4.10 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 molds to separate it from whey (Acharya, 2010).

2.4.11 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).

2.4.12 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) 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 Mehmet, 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).

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

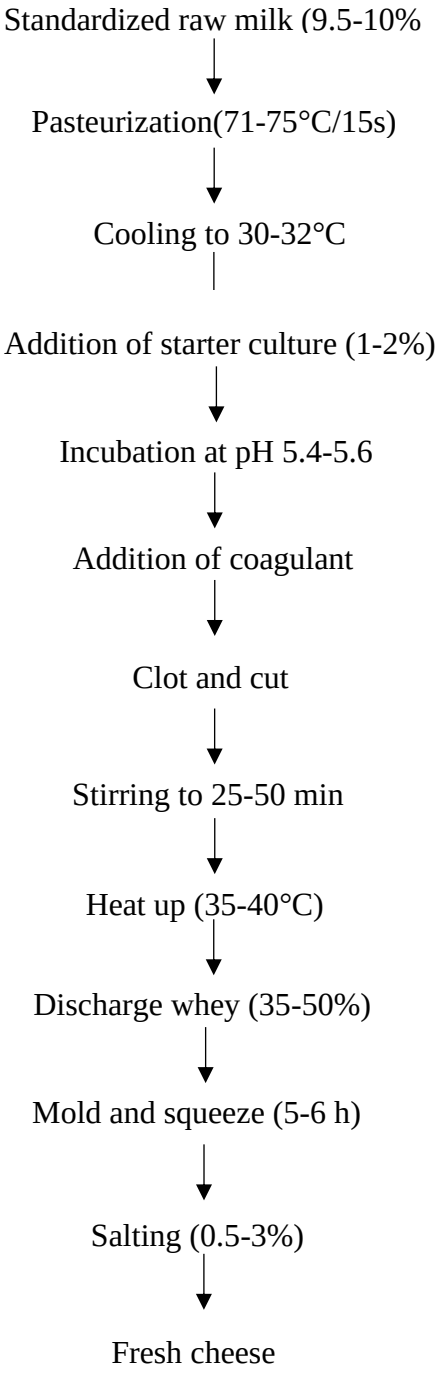

Fig 2.1 Processing steps for fresh cheese making

Source: Zheng *et al.* (2021)

2.5 Plant proteases as rennet substitute

The use of plant coagulants in cheese production has various benefits, including being secure, affordable, simple to make, allowing straightforward processes, and being utilized to produce cheese for ecological markets (Roseiro *et al.,* 2003). Plant proteases are divided into several groups according to the catalytic mechanism they utilize during hydrolytic process. Aspartic, serine, and cysteine 20 proteases make up the three main types of milk clotting proteases (Ben Amira *et al*., 2017). In certain parts of the world, plant proteases such as papain, bromelain, ficin, oryzasin, cucumisin, sodom apple, and *Jacaratia corumbensis* are used to make cheese (Duarte *et al.,* 2009). The main disadvantage of the majority of plant rennet is the emergence of cheese textural flaws and an increase in bitterness during storage and/or ripening (Egito *et al.,* 2007b).

2.5.1 Production of plant proteases

Several biological processes that take place throughout a plant's life cycle depend on plant proteases. One of the most critical steps in which proteases are crucial is plant germination, which entails the hydrolysis and mobilization of additional proteins stored in seeds and cereal grains (Martinez *et al*., 2019). Almost every component of the plant, including seeds, flowers, and latex, has been utilized to identify and study the proteases that are used as milk coagulants. To assure a constant supply of plant proteases, these enzymes can be acquired from their natural source or through in vitro cultivation (Gonzalez-Rabade *et al.,* 2011).

The choice of an appropriate protease extraction method is prerequisite for studying its properties. However, there are several methods for extracting plant protease. These range from a basic solvent-based extraction method to a more complex method involving protease purification. Plant protease extraction is complex as there is no single technique that will work for all the plant species. Since proteins are heterogeneous polymers, even little variations in the hydrogen ion concentration can significantly alter their characteristics. Thus, it is crucial to keep consistent pH in the protein environment. A simple extraction process involves the use of buffer solutions (sodium phosphate, sodium citrate, sodium acetate, potassium phosphate etc.) followed by salt precipitation for partial purification (Yahya *et al.,* 2019).

Several factors influence the protein solubility, including salt concentration in the solution. Salt stabilizes the charged groups on a protein molecule, drawing protein into the

solution and increasing its solubility at low concentrations which is referred as "salting-in". A point of maximum protein solubility is attained at greater salt concentrations, which reduces the quantity of water available to solubilize protein. Protein precipitates when there are hardly any water molecules left to interact with the protein molecules. This process of protein precipitation in presence of excess salt is referred to as "salting-out". Protease purification has been achieved by salting-out using a range of salts. Ammonium sulphate has been the most commonly used salt because of its high solubility and high ionic strength (Rachana and Jose, 2014).

2.5.1.1 Production from natural sources

To obtain plant proteases from organic sources, different plant organs including flowers, seeds, roots, and leaves can be aqueously macerated (Shah *et al*., 2014). The crude extract can be further processed to produce partially purified or pure enzyme, depending on the level of purification. Using ammonium sulfate as a precipitant, active proteases can be produced in huge quantities (Barros *et al*., 2001).

2.5.1.2 In vitro production

Plant cells can create the same chemical substances both in vitro and in vivo because they are totipotent. The two procedures produce different amounts of enzymes. Plant proteases developed in vitro often yield lower yields than those obtained under in vivo conditions (Gonzalez-Rabade *et al*., 2011). Proteases with different activities are produced by different parts of plant (Pérez *et al*., 2013). Proteases have been produced in vitro using various methods such callus and cell suspension cultures. For instance, *Centaurea calcitrapa* cell suspension culture (Raposo and Domingos, 2008), *Silybum marianum* callus culture (Cimino *et al*., 2006), and *Cynara cardunculus* callus culture (Oliveira *et al.*, 2010) are only a few examples. Techniques used in vitro have a number of benefits. The low enzyme yield and the challenges of extracting enzymes from natural sources may be resolved by in vitro enzyme synthesis. In vitro approaches can also be used to address issues with heterogeneity of the product made from plant parts and issues caused by climatic and seasonal variables (Gonzalez- Rabade *et al*., 2011).

2.5.3 Classification of proteases

Proteases are a broad and varied collection of hydrolytic enzymes that are categorized according to their mode of action, the makeup of the enzyme active site, and particular reaction processes (Ward, 2011). Proteases belong to a vast family and are classified as

endopeptidases and exopeptidases depending on where in the peptide chain they cleave. Serine proteinase, cysteine proteinase, aspartic proteinase, and metalloproteinases are the four categories of endopeptidases according to the reactive groups at the active site involved in catalysis (Barrett, 1994). However, plant proteases used as milk coagulants have only been reported from the first three types and none from metalloproteases (Bruno *et al*., 2006).

2.5.3.1 Serine protease

Eukaryotes and prokaryotes both include serine proteases, one of the major classes of proteolytic enzymes. Exopeptidases, endopeptidases, oligopeptidases, and omegapeptidases are only a few of the functions they display (Rawlings and Barrett, 1994). A number of biochemical and physiological characteristics are shared by serine proteases, which have a serine residue in their active site. The chymotrypsin-like, subtilisin-like, alpha/beta hydrolase, and signal peptidase groups are among the primary categories found in humans (Borgono *et al*., 2007).

2.5.3.2 Aspartic protease

The endopeptidases that rely on aspartic acid residues for their catalytic activity are referred to as acidic proteases or aspartic acid proteases. Three families of acidic proteases have been identified, including pepsin (A1), retropepsin (A2), and pararetrovirus enzymes (A3). The majority of aspartic proteases exhibit their peak activity between pH 3 and 4 (Kaur, 2011). Artichoke (Cynara scolymus L.), milk thistle (Silybum marianum L. Gaertn), Onopordumturcicum (Tamer), rice kernels, and Centaurea calcitrapa (Domingos *et al*., 2000) have all been found to have aspartic proteases with milk clotting activity.

2.5.3.3 Cysteine protease

Prokaryotes and eukaryotes both include cysteine proteases, also referred to as thiol proteases (e.g., bacteria, parasites, plants, invertebrates and vertebrates). A cysteine group is part of the active site's catalytic mechanism in these enzymes. Papain and similar plant proteases like chymopapain, caricain, bromelain, actinidin, ficin, and aleurain make up the family of enzymes known as cysteine proteases (Turk *et al*., 1997). Due to their ability to remain active throughout a broad range of temperatures and pH levels, cystine proteases have enormous potential in the food, biotechnology, and pharmaceutical industries (Gonzalez-Rabade *et al*., 2011).

Techniques used in vitro have a number of benefits. The low enzyme yield and the challenges of extracting enzymes from natural sources may be resolved by in vitro enzyme synthesis. In vitro approaches can also be used to address issues with heterogeneity of the product made from plant parts and issues caused by climatic and seasonal variables (Gonzalez- Rabade *et al*., 2011).

