EFFECT OF ACTINIDIN AND CHYMOSIN ON BIOPHYSICOCHEMICAL AND SENSORY CHARACTERISTICS OF CHEESE DURING MATURATION

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Effect of Actinidin and Chymosin on Biophysicochemical and Sensory Characteristics of Cheese during Maturation

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

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Approval Letter

This ***dissertation*** entitled ***Effect of Actinidin and Chymosin on Biophysicochemical and Sensory Characteristics of Cheese during Maturation*** presented by **Bindu Shrestha** has been accepted as the partial fulfilment of the requirement for the **B. Tech. degree in Food Technology**.

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Abstract

Cheese is a biochemically dynamic dairy product that undergoes significant changes during ripening. The primary objective of the present research was to study the effect of actinidin and chymosin on biophysicochemical and sensorial characteristics of cheese during ripening. The optimum temperature and pH of milk in the cheesemaking for maximum milk clotting activity (MCA) were determined by response surface methodology. Two batches of cheeses were prepared using actinidin and chymosin and were ripened at 8-10ºC and relative humidity of 80-85% to study the different ripening characteristics for 60 days with the interval of 15 days.

From RSM analysis the optimal condition for MCA in cheesemaking were 55ºC and pH 6.25 of milk. The chemical composition such as protein, fat, salt and ash increased during the ripening days except that for moisture and pH which showed decreasing trend. Changes in nitrogenous fractions showed that proteolysis increased in both cheeses as ripening proceed with the value significantly ($P<0.05$) higher for actinidin cheese. The free amino acid of actinidin cheese was significantly ($P<0.05$) higher than that of chymosin cheese after 15 days. The free fatty acid of actinidin cheese was significantly ($P<0.05$) lower throughout the ripening period. The total viable count and *Lactobacillus* counts were significantly ($P<0.05$) higher in actinidin cheese. The sensory analysis of both cheeses revealed significant ($P<0.05$) difference in appearance, flavor, odor, aftertaste and overall acceptance after 30 days whereas texture showed significant ($P<0.05$) difference from initial day of ripening. Actinidin cheese developed a bitter flavor and aftertaste after 30 days. Hence, actinidin cheese should be consumed within 30 days.

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List of Abbreviations

Abbreviations	Full form
AC	Affinity chromatography
ADV	Acid degree value
ANOVA	Analysis of Variance
AOAC	Association of official analytical chemist
AP	Aqueous phase
BSA	Bovine Serum Albumin
CA	Catalytic activity
CCRD	Central composite rationalized design
CDFT	Central Department of Food Technology
CE	Crude extract
CFU	Colony forming unit
EAE	Enzyme-assisted extraction
EDTA	Ethylenediaminetetraacetic acid
FAA	Free amino acid
FAO	Food and Agriculture Organization
FDB	Fat on dry basis
FDM	Fat in dry matter

Abbreviations	Full form
FFA	Free fatty acid
GDL	Glucono delta lactone
GFC	Gel filtration chromatography
HPLC	High performance chromatography
IDE	International Dairy Federation
IP	Intermediate phase
LAB	Lactic acid bacteria
MAE	Microwave-assisted extration
MCA	Milk clotting activity
MFFS	Moisture in fat free substances
MWCO	Molecular weight cut-off
NF	Nanofiltration
NSLAB	Non-starter lactic acid bacteria
PA	Protease activity
PAB	Propionic acid bacteria
PCA	Plate count agar
PDA	Potato dextrose agar

Abbreviations	Full form
PGE	Pregastric esterases
PLE	Pressurized liquid extraction
PPM	Parts per million
PTA	Phosphotungstic acid
RCT	Rennet coagulation time
RSM	Response surface methodology
RP	Reverse phase
SE	Solvent extraction
SFE	Supercritical fluid extraction
SNF	Solid not fat
SWE	Subcritical water extraction
TCA	Trichloroacetic acid
TOC	Time of coagulation
TPP	Three phase partitioning
UAE	Ultrasonic-assisted extraction
UF	Ultra filtration
WSN	Water soluble nitrogen