2.5.4 Applications of proteases

Based on the structures or characteristics of the active site of the proteins, proteases are proteolytic enzymes that catalyze the breakdown of proteins (Raju *et al*., 2013). The hydrolysis of proteins in food technology is one of the most pertinent areas of application, and this has greatly increased the usage of proteomics. The objective is to use peptide maps to categorize the various proteins that result from a given protease hydrolysis (Tavano *et al*.,2018). derived from plant sources have a great potential as processing aids in the manufacture of food (e.g., innovative dairy products, meat tenderizers, and protein hydrolysates) and medicine (e.g., digestive and anti-inflammatory medicines) (Huang *et al*., 2008; Katsaros *et al*., 2010). Now, industries can synthesize proteases, allowing for their cost-effective usage in industrial processes (Elizabeth *et al*., 2003).

2.5.5 Evaluation of enzymatic activities of plant protease

Milk clotting activity (MCA) is the ability of enzyme to hydrolyze specifically the κ-casein from milk and is most important property of proteases used in cheese production (Shah *et al.*, 2014). Concerning cheese production using plant rennet, the initial goal was always the production of coagulants with a maximum specific coagulant activity. This activity depends on several factors, such as the plant source, the part in the plant, as well as the type and the concentration of protease. In order to detect the optimum of activity, different comparison studies were carried out between extracts of different parts in the same plant. Results of Rajagopalan *et al.* (2019) revealed that the highest clotting activity was observed in the extract of latex followed by extracts of stems, leaves, and flowers of *Calotropis gigantea*, in descending order. In fact, aqueous extracts from flowers have been widely used as substitutes of animal rennet (AR) in some artisanal Italian Spanish and Portuguese cheeses. Protease is an enzyme that catalyzes proteolysis, breakdown of protein in smaller polypeptides or single amino acids by cleaving the peptide bonds that connect two amino acids. They follow a hydrolytic reaction mechanism. This potential is known as protease activity (PA) (Dhillon *et al.*, 2017).

2.6 Calotropis gigantea

Calotropis gigantea sometimes known as milkweed or swallow-wort are latex-producing plants belonging to the Asclepiadaceae family. They are also known for their medicinal properties (Singh *et al*., 1996; Rastogi *et al.,* 1991). The plant is a member of the Kingdom: Plantae, Order: Gentianales, Family: Apocynaceae, Subfamily: Asclepiadoideae, Genus: Calotropis, Species: C. gigantea. Aank often referred to as giant milkweed, is a typical wasteland herb. This plant is indigenous to Bangladesh, China, India, Indonesia, Thailand, Malaysia, Pakistan, Sri Lanka, Indonesia, and the Philippines. The plant has milky stems, oval, light green leaves, and clusters of either white or lavender-colored way flowers. Typically, plants are found strewn over dry coastal regions, on beaches, by the sides of roads, and in disturbed urban lots (Kumar *et al*., 2011).

A proteolytic enzyme from different parts of the Calotropis gigantea can coagulate milk. The enzyme from latex exhibits high caseinolytic as well as a milk-clotting activity when compared to other parts. Significant milk clotting index was presented by the enzyme of latex followed by stem, flower, and leaf in decreasing order. Proteolytic enzymes from all parts showed optimum activity at 37°C, pH 5.5 and 10 mM calcium chloride concentration with excellent pH (4.5–6.5) and thermal stability (30–60°C). (Rajagopalan *et al*., 2014).

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

2.6.1 Purification of plant proteases: Three phase partitioning (TPP)

The common methods for the purification of plant protease are chromatographic techniques and often include ammonium sulphate precipitation, column chromatography, gel-filtration and affinity chromatography. Chromatographic techniques are expensive, time consuming and involves several steps. Furthermore, the scale up for chromatographic techniques is also

difficult. Thus, an alternative method, such as TPP, for proteases purification is required to solve the drawbacks of chromatographic technique (Gagaoua *et al.*, 2015).

TPP is a simple, quick, and efficient process for the separation and purification of proteins from complex mixtures. It was first described by Tan and Lovrien (1972) and was intensively used to purify several target macromolecules and has now become a versatile and a common bio separation tool with a wide area of application (Gagaoua and Hafid, 2016). This three-stage batch bio separation process combines salting out, isoionic precipitation, osmolytic, kosmotropic protein precipitation, and alcohol precipitations to extract, purify, and concentrate proteins for application in small or large industrial operations (Saxena *et al.*, 2007). The principle of this fast-emerging tool consists in mixing the crude protein extract with solid salt (mostly ammonium sulphate $(NH_4)_2SO_4$) and an organic solvent, usually *t*-butanol in order to obtain three phases. The desired enzymes or proteins are selectively partitioned to one phase after centrifugation at 4°C while other contaminants such as pigments and lipids to the other one. The upper organic phase containing nonpolar compounds (pigments, lipids, etc.) is separated from the lower aqueous phase that containing polar compounds (proteins, carbohydrates etc.) by an interfacial protein precipitate (Gagaoua *et al.*, 2015).

In TPP, *t*-butanol is first added to the aqueous solution of protein to about 1 to 2 ratio. It is believed that this results in the protein equilibrating with the solvent (water) and the co-solvent. Thus, the protein becomes partially hydrated and partially *t*-butanol, in proportion to the relative abundance of the solvents in the mixture. Upon addition of ammonium sulphate, water is absorbed by the salt ions (especially the SO_4^{2-} ions) as these become hydrated. The salt appears to preferentially sequester water since it has a stronger affinity for water than for *t*-butanol. As a result, the solution separates into two phases in the absence of protein because part of the water becomes unavailable to the *t*-butanol. If protein is present, it equilibrates with the new proportions of solvent and co-solvent available to it. Thus, the partitioning process is affected by the hydrophobicity, the molecular weight, the charge and isoelectric point (pI) of protein and also by the physical conditions of the phase system (Dennison, 2021). Selectively concentrating enzymes in one phase and partitioning contaminants in a different phase enhances recovery, making scaling up and down easier in TPP (Rajagopalan and Sukumaran, 2018).

2.6.2 Effect of ammonium sulphate

The most common salt used for salting out proteins is ammonium sulphate, or $(NH_4)_2SO_4$, since it is affordable, easily accessible, mild on proteins, stabilizes certain proteins, and has a high solubility. Furthermore, it has been demonstrated that the intermolecular interactions in macromolecules like protein structures, which are near the endpoints of their respective Hofmeister series, may be stabilized by NH_4^+ and SO_4^{2-}. $(NH_4)_2SO_4$ saturation is crucial and plays a major role in TPP as it is responsible for protein–protein interaction and precipitation. It causes protein precipitation by salting-out mechanism. The efficiency of the salting out is firstly dependent on the amount of $(NH_4)_2SO_4$ and second on the ionic strength of the solution. For instance, at higher salt precipitation, water molecules are attracted by salt ions result in stronger protein interactions and the protein molecules precipitate through hydrophobic interactions (Gagaoua *et al.*, 2014).

2.6.3 Effect of *t*-butanol

TPP is based on the use of *t*-butanol. It is a C-4 non-ionic kosmotrope which is very soluble and miscible in water, but after the addition of solid salt, becomes hydrated and acts as a differentiating solvent (Özer *et al.*, 2010). It does not cause denaturation of the partitioned enzyme as it is unable to permeate inside the folded three-dimensional structure of protein due to its larger molecular size. At temperatures between 20 and 30°C, it exhibits strong kosmotropic and crowding effects that improve enzyme partitioning and may have an impact on stabilizing the isolated protein (Dennison, 2021).

2.6.4 TPP as an emerging technique for protease purification

TPP has been used to purify many proteins and enzymes with remarkable levels of recovery and purity. For example, it was widely used to purify invertase (Akardere *et al.*, 2010), pectinase (Sharma and Gupta, 2001), α-galactosidase (Dhananjay and Mulimani, 2009), trypsin inhibitor (Wati *et al.*, 2009), lactase (Rajeeva and Lele, 2011), catalase (Duman and Kaya, 2013) and many others. Proteases, which hold the first place in the world market of enzymes, play an important role in biotechnology. Dhananjay and Mulimani (2008) purified invertase and α-galactosidase from crude extract of *Aspergillus oryzae* using TPP. Proteases are extensively utilized in several production stages in the food industry. Although the majority of these enzymes are derived from microorganisms, vegetable proteases, which are obtained from higher plant organs, have recently received a significant amount of attention

as potential proteolytic enzymes in the food industry. Most of them are cysteine proteinases, namely papain, bromelain, zingibain, and ficin. Various proteases which are separated by TPP are shown in the Table 2.4.

Table 2.4 Some proteolytic enzymes purified using Three Phase Partitioning

Protease	Source	Reference
Ficin	*Ficus carica* latex	Gagaoua *et al.* (2014)
Zingibain	*Zingiber officinale roscoe* rhizome	Gagaoua *et al.* (2015)
Cucumisin	*Cucumis melo* var. *reticulatus* juice	Gagaoua *et al.* (2017)
Papain	*Dried papaya peels*	Chaiwut *et al.* (2010)
Protease	*Calotropis procera* latex	Rawdkuen *et al.* (2010)
Bromelain	*Ananas comosus* crown	Gul *et al.* (2022)

2.6.5 Advantage and disadvantage of TPP

TPP is a quick and easy method of purification that has proven to be quite efficient for concentrating a variety of protein solutions, particularly proteases. Through this method, certain enzymes are stabilized, and others are inhibited by *t*-butanol, allowing for crude extract preliminary fractionations. TPP increase enzymatic activity. In contrast, it was said to denature quaternary-structured proteins, such as hemoglobin, which is advantageous for separating blood proteins (Gagaoua and Hafid, 2016).