Part I

Introduction

1.1 General introduction

Cheese is a fermented dairy based food product available in a diversity of flavors, texture and forms. It is made in almost every country all over the world and has steadily gained in popularity because of the increased use of cheese as an ingredient in a variety of prepared foods (Ye *et al.*, 2009). It is an excellent source of protein, fat, and minerals (Fuwei *et al.*, 2009). It also contains vitamins such as vitamins A, B_2, B_6 and B_{12}. Cheese is an essential part of a balanced diet in moderation because of its high protein and calcium content (Considine, 2012). It is made by coagulating milk casein with rennet or similar enzymes in the presence of lactic acid, then removing some of the moisture by cutting, cooking, and pressing, then shaping in a mould and ripening at the appropriate temperature and humidity (Walstra *et al.*, 2006). The ruminant stomach, particularly the calf stomach, is the source of rennet. Rennet comprises the major enzyme component chymosin (EC 3.4.23.4), which is responsible for the particular cleavage of the κ-casein Phe_{105}-Met_{106} bond, resulting in the breakdown of casein micelles and milk coagulation (Fox *et al.*, 2017).

Cheese can be consumed fresh, but it is mostly utilized after ripening in brine solution. It is placed in cold storage at temperature of 4-8°C for a duration of 1 to 3 months to develop its flavor. During the ripening process, physical, chemical, biochemical, microbiological, and sensorial changes occur. The principal and most complex biochemical reaction that occurs throughout the ripening process of most cheese varieties is proteolysis (McSweeney, 2004). Proteolysis plays an important part in the development of cheese characteristics. The enzymes involved, the type of cheese, and the environmental conditions during cheese ripening all influence the rate, extent, and pattern of proteolysis and it contributes to cheese ripening through direct flavor formation (Guizani *et al.*, 2002; Topçu and Saldamli, 2006).

However, the need for alternate milk coagulating sources is increasing as cheese production increases and rennet availability decreases (Chazarra *et al.*, 2007). Other factors driving the search for rennet alternatives are rising calf rennet prices, ethical concerns about the production

of such enzyme, religious beliefs, a restriction on genetically engineered foods, and vegetarianism (Roseiro *et al.*, 2003). Milk coagulation can be accomplished by a variety of proteolytic enzymes, including microbial proteases (*Rhizomucor miehei*, *Cryphonectria parasitica)* and plant proteases (Andrén, 2002). Plant proteases have strong catalytic activity and can coagulate milk in a variety of pH and temperature (Mazorra-Manzano *et al.*, 2018). Some plants having milk clotting protease are *Cynara cardunculus* (Gomes *et al.*, 2019), *Ficus carica* (Hachana *et al.*, 2021), *Zingiber officinale (Gagaoua et al.,* 2015*)*, *Actinidia deliciosa* (Mahdian Dehkordi *et al.*, 2022; Nicosia *et al.*, 2022), *Carica papaya* (Maskey and Shrestha, 2020*)*, *Ananas comosus* (Kartawiria *et al.*, 2019), *Calotropis procera* (Silva *et al.*, 2020), *Withania coagulans* (Nazish et al., 2022*).* Plant proteases, rather than animal proteases and genetically modified organisms-derived enzymes, have the benefit of being a rich source of bioactive chemicals, antioxidant capabilities, and binding qualities (Park *et al.*, 2014; Gupta *et al.*, 2015).

Kiwifruit *(Actinidia deliciosa)* contains milk clotting enzyme, actinidin (EC3.4.22.14). Actinidin is a cysteine protease which has a molecular mass of around 24 kDa, and it consist of 220 amino acid residues (Carne and Moore, 1978). It has a promising milk clotting activity to proteolytic activity ratio that is just 30% lower than rennet (Grozdanovic *et al.*, 2013). The pH and temperature profile for optimal activity is compatible with cheesemaking conditions, and β-casein is the initial target substrate during casein hydrolysis, followed by κ-casein at Arg_{97}-His_{98} and Lys_{111}-Lys_{112} (Lo Piero *et al.*, 2011). Actinidin may hydrolyze casein fractions in the presence of up to 5% cream fat (Puglisi *et al.*, 2012). Furthermore, the texture of curd produced with crude kiwifruit extract is equivalent to that produced with chymosin (Mazorra-Manzano *et al.*, 2013).

Three-phase partitioning (TPP) is rapidly developing as a revolutionary bio-separation approach that is being quickly developed for the separation and purification of target macromolecules, principally enzymes, from various biological resources (Ramos and Malcata, 2017). TPP has been used for a variety of purposes, including enhancing the catalytic activity of enzymes and stabilizing their structure (Chew *et al.*, 2019). In addition to increased concentration, TPP purification has been shown to be equivalent to chromatographic approaches

(Saxena *et al.*, 2007). Some plant proteases that are separated by TPP are Ficin from *Ficus carica* latex (Gagaoua *et al.*, 2014), Zingibain from *Zingiber officinale* Roscoe rhizomes (Gagaoua *et al.*, 2015), Papain from dried papaya leaves (Chaiwut *et al.*, 2010), Actinidin from kiwifruit (Maskey and Karki, 2023), Bromelain from pineapple crown (Gul *et al.*, 2022).

1.2 Statement of the problem

The global expansion in cheese production, along with a decreasing availability of natural animal rennet, is driving up demand for alternative milk-coagulating sources (Ben Amira *et al.*, 2017)**.** The rising cost of calf rennet, as well as ethical issues about the production of such enzymes for general cheesemaking, have led to systematic investigation on the possibility and suitability of their substitution by other enzymes of plant origin. The expensive cost of rennet, religious concerns, diet, or a restriction on recombinant calf rennet, interest is being drawn to the use of microbial coagulants and plant-derived coagulants (Nawaz *et al.*, 2011)**.**

Actinidin, a kiwifruit protease enzyme exhibited promising characteristics as a milk-clotting agent in cheese technology (Katsaros *et al.*, 2010; Puglisi *et al.*, 2014; Bin *et al.*, 2017). The use of actinidin as a coagulant enzyme in cheesemaking has several advantages, including a high milk clotting activity/protease activity ratio and the ability of specific hydrolyzing (Serra *et al.*, 2020). Actinidin also showed comparable relative activity to chymosin (Alirezaei *et al.*, 2011)**.** Kiwifruit protease, however, is underutilized in Nepal due to expensive chromatographic techniques.

When a potential rennet substitute is studied, it is particularly important to evaluate adequately the degradation pattern of the casein because of the effects on chemical composition, texture, consistency, flavor and yield of the final cheese (Fox, 1989). However, limited literature is currently available about the influence of purified kiwifruit protease on proteolysis pattern, lipolysis, chemical composition and ripening of cheese despite the fact actinidin was reported to give similar enzymatic activity as chymosin. Hence, this study is carried out to determine the influence of purified kiwifruit protease on biophysicochemical and sensory properties of cheese during ripening.

1.3 Objectives

The objective of research can be divided into two parts:

1.3.1 General objective

The general objective of this study was to determine the effect of actinidin and chymosin on biophysicochemical and sensory characteristics of cheese during maturation.

1.3.2 Specific objectives

The specific objectives of this study were as follows:

1. To extract and purify kiwifruit protease using TPP method.
2. To optimize the temperature and pH of milk for actinidin by response surface methodology for cheesemaking.
3. To prepare cheeses by using actinidin and chymosin in optimized condition.
4. To analyze the physicochemical properties of prepared cheeses such as moisture, pH, protein, fat, salt, ash and yield during ripening.
5. To examine the proteolysis of prepared cheeses during ageing.
6. To analyze the total free amino acid of prepared cheeses throughout ripening.
7. To determine the lipolysis of prepared cheeses during maturation.
8. To study the microbiological properties such as total viable count, yeast and mold, *Lactobacillus* and *Streptococcus* counts of prepared cheeses throughout ripening.
9. To evaluate the sensory quality of prepared cheeses during ageing.