In addition, little quantities of $(NH_4)_2SO_4$ may need to be removed by dialysis. Similarly, some enzymes may lose their activity in the presence of high amount of *t*-butanol. Few studies have also reported that protein structure may be altered by *t*-butanol. IgG antibodies and proteins that are less abundant in the solution (less than 5 g) cannot be isolated using TPP. Otherwise, a problem with tissue homogenization is the possible generation of artefacts, either by proteolysis or by other protein/protein interactions (Gagaoua and Hafid, 2016).

Part III

Materials and methods

3.1 Materials

3.1.1 Sample

The latex of *aank* (*Calotropis gigantea*) was collected from different locations of Dharan.

3.1.2 Milk

Cow milk was collected from local farm of Hattisar, Dharan-14.

3.1.3 Rennet

Rennet (CHR.HANSEN, Denmark) was collected from Trishul Trade Links (Sarawagi Group), Kathmandu.

3.1.4 Chemicals and apparatus

The chemicals and apparatus required in this study are listed in Appendix A.1 and A.2 respectively.

3.2 Methodology

3.2.1 Extraction of *aank* protease

Extraction of protease was carried out according to Blandine *et al*. (2016). The *aank* latex and distilled water were mixed into 1:1 ratio and it was then centrifuged (2500 rpm, 10 min at 4°C) for separation of gums. The supernatant was collected and mixed with ammonium sulphate at 25% saturation. It was centrifuged (5000 rpm, 5 min at 4°C) and the supernatant was collected followed by precipitation with ammonium sulphate at 75% saturation. Finally, it was centrifuged (15000 rpm, 10 min at 4°C). The resulting pellets were dissolved in buffer and dialyzed overnight against three changes of the sodium phosphate buffer (50mM, pH 7). The dialysis of the extract was performed using dialysis membrane (MWCO 12-14 kDa). The crude extract was analyzed for protein content, caseinolytic activity and milk clotting activity. The crude extract was then taken for purification.

3.2.2 Purification of protease

Purification of the crude extracts were carried out by three phase partitioning (TPP) as described by Gagaoua *et al.* (2015). The crude dialyzed extract was added to an equal amount of *t*-butanol after being saturated with ammonium sulphate. The mixture was vortexed and permitted to stand for 1 h at room temperature. Using a Pasteur pipette, the top layer of *t*-butanol was removed. The interfacial phase (IP) was dissolved in sodium phosphate buffer (50mM, pH 7). The IP and aqueous phase (AP) were then dialyzed overnight with three changes of the same buffer. The total recovery profile (specific activity, purification fold and yield recovery) of both IP and AP was analyzed.

3.2.2.1 Optimization of salt concentration

The impact of ammonium sulphate concentrations (30%, 40%, 50%, 60%, 70%, and 80%) on the crude extracts for three phase partitioning was investigated at 1:1 ratio of crude extract to *t*-butanol, in accordance with Gagaoua *et al.* (2015).

3.2.2.2 Optimization of extract to solvent ratio

Several crude extracts to *t*-butanol ratios (1:0.5, 1:0.75, 1:1, 1:1.25, 1:1.5, and 1:2) were used to study the effect on purification at optimum ammonium sulphate saturation according to Gagaoua *et al.* (2017).

3.2.2.3 Optimization of pH of extracts

At optimum salt concentration and solvent ratio, the impact of varied pH values on the partitioning behavior of proteases were investigated at pH 4, 5, 6, 7, 8, and 9 following the procedure given by Gagaoua *et al.* (2017).

3.2.3 Determination of protein content

Quantitative determination of protein content of the extracts was estimated according to Bradford (1976) method (Appendix C). The protein content was determined using the BSA standard curve given in Fig. D.1.

3.2.4 Determination of caseinolytic activity (CA)

The CA of the proteases was determined using a method performed by Rajagopalan *et al*. (2014). Equal volume of casein and diluted enzyme was incubated at 37ºC for 30 min. The reaction was stopped by adding 3 ml of 10% trichloroacetic acid (TCA), which was incubated for 1 h on ice. The mixture was centrifuged at 5000 rpm for 10 min. The absorbance of supernatant was determined at 280 nm. The amount of enzyme needed to release 1 µg tyrosine under standard assay is referred to as one unit of CA. The standard curve for determination of CA is given in Fig. E.1.

3.2.5 Determination of milk-clotting activity (MCA)

The milk clotting activity will be determined following the procedure given by Berridge (1952). One milk coagulation unit is the amount of enzyme needed to coagulate 10 ml of reconstituted skim milk at 30°C for 100 s (U). It can be calculated as,

$$\text{MCA (U/ml)} = \frac{2400 \times V_S}{\text{Time} \times V_E}$$

Where, V_S = volume of substrate (milk) and V_E = volume of enzyme (sample)

3.3 Experimental design

The effect of two independent variables, namely temperature and pH of milk on MCA of purified *aank* latex protease 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 time of coagulation (TOC) and milk clotting activity (MCA). The experimental design, data analysis and model building were performed using "Design Expert" software (Version 13.0.5, Stat-Ease Inc., USA).

Table 3.1 Different constraints of optimization for *aank* protease

Name	Goal	Range
Temperature of milk	To be in range	40-55℃
pH of milk	Target = 6	5.8-6.5
Time of coagulation (TOC)	To be minimized	To be determined
Milk clotting activity (MCA)	To be maximized	To be determined

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

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

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.

3.3.1 Analysis of data

The independent process variables were correlated using a second order quadratic model. Multiple regression analysis with Design Expert software was used to determine the second order polynomial coefficient for each term of the equation. The statistical significance of the terms was investigated using analysis of variance for the response after the data was fitted to the selected model (Maskey and Karki, 2022).

3.4 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 *aank* protease was labelled as cheese B. Milk was heated until it reached temperature of 80-85℃. For cheese A, the rennet was added at the rate of (2.5 g/100 L) after the milk attained pH 6 by 2% food grade GDL i.e., (glucono delta lactone) addition at temperature of 37℃. For cheese B, pH 6 and temperature 55℃ of the milk were adjusted and the *aank* protease was added at the rate of 0.5% of the milk. The milk was stirred gently and allowed to coagulation for 35-40 mins. The curd was then cut by a stainless-steel knife to separate the whey and it was further cooked at 45-50℃ for 25-30 mins. 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℃ (Zheng *et al.*, 2021).

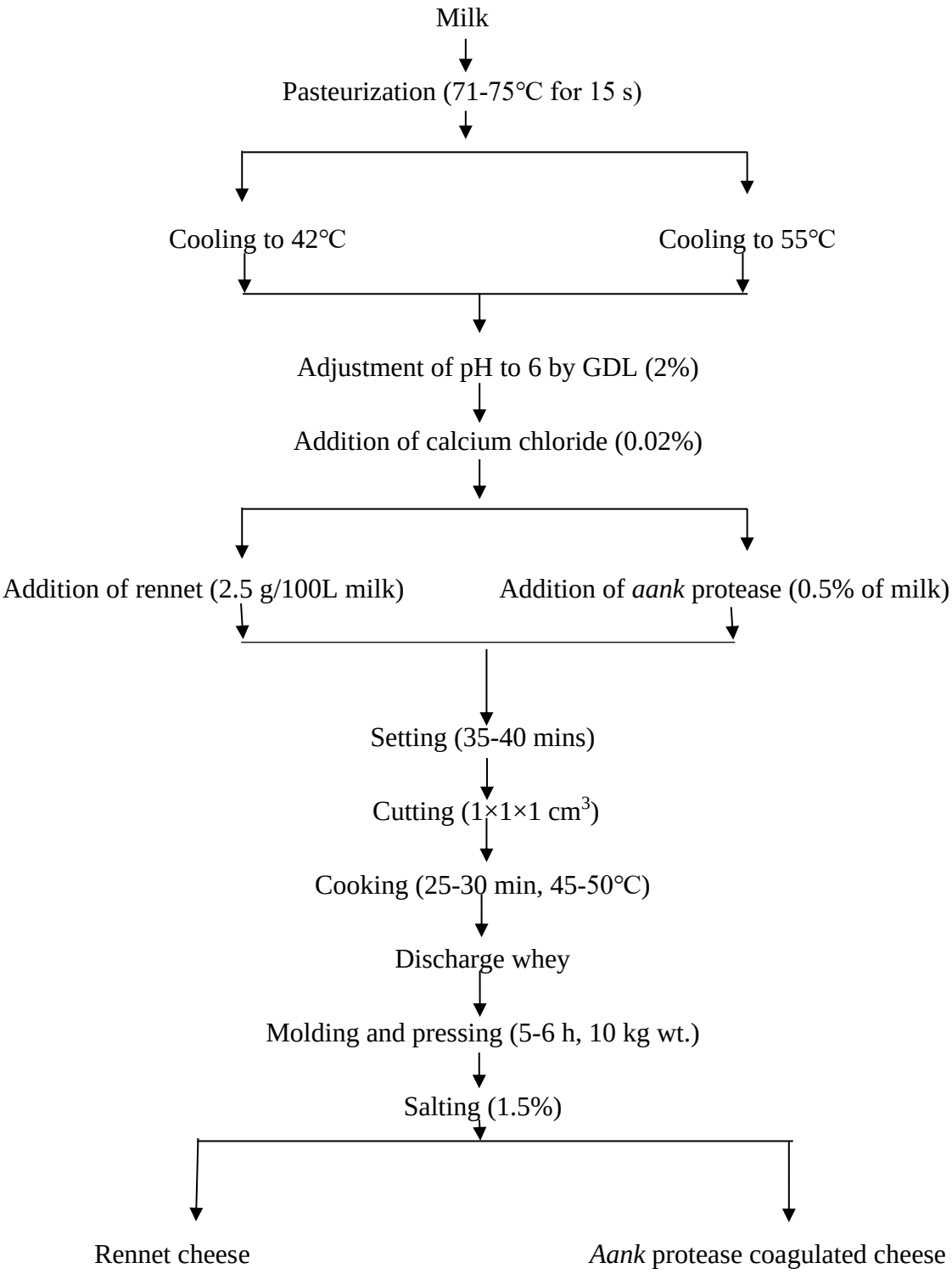

Fig 3.1 Preparation steps of fresh cheese

3.5 Physicochemical analysis of milk and cheese

3.5.1 Determination of fat in milk and cheese

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

3.5.2 Determination of SNF in milk

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

3.5.3 Determination of pH in milk and cheese

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

3.5.4 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).