1.4 Significance of the work

Cheese is a staple in many cultures' diets, valued for its sensory features, bioactive components, and nutritional benefits (Mazorra-Manzano *et al.*, 2018). Because of its high specificity for cleaving the caseinomacropeptide, which causes casein micelle destabilization and thus stimulates milk coagulation, chymosin has been used as the major enzyme component of calf rennet in cheese manufacturing (Jacob *et al.*, 2011). Plant proteases, on the other hand, have become an appealing alternative as a novel milk clotting agent in cheese manufacturing due to

ethical, cultural, and religious views. Also, the use of plant protease in cheese production will help enhance acceptance among vegetarians and other religious groups (Rajagopalan *et al.*, 2013).

As a result, the search for novel plant-based milk coagulating enzymes to make them industrially useable continues, in order to meet the rising demand for diverse and excellent cheese production on a global scale. The present study utilizes a plant-based substitute to chymosin. Further, this study employed the three-phase partitioning (TPP) method for purification of kiwifruit protease, which could be a key strategy for its application in a wide spectrum for the concentration, and purification of enzymes. The TPP purified kiwifruit protease could be more economical and accessible source of enzyme for cheese manufacturing, assisting to decrease the cost of cheese production and assuring easy purchasing access.

Ripening is, relatively, an expensive process for the cheese industry and reducing maturation time without destroying the quality of the ripened cheese has economic and technologic benefits. During cheese ripening, microbiological and biochemical changes occur that result in the development of flavor and texture characteristics of the variety (McSweeney, 2004). Thus, this study helps to know the physiochemical, microbiological and sensory properties of the prepared cheeses during ripening

1.5 Limitations of the work

- Texture profile analysis (TPA) of cheese was not conducted.
- Peptide analysis of cheese was not performed.

Part II

Literature review

2.1 Overview of cheese

Cheese is a general term for the group of fermented dairy products produced all over the world in a wide variety of flavors, textures, and shapes (Singh *et al.*, 2003). It is a concentrated protein gel that comprises fat and moisture. Its manufacture generally involves gelation of cheese milk, dehydration of the gel to form a curd and treatment of the curd (cheddaring, texturization, salting, moulding and pressing) (Johnson and Law, 2010). An important part of the cheesemaking process is the conversion of a milk (liquid) into a solid material (the curd), which contains the casein and fat of the milk, but it usually removes most of the water, the whey proteins and part of the lactose content (Law and Tamime, 2010).

Cheese that ranges in texture from semi-solid to stone hard and in flavor from exceedingly mild to very sharp, has been made over the years by manufacturing and curing. Because of this, cheeses' nutrients, flavors, textures, and cooking qualities vary greatly, allowing them to satisfy a variety of sensory and nutritional (Khanal *et al*., 2019). Cheese output in the world is ~19 × 10^6 tonnes per year (35% total milk production) and has expanded at a 4% annual rate over the last 30 years. Within the dairy industry, cheese is the most rapidly growing product. The rising intake of cheese can be attributed to a variety of factors, including a positive dietary image, ease and flexibility of use, and a wide range of flavors and textures (Fox and McSweeney, 2017).

2.2 Classification of cheese

Because of the diversity in cheeses, classification is challenging, but the most widely accepted approach is one based on moisture content, with further subdivision based on milk type and the function of microorganisms in cheese ripening. The cheese's softness or hardness is therefore directly related to its moisture level, with greater moisture cheeses being softer than low moisture cheeses (Beresford *et al.*, 2001).

McSweeney *et al.* (2004) stated that there is no authoritative list of cheese varieties. According to Fox (1993), there are more than 2000 different cheese varieties that exist in the

world. Walter and Hargrove (1972) described about 400 variants, while Burkhalter (1981) categorized 510 varieties. According to Walter and Hargrove (1972), there are probably only about 18 distinct types of natural cheeses, none of which are made in the same way, i.e., they differ in terms of setting the milk, cutting the coagulum, stirring, heating, draining, pressing, and salting of the curds, or ripening of the cheese. There are three types of cheese classification scheme (McSweeney *et al.*, 2004).

- Texture
- Coagulation method
- Ripening indices

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

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

Classification of cheese on the basis of texture as given by Codex Alimentarious, FAO/WHO, has been shown in the Table 2.1.

Table 2.1 Classification of cheese according to Codex Alimentarious

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

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

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

The classification of cheese on the basis of composition, firmness and maturing agents have been shown in the Table 2.2.