3.5.5 Determination of acidity in milk and cheese

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

3.5.6 Determination of calcium in cheese

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

3.5.7 Determination of moisture in cheese

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

3.5.8 Determination of ash in cheese

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

3.5.9 Determination of cheese yield

The cheese yield was calculated by weighing the curd as described by Nasr *et al.* (2016). The percentage of cheese yield was calculated as follow:

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

3.6 Statistical analysis

Data are reported as mean ± standard error. The data were analyzed using SPSS version 26 and graphs were constructed in Microsoft Excel 2021 at 5% level of significance.

Part IV

Results and discussion

The crude *aank* latex protease was purified using three phase partitioning with the optimization of the parameters such as salt concentration, extract to solvent ratio and pH. The total recovery profile (specific activity, purification fold and yield recovery) of the purified enzyme was analyzed. 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 and TPP purified *aank* protease were analyzed for their physico-chemical properties.

4.1 Three phase partitioning of *aank* protease

TPP technique was used to partition crude extract from *aank* latex using salt (ammonium sulphate) and solvent (*t*-butanol). According to Gagaoua et al. (2017), contaminants mainly partition in the upper solvent phase and the lower AP (aqueous phase), while the pure form of the enzyme is typically recovered from IP (intermediate phase). Since enzyme activity in this investigation was focused in the IP fraction, this fraction was examined to standardize the best conditions for protease recovery and fold purification. Different ammonium sulphate concentrations and *t*-butanol ratios on CE (crude extract) were used to optimize TPP.

4.1.1 Effect of ammonium sulphate concentration

From analysis of crude extracts saturated with 30%, 40%, 50%, 60%, 70% and 80% ammonium sulphate, it was concluded that 60% saturation had the best effect based on its high activity with regards to % activity recovery and purification fold which is shown in the Fig. 4.1. To obtain a maximum recovery optimization of ammonium sulphate concentration is very crucial. At 30%, 40%, 50%, 70%, 80% salt saturations, low recovery percentage and purification fold were found as compared to 60% salt saturation. Beyond 60% there was a decrease in the % recovery of the *aank* protease. Hence, 60% w/v $(NH_4)_2SO_4$ concentration was selected as the optimum value for the next set of experiments. This salt concentration was sufficient enough to concentrate the protease. In research conducted by Gagaoua *et al.* (2014), maximum purification fold and activity recovery was obtained at 40% salt concentration for papain.

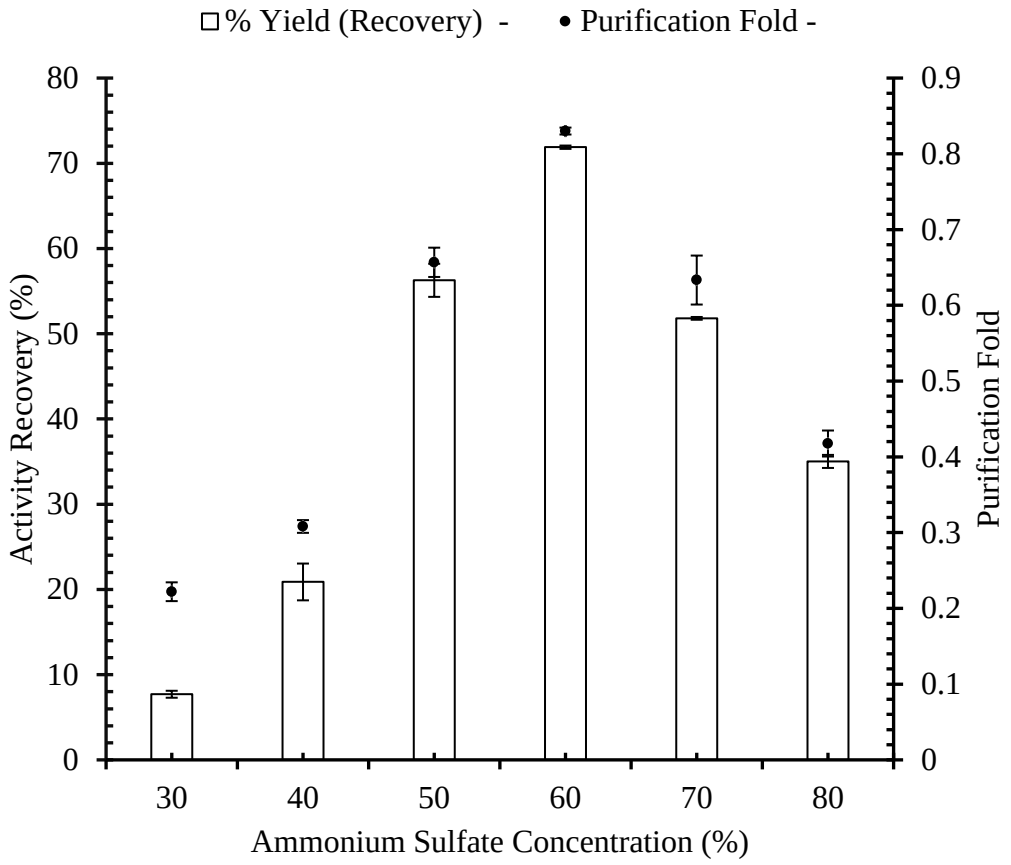

Fig. 4.1 Effect of varying saturations of ammonium sulphate on the degree of purification and activity recovery of *aank* protease. The crude extract was brought to different levels of saturation and *t*-butanol was added in the ratio 1:1 (v/v) with respect to the volumes of the aqueous extract.

4.1.2 Effect of ratio of *t*-butanol to CE

Different ratios of CE of *aank* latex to *t*-butanol, at 60% ammonium sulphate concentration, were evaluated to obtain high recovery and purification. 1:1.25 ratio of CE to t-butanol resulted in high recovery percentage and fold purification (Fig. 4.2). Further increase in butanol volume resulted in significant reduction in enzyme activity. It can be seen that the highest recovery was obtained at the ratio of 1:1.25 and activity recovery decreased with subsequent increase in crude extract to *t*-butanol ratio.

At 1:0.5 there was less activity recovery (%) which may be due to smaller amount of *t*-butanol does not adequately synergize with $(NH_4)_2SO_4$ (Özer *et al.*, 2010). All the reports published on TPP have used *t*-butanol as the organic co-solvent for partitioning and has been

generally reported to give best results (Gagaoua *et al.*, 2014; Wati *et al.*, 2009). Therefore, *t*-butanol was chosen as the organic co-solvent for partitioning of *aank* protease. T -butanol is miscible in water, but after the addition of enough salt, like $(NH_4)_2SO_4$, the solution gets separated into phases. *t*-butanol does not easily permeate inside the folded protein molecules due to its size and branched structure and hence does not cause denaturation (Rajagopalan and Sukumaran, 2018). If the volume of *t*-butanol is not optimum, it may not effectively synergize with ammonium sulphate for protein partitioning (Gagaoua *et al.*, 2017).

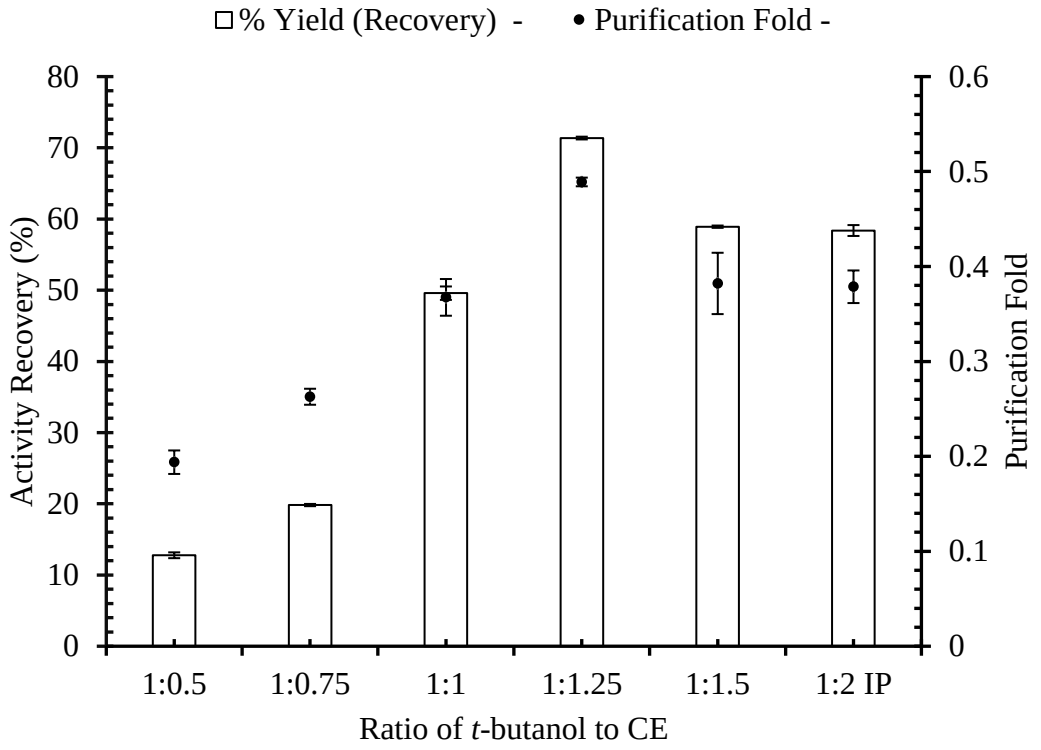

Fig. 4.2 Optimization of crude extract to *t*-butanol ratio for the recovery of *aank protease* for CA. Various amount of *t*-butanol was added to crude extract and saturated with 60% ammonium sulphate.

4.1.3 Effect of pH on enzyme activity

The behavior of the effect of crude extract with different pH values ranging from 4 to 9 on the partitioning of *aank protease* is illustrated in Fig. 4.3. It depicts that 60% (w/v) $(NH_4)_2SO_4$ saturation with 1:1.25 ratio of crude extract to *t*-butanol (v/v) at pH 6 gave a maximum fold purification and activity recovery of the enzyme.

It can be stated that the purification efficiency was low in the acidic as well as basic range as compared to the neutral range. The possible reason for this type of behavior may be that in acidic pH, there are more H^+ ions which compete over protease interaction with the solvent molecules and this does not allow the enzyme to interact due to which there is a decrease in the recovery at acidic pH. In research conducted by Gagaoua *et al.* (2015), maximum purification fold and activity recovery was obtained at pH 6.

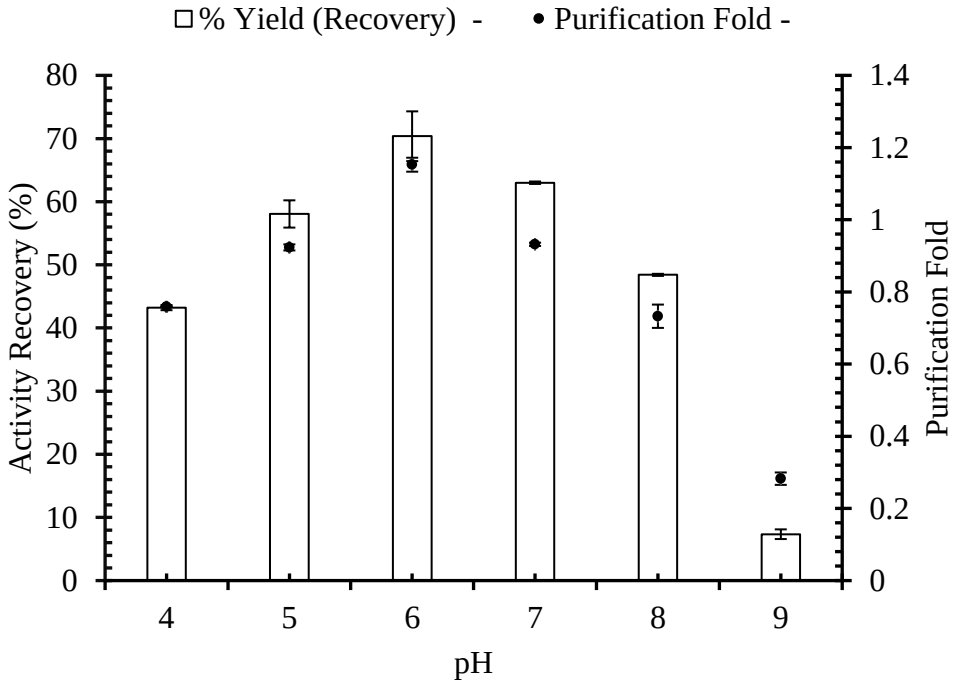

Fig. 4.3 Influence of pH on the degree of purification and activity recovery of *aank* protease. Ammonium sulphate (60% w/v) was added to the crude extract. The pH of the medium was adjusted to different pH values. This was followed by addition of *t*-butanol in a ratio of 1:1.25 (v/v).

4.1.4 Overall TPP recovery profile of *aank* protease

Overall TPP recovery profile of protease from *aank* latex at 60% ammonium sulphate precipitation and 1:1.25 ratio of CE to *t*-butanol at pH 6 based on caseinolytic activity is represented in Table 4.1. From these data, it appears that TPP process is suitable for economic and speedy recovery of milk clotting proteases from *aank* protease. Increased recovery percentage at optimal conditions could be attributed to the synergistic effect of ammonium sulphate concentration and *t*-butanol volume (Rajagopalan and Sukumaran, 2018).

Table 4.1 TPP recovery profile of protease from *aank* latex based on CA.

Enzyme Extract	CA (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Activity Recovery (%)	Purification Fold
Crude	472.93± 0.8	62.70± 0.31	7.54± 0.07	100± 0.0	1± 0.0
IP	332.77± 0.2	51.75± 0.61	6.43± 0.01	70.36± 0.96	1.15± 0.15

4.2 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 *aank* protease varied from 5-41 s and 585.37-4800 units respectively as shown in Table 4.2. Table B.1 and B.2 show the coefficients of the model and other statistical attributes of TOC whereas Table B.3 and B.4 show that of MCA.

Table 4.2 Product responses by independent variables

Run	Space Type	Factor 1	Factor 2	Response 1	Response 2
		A: Temperature (°C)	B: pH	TOC (s)	MCA (Units/ml)
1	Center	48	6.2	27	888.89
2	Factorial	55	5.8	5	4800
3	Factorial	40	5.8	19	1263.16
4	Center	48	6.2	25	960
5	Axial	58	6.2	14	1784.79
6	Factorial	55	6.5	35	685.71
7	Axial	48	6.6	40	600
8	Factorial	40	6.5	41	585.37
9	Center	48	6.2	26	923.08
10	Axial	48	5.7	6	4000
11	Axial	37	6.2	35	685.71
12	Center	48	6.2	26	923.08
13	Center	48	6.2	28	857.14

The coded equations for *aank* protease are:

$$TOC = 26.40 - 6.21\ A + 12.51\ B + 2.00\ AB - 0.6375\ A^2 - 1.39\ B^2 \text{ ---------- } 4.1$$

$$MCA = 910.44 + 648.94\ A - 1200.05\ B - 859.12\ AB + 178.89\ A^2 + 711.26\ B^2 \text{ ----------} 4.2$$

where A and B are the coded values of temperature 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) and 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) and pH of milk (B^2) had no 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 no significant (P>0.05) positive 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 no significant (P>0.05) positive effect and the quadratic term of pH of milk (B^2) had significant (P<0.05) positive effect on MCA as given by Table B.4. The interaction term of temperature of milk and pH of milk (AB) had significant (P<0.05) negative effect on MCA.