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

Types of cheese	Examples
1. Soft cheese (50-80% moisture)	
a. Unripened low fat	Cottage, Quark, Baker
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 external 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 ripened)	Limburger, Brick, Trappist, Port, du salut, St paulin Oka
c) Ripened primarily by internal bacterial fermentation but may also have some surface growth	Miinster, Bel Paese, Tilsiter
d) Ripened internally by bacterial fermentation	Pasta filata provolone, low moisture Mozzarella
3. Hard cheese (39% moisture maximum)	
a) Internally ripened by bacterial fermentation	Cheddar, Colby, Caciocavallo
b) Internally ripened by bactecterial fermentation (CO_2 production resulting in holes or eyes)	Swiss (Emmental), Gruyere, Gouda, Edam, Samsoe
c) Internally ripened by mold growth	Stilton
4. Very Hard Cheese (34% moisture maximum)	Asiago Old, Parmesan, Parmigiano Grana, Romano, Sardo
5. Whey Cheese	
a) Heat and acid denaturation of whey protein	Ricotta (60 % moisture)
b) Condensing of whey by heat and water evaporation	Gjetost (goat milk whey, 13% moisture), Myost, Primost (13-18 % moisture)

6. Spiced Cheese	Noekkelost - cumin, cloves

Source: Bamforth and Ward (2014)

The classification of cheese on the basis of mode of coagulation has been shown in the Table 2.3.

Table 2.3 Classification of cheese groups on the basis of mode of coagulation

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

Source: Lawrence *et al.*, (2004)

2.3 Pretreatments of milk for cheesemaking

Pretreatments have profound impact on production schedule of cheese, the effectiveness of cheese production, physicochemical, microbiological and organoleptic characteristics of cheese and shelf life. The various treatments employed (Walstra *et al.* 2006) are:

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

2.3.1 Chilling and cold storage

In advanced milk countries, most raw milk is cooled to 4°C and stored on the farm in refrigeration bulk tanks and in insulated or refrigerated storage tanks in cheese plants prior to its conversion into cheese. Raw milk tends to be colder for a much longer period of time than when milk is handled in cans. Such a long-term holding practice of cooled raw and/or processed milk not only increases the likelihood of psychrotrophic development, but also changes the physicochemical status of milk components, particularly casein and minerals. In fact, this has been shown to change milk behaviour during subsequent cheesemaking (Upadhyay, 2003).

2.3.2 Lactoperoxidase treatment

Lactoperoxidase is an enzyme that occurs in raw milk, colostrum, saliva and other biological secretion. The enzyme reacts with thiocyanate in the presence of hydrogen peroxide and forms antimicrobial compounds. This is termed as the lactoperoxidase system (LPS). Fresh milk contains 1 to 10 mg of thiocyanate per liter, which is not sufficient to activate the LPS. Hydrogen peroxide, the third component of the LPS, is not present in fresh milk due to the action of natural catalase, peroxidase or superoxide dismutase. Approximately, 8 to 10 mg hydrogen peroxide per liter is required for LPS. In the LPS reaction, thiocyanate is oxidized to the antimicrobial hypothiocyanite which exist in equilibrium with hypothiocyanous acid (Davidson and Zivanovic, 2003). The lactoperoxidase method can be used for the preservation of raw milk as an alternative to chilling and to solve the problem posed by psychrotrophs in stored milk. Unless lactoperoxidase is inactivated by appropriate heat treatment (e.g.,78°C/15 s), the ability of starter organisms to produce H_2O_2 results in the reactivation of the lactoperoxidase system. There is, therefore, a potential for the reactivation of the LPS, leading to problems in the production of cheese due to the suppression of starter activity (FAO, 1999).

2.3.3 Thermization

The thermization process is a sub pasteurization heat treatment of milk at 62-65°C for 10-20 s, followed by refrigeration. It is used as a pre pasteurization treatment of raw milk to safeguard milk quality during prolonged storage in insulated silos. Research has shown that this process effectively reduces both total and psychrotrophic bacterial counts, enabling thermized milk to

be stored for up to 3 days longer at 8°C. In this way, detectable sensory changes and the consequent decrease on shelf-life are limited (Swart *et al.*, 2003).