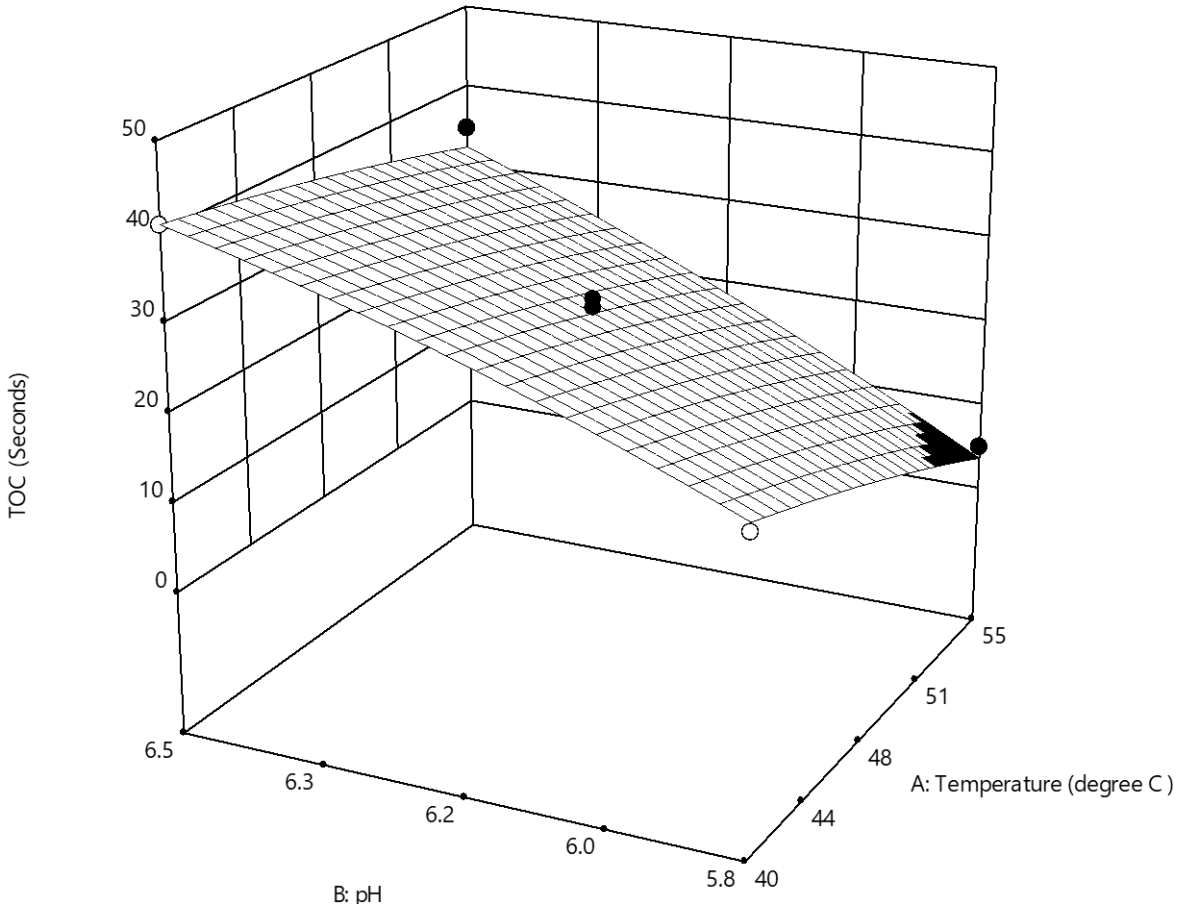

Fig. 4.4 Response surface plot of TOC of *aank* protease as a function of pH and temperature of milk.

In Fig. 4.4, it was found that TOC decreased gradually with higher temperature while increased greatly with increase in pH in confirmation with Fig. 4.5 and 4.6. Maskey and

Karki (2022) also reported that the TOC of kiwifruit protease increased gradually with the increase in pH, while decrease in TOC was seen with the increase in temperature of milk.

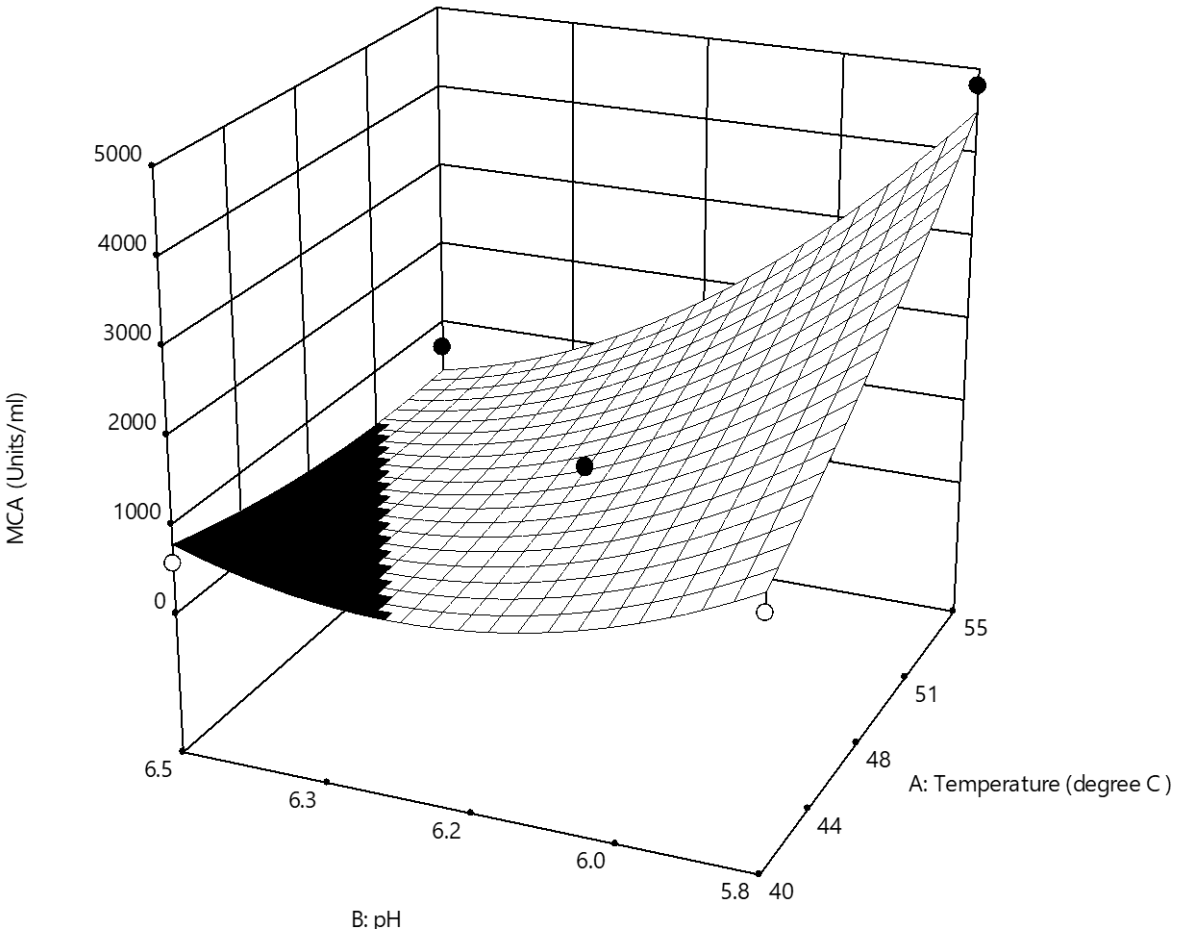

Fig. 4.5 Response surface plot of MCA of *aank* protease as a function of pH and temperature of milk.

In Fig. 4.7, it was found that MCA increased gradually with higher temperature while decreased greatly with increase in pH in confirmation with Fig. 4.8 and 4.9. Maskey and Karki (2022) also reported that the TOC of kiwifruit protease increased gradually with the increase in pH, while decrease in TOC was seen with the increase in temperature of milk.

4.2.1 Optimization of *aank* protease

A numerical response optimization technique was applied to determine the optimum combination of temperature of milk and pH of milk for the minimum TOC and maximum MCA, which is shown in Table 4.3.

Table 4.3 Different constraints for optimization of *aank* protease

Name	Goal	Lower Limit	Upper Limit
Temperature	is in range	40	55
pH	is target = 6.0	5.8	6.5
TOC	minimize	5	41
MCA	maximize	585.37	4800

The optimum combinations were found to be milk pH 6 and 55℃ of milk temperature, along with 0.736 desirability as shown in Table C.1. The TOC of 12.91 s, and MCA of 2779.65 units were obtained under this optimum condition.

4.2.2 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 4.4.

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

Response	Conditions		Predicted value	Mean Observed value
	Temperature of milk (℃)	pH of milk		
TOC	55	6	12.9108	10.3333
MCA	55	6	2779.65	2527.27

4.3 Physicochemical properties

4.3.1 Chemical composition of raw cow milk

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

Table 4.5 Proximate composition of raw cow milk

Parameter	Cow milk
Moisture (%)	85.34±0.11
Fat (%)	2.88±0.04
Protein (%)	3.88±0.05
SNF (%)	8.11±0.03
Acidity (%)	0.13±0.02
pH	6.6±0.06

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

The resulted presented in Table 4.5 revealed that the moisture, fat, protein, SNF, acidity and pH in cow milk were 85.3%, 2.8%, 3.8%, 8.1%, 0.13% and 6.6 respectively. The values are similar to those reported by Walstra *et al.*, (2006) and any variation may be due to cow breed, milking conditions and milking time.

4.3.2 Chemical composition of fresh cheese

The chemical composition of the fresh cheeses made from rennet and *aank* protease has been shown in Table 4.6. The moisture percentage is in the line with the findings of Johnson *et al.* (2001) and the findings of Nawaz *et al.* (2011) but higher than that of Rana *et al.* (2017). The results were higher than the findings of Islam (2006), who reported moisture content of cows' milk cheese as 46.8%. The variation in moisture might be due to the difference in milk composition, activity of coagulant and processing techniques. Among the two cheeses the average moisture was more in *aank* protease. Variation in moisture might be attributed due to the difficulty in whey drainage, resulting from clogging of the drainage screen by fine particles. Similar views were expressed by Hill *et al.* (1982). Moisture content in soft cheeses made using *aank* protease were significantly higher ($P<0.05$) than that of rennet (Table F.1). Molecular forces involved in the coagulation of casein by plant proteases resulted into a greater water binding capacity of protein matrix of cheese which could be the reason of

variation (Koirala, A. 2021). 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).

Table 4.6 Chemical composition of rennet and *aank* protease coagulated fresh cheeses

Parameters	Cheese samples	
	Rennet	*Aank* protease
Moisture (%)	49.8^{a}±0.01	51.07^{b}±0.02
Fat %	25.2^{a}±0.26	23.33^{a}±0.9
Protein %	19.99^{a}±0.02	20.38^{a}±0.11
Ash %	2.23^{a}±0.01	2.33^{a}±0.02
pH	5.64^{b}±0.01	5.99^{a}±0.02
Calcium (mg/100 g)	652.73^{b}±0.45	636.9^{a}±1.99

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

The fat level correlated with findings of Nawaz *et. al.* (2011). The results were similar to the findings of Ghosh and Singh (1990) and Islam (2006). Among the two samples, the average fat was higher in rennet than *aank* protease coagulated cheese but no significant difference ($P>0.05$) was found (Table F.2). The fat is one of the leading factors in determining the characteristic body, texture and flavor of cheese (Abd El- Gawad *et al.*,2007). The lower value fat contents recorded in cheese made using plant latex might be due to the fact that it takes more time for coagulation as compared to rennet. This may be responsible for the retention of fat in the final product (Khan and Masud, 2013.