2.3.4 Bactofugation

Bactofugation is a process which is usually applied to remove spores from products that are low pasteurized. This may involve removal of spores of *Bacillus* spp. or *Clostridium* and related species from cheese milk. Spores are quite small, but the density difference with plasma is larger than that of bacteria, and at separation temperatures of 60 to 65°C, a substantial proportion can be removed, generally 90 to 95%. By using two bactofuges in series, a reduction by over 99% can generally be attained. It should be noted that bactofugation is by no means equivalent to sterilization: the product still contains heat-resistance bacteria and a small number of spores. The process may serve to give pasteurized beverage milk a longer shelf life or to obtain cheese that is not subject to the defect of late blowing without inducing unfavorable change in the milk (Walstra *et al.*, 2006).

2.3.5 Microfiltration

Without the application of heat, microfiltration effectively removes the indigenous microorganisms from milk. Microfiltration seems to offer the ideal approach for evaluating the contribution of non-starter lactic acid bacteria (NSLAB) to cheese maturation without complicating changes in milk protein or enzymes induced by heat, except those on the membrane of fat globules. Microfiltration was introduced some years ago in the so called 'Bactocatch' process as an alternative to bactofugation (Upadhyay, 2003).

2.3.6 Standardization

Standardization of cheese milk is necessary due to variation in the composition of milk and to ensure that the final product meets the legal requirements. Fat and protein are the two components that constitute the main body of cheese. Therefore, it is reasonable to standardize the milk at the ratio of fat/protein, which gives good quality of cheese and even quality of production during all seasons. The adjustment of the casein/fat ratio (C/F) in cheese milk in the 0.69 to 0.70 range produces a cheese with better body-texture characteristic (Scott *et al.*, 1998).

2.3.7 Pasteurization

Pasteurization in cheese milk serves to kill pathogenic and harmful organisms. A more intense pasteurization causes part of the serum proteins to become insoluble, leading to an increase in cheese yield, it decreases the renetability and the syneresis, it inactivates xanthine oxidase, thereby increasing the risk of bacterial spoilage (Walstra *et al.*, 2006). Pasteurization temperature-time combination is dependent on the cheese variety and mode of pasteurization employed. According to Scott (1986), three systems of pasteurization practiced in most countries are:

1. Flash heating (no holding) to temperature of 75-95°C
2. HTST: 71-75°C /14-40 s
3. LTLT: 61-65°C / 20-40 min.

2.3.8 Homogenization

In manufacturing, most of the cheese verities such as cream, blue and soft varieties, homogenization is not generally practiced. Homogenization of milk is done for:

- Reducing the loss of fat in whey (incorporation of small fat globules in the curd) and thus increasing the yield of cheese.
- Improving the texture of cheeses by making the curd smoother and finer, ensuring greater retention of moisture.
- Producing 'white cheese' from cows' milk similar to that of goat milk cheese (Banks *et al.*, 1992).

2.3.9 Lactose hydrolysis

It has been reported that the use of lactose hydrolyzed milk for cheesemaking has the following effects on production:

- Faster production of acid as growth of starter is stimulated.
- Before renneting and cheddaring, milk ripening time could be reduced by 25-30%.
- The moisture content of cheese could be increased slightly.
- Whey obtained in the process of cheesemaking could be used for syrup production as it contains increased glucose and galactose level (Upadhyay, 2003).

2.3.10 Concentration

The production of cheese is essentially a controlled dewatering process in which the milk fat and casein are concentrated around 6-12 times, depending on the variety of cheese produced. The concentration of milk for cheesemaking can be achieved (Bylund, 1995) by:

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

2.4 Additives in cheese milk

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

2.4.1 Calcium salt

Calcium plays an important role in the secondary phase of rennet action. As a result, the calcium balance between soluble, colloidal, and complex is very important for effective coagulation (Lucey and Fox, 1993). Calcium can be added to milk in several forms, such as calcium chloride ($CaCl_2$), up to 0.02 percent, dibasic calcium phosphate with pepsin (0.01 percent), lime water, and calcium lactate (Upadhyay, 2003). The beneficial effects of $CaCl_2$ addition on rennet coagulation time (RCT) and gel strength are thought to be the result of an increase in Ca^{++}, an increase in colloidal calcium phosphate (CCP), and a drop in pH (De, 1980). Micellar calcium plays an important role in increasing the melt and other associated functional qualities of Mozzarella cheese (Joshi *et al.*, 2004).

2.4.2 Cheese color

It is usual practice to add additional color to pale colored milk to give cheese an attractive and appetizing appearance. Riboflavin and carotenoids are two important colors found in milk but lost in whey. The color of annatto cheese of vegetable origin is widely used at the rate of 88 g/1000 kg (Kosikowski and foods, 1982).

2.4.3 Inhibitory salts

To avoid the development of gas producing species such as coliform/aerogenes classes of bacteria responsible for early blowing defect in cheese and butyric acid bacteria responsible for late blowing defect in cheese, Swiss inhibitory salts (saltpeter) are applied to milk in the manufacture of less acidic cheese such as Edam, Gouda. A quantity of 10 to 100 ppm nitrite or 2 to 5 ppm nitrate is adequate to suppress spore development. The main disadvantage of using nitrate in cheesemaking is color defects and potentially carcinogenic effect. Lysozyme has been used as an alternative for saltpeter to suppress clostridia species (Farkye and Fox, 2004).

2.4.4 Starters

The starter culture is a very important factor in cheesemaking. According to McSweeney (2004), two principal types of culture are used in cheesemaking:

- Mesophilic cultures with a temperature optimum between 25°C and 40°C such as *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris,* and *Leuconostoc* spp.

- Thermophilic cultures, which develop at up to 50°C such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

The acidification of milk is the most important step in the cheesemaking process. Acidification is necessary for flavor and texture development; it promotes coagulation; and lowering the pH limits the growth of pathogens and spoilage organisms. It is normally obtained by fermenting lactose with starter bacterial cultures to produce lactic acid, while some fresh cheeses, such as cottage cheese, can be acidified by adding acid directly and do not require starter. Most cheeses are now manufactured using selected starters, yielding predictable and desirable results. The principal microorganisms used in cheese production are *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus,* and *Lactobacillus delbrueckii subsp. bulgaricus* (Fernandes, 2009).

According to Walstra *et.al* (2006), three characteristic abilities of starter cultures are of primary importance in cheesemaking:

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

Direct acidification with acid (usually lactic acid) or glucono delta lactone (GDL) is an alternative to biological acidification and is widely used commercially in the production of Cottage, Quark, Feta-type cheese, and Mozzarella from ultrafiltration (UF) concentrated milk. Direct acidification is more controllable than biological acidification and is not subjected to bacteriophage infection, unlike starters. However, enzymes from starter microorganisms are required for cheese ripening, therefore artificial acidification is used mainly for cheese varieties where texture and functionality are more important than flavor (Park and Haenlein, 2013).

2.4.5 Salt

Salt in cheese serves two important functions: preservation and direct contribution to flavor and quality. The depressing effect of sodium chloride (NaCl) on the water activity of the cheese and the increase in osmotic pressure of the aqueous phase cause dehydration of bacterial cells, killing them or, at least, preventing their growth. NaCl adds directly to the saltiness of cheese, a flavor that is generally highly appreciated. The flavor of salt-free cheese is watery and insipid. NaCl indirectly contributes to cheese flavor by influencing microbial and enzymatic activity, which in turn impact lactose metabolism, cheese pH, fat and casein degradation, and the formation of flavor compounds such as peptides, free amino acids, and free fatty acids (Lawrence *et al.*, 2004).