The protein contents of both the cheeses are in line with the findings of Nawaz *et. al.* (2011). Proteolytic plant proteases are responsible for the formation of nitrogenous products of intermediate size, such as peptones, polypeptides, peptides and free amino acids. Enzymes of microorganism act on these and other substances to form products like amino acids, amines, fatty acids, esters, aldehydes, alcohols and ketones (Fox and McSweeney, 2017).

Among two samples, the higher protein content was found in *aank* protease coagulated cheese but no significant difference (P>0.05) was found (Table F.3).

The ash contents of fresh cheese are similar to the findings of Mijan *et al.* (2010). Khan and Masud (2013) and Patel and Gupta (1986), who had reported that the ash contents of cheese ranged from 2.50 to 3.20%. The possible reason for the less ash contents may be the seasonal variation in the composition of milk. Ash content in soft cheeses made using *aank* protease were significantly higher (P<0.05) than that of rennet (Table F.4).

The pH is the most vital indicator of food quality and safety. pH of food such as milk and milk products are measured to ensure the quality of foodstuff (Razzaq, 2003). The functional properties of cheese are greatly influenced by the pH (Rowney *et al.*, 1999). Among two samples the pH was found significantly higher (P<0.05) at rennet cheese (Table F.5) which may be due to the processing techniques, effect of coagulants, incubation and coagulation time and temperature.

The calcium content of cheeses ranges from 630-655 mg/100 g of cheese. Among the two samples the calcium content was significantly more (P<0.05) in rennet cheese (Table F.7). According to Keller *et al.* (1973), calcium content inversely correlates to the moisture content of the cheese. Similarly, Joshi *et al.* (2004) reported that caseins are more hydrated as the level of bound calcium decreases in milk.

4.3.3 Cheese yield

Optimum yield of cheese is of vital importance for cheese in cheese making operation. Emmons and Binns (1990) reported that accurate estimates of cheese yield were of great importance in establishing the relationship between composition of milk and yield of cheese and in assessing the efficiency of an operation in converting milk into cheese. The yield of fresh cheeses is shown in Table 4.7. There was no significant difference (P>0.05) in the yield of the cheese samples (Table F.8). The yield of all samples was lower than the findings of Mahajan and Chaudhari (2014). The variation may be due to difference in milk composition and processing technique. The yield reduction may be due to variation in cheese making technique resulting in low casein and/or fat retention. Among the two cheeses, the *aank* cheese has more percentage of yield than of rennet cheese. The higher yield in cheese prepared with plant protease may be attributed to the longer coagulation time resulting in more moisture contents which increased the yield.

Table 4.7 Yield percentage of rennet and Aank cheese

Cheese	Yield (%)
Rennet	21.71^{a}±1.32
Aank latex protease coagulated	22.75^{a}±1.11

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

Part V

Conclusions and recommendations

5.1 Conclusions

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

1. The optimum conditions for three phase partitioning (TPP) purification of *aank* latex protease that yields highest activity recovery and purification fold were 60% (w/v) ammonium sulphate concentration, 1:1.25 ratio of crude extract to *t*-butanol (v/v) and pH 6.
2. Numerical optimization study revealed that the optimum condition for maximum milk clotting activity and minimum time of coagulation were 2779.65 U/ml and 12.91 s at pH 6 and temperature 55℃ of milk.
3. The fat, protein and ash content of rennet cheese and latex protease coagulated cheese were not significantly different, while other parameters were found to be significantly different.
4. The yield of rennet cheese was not significantly different than that of *aank* protease coagulated cheese.

5.2 Recommendations

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

1. Tricine SDS–PAGE analysis for determination of molecular weight of the protease could be performed.
2. Microbial analysis of cheese could be done.
3. Texture evaluation of the product can be performed.

Part VI

Summary

The main objective of this research is to extract, purify and utilize proteolytic enzyme from the latex of *aank* (*calotropis gigantea*) in fresh cheese making. The factor that influences the purification and activity of an enzyme in TPP such as ammonium sulphate saturation, crude enzyme to *t*-butanol ratio and pH range were optimized for maximum activity recovery and purification fold. The milk clotting activity (MCA), caseinolytic activity (CA) and protein content of intermediate phase (IP) and aqueous phase (AP) were determined. The optimized conditions of the TPP purified protease for maximum MCA and minimum time of coagulation (TOC) was determined using response surface methodology. The fresh cheese was compared with rennet cheese for physico-chemical analysis.

The present work showed that the TPP of crude latex extract using ammonium sulphate precipitation at 60% saturation, 1:1.25 crude extract to butanol ratio and pH 6 had the best activity at IP based on activity recovery (%) and purification fold. The activity recovery (%) and purification fold of the TPP purified *aank* protease was found to be 70.36± 0.96% and 1.15± 0.15 respectively. The enzyme showed optimum temperature at 55°C and pH 6 which gives maximum milk clotting activity (2779.65 U/ml) with minimum time of coagulation (12.91 s). The physico-chemical parameters (fat, protein and ash content) of cheese prepared from rennet and *aank* latex protease were found to be not significantly different ($P>0.05$). The yield of rennet cheese was not significantly different than that of *aank* protease coagulated cheese.

This study indicates that TPP purified *aank* protease can be used to produce fresh cheese. Therefore, *aank* latex protease can be viewed as a possible substitute for calf rennet in the manufacturing of fresh cheese.

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Appendices

Appendix A

A.1 Chemicals required

S. No.	Chemicals	Manufacturer
1	Acetic acid	Qualigens, India
2	Bovine serum albumin	Qualigens, India
3	Calcium acetate	HiMedia
4	Calcium chloride	HiMedia
5	Casein solution	Merck
6	Citric acid	HiMedia
7	Coomassie Brilliant Blue G-250	HiMedia
8	Ethanol	Qualigens, India
9	FC reagent	Qualigens, India
10	L-tyrosine	Qualigens, India
11	Phosphoric acid	HiMedia
12	Potassium phosphate buffer	HiMedia
13	Sodium acetate	HiMedia
14	Sodium carbonate	HiMedia
15	Sodium citrate	HiMedia
16	Trichloroacetic acid	Merck

A.2 Apparatus required

S. No.	Apparatus	Manufacturer
1	Beaker	Merck
2	Conical flask	Merck
3	Grinder	Baltra
4	Incubator	Ambassador P.I.D.
5	Magnetic stirrer	INTTLAB
6	Measuring cylinder	Merck
7	Spectrophotometer	Agilent Technologies
8	Centrifuge	DLAB
9	pH meter	OHAUS
8	Micropipette	Micro-lit RBO
9	Test tube	Merck
10	Vial	Merck
11	Water bath	LABLINE
12	Weighing balance	JA1 203

Appendix B

Table B.1 Model summary statistics for Time of Coagulation

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	**2.30**	**0.9672**	**0.9607**	**0.9360**	**103.30**	**Suggested**
2FI	2.02	0.9772	0.9695	0.9434	91.40	
Quadratic	1.77	0.9864	0.9766	0.9209	127.59	
Cubic	1.29	0.9948	0.9876	0.8710	208.13	Aliased

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

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1591.69	5	318.34	101.29	< 0.0001	significant
A-Temperature	308.74	1	308.74	98.24	< 0.0001	
B-pH	1252.08	1	1252.08	398.39	< 0.0001	
AB	16.00	1	16.00	5.09	0.0587	
A^2	2.83	1	2.83	0.8995	0.3745	
B^2	13.39	1	13.39	4.26	0.0779	
Residual	22.00	7	3.14			
Lack of Fit	16.80	3	5.60	4.31	0.0961	not significant
Pure Error	5.20	4	1.30			
Cor Total	1613.69	12				

Table B.3 Model summary statistics for Milk Clotting Activity

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	841.50	0.6777	0.6132	0.3595	1.407E+07	
2FI	677.32	0.8121	0.7494	0.5277	1.038E+07	
Quadratic	**282.11**	**0.9746**	**0.9565**	**0.8212**	**3.928E+06**	**Suggested**
Cubic	54.36	0.9993	0.9984	0.9742	5.660E+05	Aliased

Table B.4 Analysis of variance (ANOVA) for Response Surface Quadratic Model of Milk Clotting Activity

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.141E+07	5	4.283E+06	53.81	< 0.0001	significant
A-Temperature	3.369E+06	1	3.369E+06	42.33	0.0003	
B-pH	1.152E+07	1	1.152E+07	144.76	< 0.0001	
AB	2.952E+06	1	2.952E+06	37.10	0.0005	
A^2	2.226E+05	1	2.226E+05	2.80	0.1383	
B^2	3.519E+06	1	3.519E+06	44.22	0.0003	
Residual	5.571E+05	7	79584.27			
Lack of Fit	5.510E+05	3	1.837E+05	120.81	0.0602	not significant
Pure Error	6081.03	4	1520.26			
Cor Total	2.197E+07	12				

Appendix C

Table C.1 Solutions of optimization result

S. No.	Enzyme concentration	Temperature	pH	Time of Coagulation	Milk Clotting Activity (MCA)	Desirability
1	**55.000**	**5.996**	**12.911**	**2779.653**	**0.736**	**Selected**
2	55.000	6.000	13.066	2753.144	0.736	
3	55.000	5.991	12.661	2822.412	0.736	
4	55.000	5.975	11.957	2944.190	0.734	
5	55.000	5.966	11.527	3019.379	0.732	
6	55.000	5.930	9.881	3313.638	0.714	
7	40.000	6.442	39.783	651.589	0.039	

Appendix D

Calibration curve for protein content

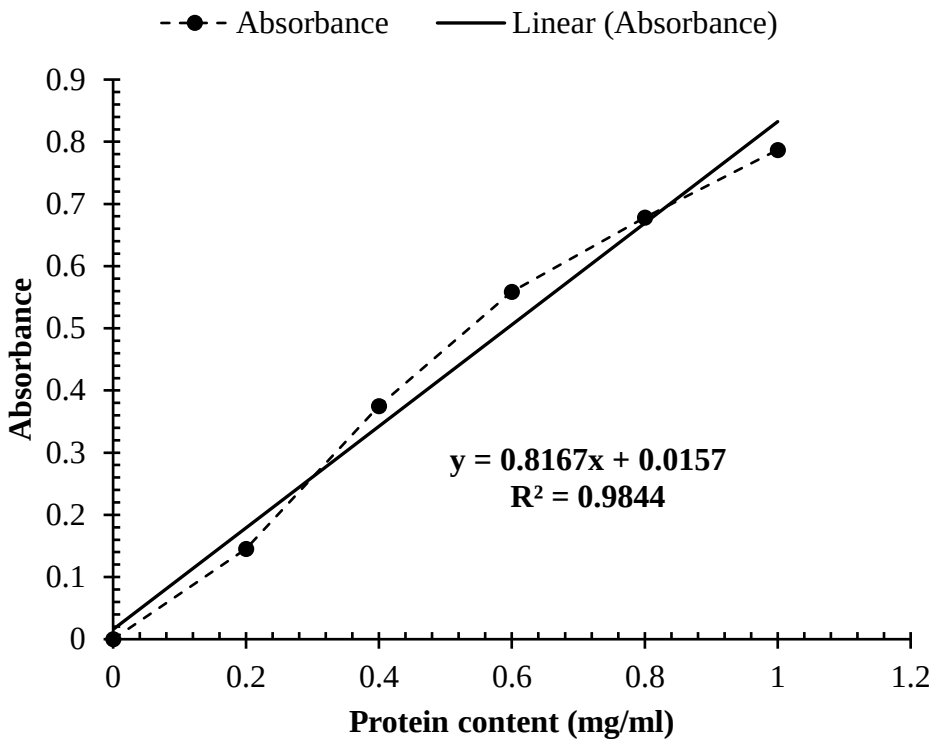

Fig. D.1 BSA standard curve for Bradford protein assay

Appendix E

Calibration curve for caseinolytic activity

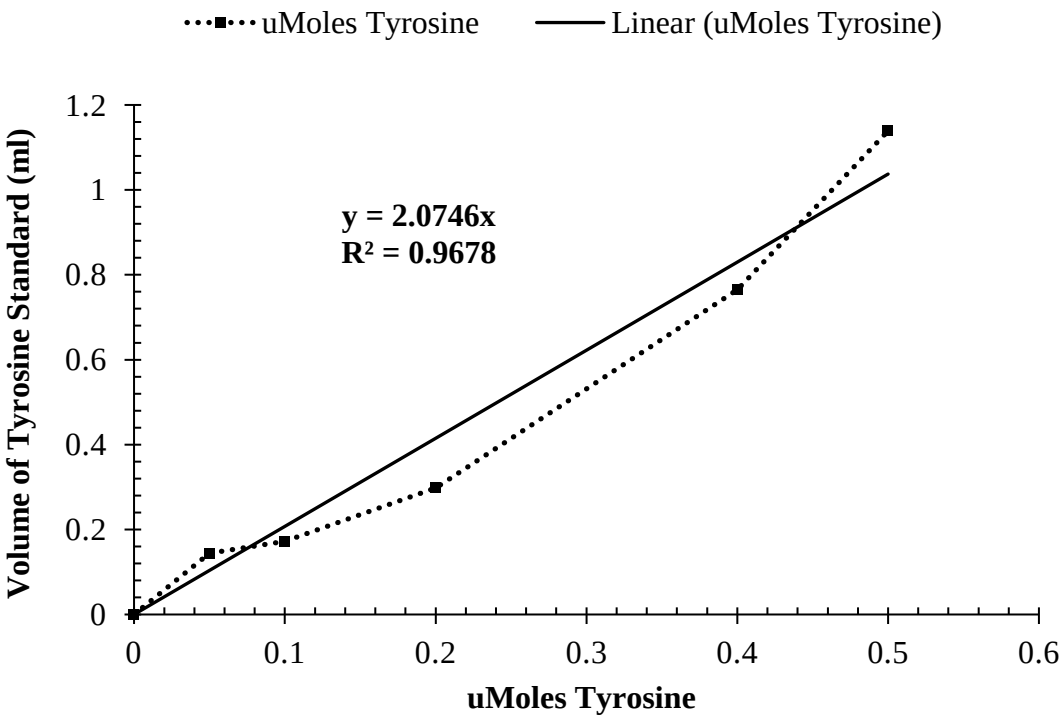

Fig. E.1 Standard curve of L-Tyrosine for caseinolytic activity

Appendix F

Statistical analysis (T-test Tables)

Table F.1 T-Test: Two-Sample Assuming Equal Variances for Moisture content

	Rennet	*Aank*
Mean	49.8	51.07
Variance	0.0001	0.0004
Observations	3	3
Pooled Variance	0.00025	
Hypothesized Mean Difference	0	
df	4	
t Stat	-98.373777	
P(T<=t) one-tail	3.2011E-08	
t Critical one-tail	2.13184679	
P(T<=t) two-tail	6.4023E-08	
t Critical two-tail	2.77644511	

Table F.2 T-Test: Two-Sample Assuming Equal Variances for Fat

	Rennet	*Aank*
Mean	25.2	23.33
Variance	0.1352	1.62
Observations	2	2
Pooled Variance	0.8776	
Hypothesized Mean Difference	0	
df	2	
t Stat	1.99615058	
P(T<=t) one-tail	0.09201413	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.18402827	
t Critical two-tail	4.30265273	

Table F.3 T-Test: Two-Sample Assuming Equal Variances for Protein

	Rennet	*Aank*
Mean	19.99	20.38
Variance	0.0008	0.0242
Observations	2	2
Pooled Variance	0.0125	
Hypothesized Mean Difference	0	
df	2	
t Stat	-3.488266	
P(T<=t) one-tail	0.0366328	
t Critical one-tail	2.9199856	
P(T<=t) two-tail	0.0732655	
t Critical two-tail	4.3026527	

Table F.4 T-Test: Two-Sample Assuming Equal Variances for Ash

	Rennet	*Aank*
Mean	2.225	2.33
Variance	0.00045	0.0008
Observations	2	2
Pooled Variance	0.000625	
Hypothesized Mean Difference	0	
df	2	
t Stat	-44.2	
P(T<=t) one-tail	0.0002557	
t Critical one-tail	2.9199856	
P(T<=t) two-tail	0.0005115	
t Critical two-tail	4.3026527	

Table F.5 T-Test: Two-Sample Assuming Equal Variances for pH

	Rennet	*Aank*
Mean	5.64	5.99
Variance	0.0002	0.0008
Observations	2	2
Pooled Variance	0.0005	
Hypothesized Mean Difference	0	
df	2	
t Stat	29.06888	
P(T<=t) one-tail	0.000591	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.001181	
t Critical two-tail	4.302653	

Table F.6 T-Test: Two-Sample Assuming Equal Variances for Calcium

	Rennet	*Aank*
Mean	652.73	636.9
Variance	0.405	7.9202
Observations	2	2
Pooled Variance	4.1626	
Hypothesized Mean Difference	0	
df	2	
t Stat	7.758872	
P(T<=t) one-tail	0.008104	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.016209	
t Critical two-tail	4.302653	

Table F.7 T-Test: Two-Sample Assuming Equal Variances for Yield

	Rennet	*Aank*
Mean	21.71	22.75
Variance	3.4848	2.4642
Observations	2	2
Pooled Variance	2.9745	
Hypothesized Mean Difference	0	
df	2	
t Stat	-1.762652077	
P(T<=t) one-tail	0.110007618	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.220015236	
t Critical two-tail	4.30265273	

Table F.8 T-Test: Two-Sample Assuming Equal Variances for TPC

	Rennet	*Aank*
Mean	2.398730264	2.455984411
Variance	3.03989E-06	2.3811E-06
Observations	3	3
Pooled Variance	37.3421	
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 F.9 T-Test: Two-Sample Assuming Equal Variances for Yeast and Mold

	Rennet	*Aank*
Mean	1.42749	1.39652
Variance	0.00027	0.00093
Observations	3	3
Pooled Variance	22.3346	
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	