2.4.6 Rennet

Rennet is a general term for proteinase preparations used to coagulate milk. Most proteinase will coagulate milk under suitable conditions of pH and temperature (McSweeney 2007)**.** Rennet is extracted from the stomach of young calves and marketed in the form of solution with a strength of 1:10000 to 1:15000, which means that one part of rennet can coagulate 10,000-15,000 parts of milk in 40 min at 30ºC. Rennet in powder form is normally 10 times as strong as liquid rennet (Bylund, 1995). Rennet consists of mainly rennin and pepsin, principally the former being responsible for milk clotting and the latter for proteolysis (although both possess

the two characteristics). The prime enzyme used in cheesemaking is chymosin (rennin) (EC 3.4.23.4, isoelectric pH ≈ 4.65). It is an aspartate proteinase, hence an endopeptidase, which means that it can split proteins into relatively large fragments (Walstra *et al.*, 2006).

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

- **Calf rennet**

Rennet has been manufactured from calf stomach (abomasums, the fourth stomach, or the veal) for ages. The stomach is cut into strips which are extracted in slightly acidic salt solution. Calf rennet is estimated to be involved in the production of up to 80% of cheese produced worldwide (Li and Zhao, 2019). Because heating to 55-60°C destroys rennet enzymes, heating cannot be used to preserve rennet extracts. To achieve the greatest possible preservation, the extract's pH was adjusted to around 5.5, and the salt solution to 15-20%. Rennet must be stored cool and kept away from light. The enzymes are destroyed by alkali and strong acid. The enzyme chymosin (rennin) is responsible for the majority of calf rennet's coagulation activity. However, another enzyme, bovine pepsin, is responsible for some of the coagulation activity. Pepsin levels increase whereas chymosin (rennin) levels decrease as the calf matures (Ling, 1946).

- **Microbial rennet**

The rapid growth in cheese industry, as well as the shortage of calf rennet, has stimulated the research for milk clotting enzyme from alternate sources (Fox and McSweeney, 2017). Chymosin produced using recombinant DNA technology was first introduced to cheesemakers for testing in 1988 (Mohanty *et al.*, 1999). Many microbial enzymes, such as proteinases, are known as producer of rennet that can replace calf rennet. Microbial rennet accounts for 30% of total cheese produced (Abada, 2019). However, the microbial enzymes had two key drawbacks: (i) the presence of high levels of nonspecific and heat-stable proteases, which led to the development of bitterness in cheese after storage; and (ii) a poor yield. Microorganisms such as *Rhizomucor pusillus* and *Cryphonectria parasitica* (Egito *et al.*, 2007), *Thermomucor indicae-*

seudaticae N31 (Merheb-Dini *et al.*, 2010), *Nocardiopsis* sp. (Cavalcanti *et al.*, 2004) and *Aspergillus oryzae* (Hashem, 2000) are extensively used for rennet production in cheese manufacture.

- **Plant rennet**

Plant proteases hydrolyze protein molecules into peptides and amino acids. These constitute a diverse and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature, etc. The specificity of proteolytic enzymes is determined by the nature of the amino acid and other functional groups close to the bond being hydrolyzed (Sumantha *et al.*, 2006). Proteases are divided into groups on the basis of catalytic mechanism used during the hydrolytic process. The main catalytic types are aspartate, serine, cysteine, and metalloproteases (Shah *et al.*, 2014). The type and sources of milk clotting plant proteases are shown in the Table 2.4.

Table 2.4 Types and sources of milk clotting proteases

Types of proteases	Protease name	Source	Reference
Aspartic	Cardons	*Cynara cardunculus*	Silva and Malcata (2005)
	Cynarase	*Cynara scolymus*	Chazarra *et al.* (2007)
	Oryzasin	*Oryza sativa*	Asakura *et al.* (1997)
	Onopordosin	*Onopordum acanthium*	Brutti *et al.* (2012)
	Procirsin	*Cirsium vulgare*	Lufrano *et al.* (2012)
Cysteine	Actinidin	*Actinidia deliciosa*	Maskey and Karki (2023)
	Ficin	*Ficus racemose*	Devaraj *et al.* (2008)
	Actinidin	*Actinidin chinesis*	Katsaros *et al.* (2010)
	Protein extract	*Helianthus annuus*	Egito *et al.* (2007)
Serine	Cucumisin	*Cucumis melo*	Uchikoba and Kaneda (1996)
	Lettucine	*Lactuca sativa*	Lo piero *et al.* (2002)
	Neriifolin	*Euphorbia neriifolia*	Yadav *et al.* (2011)
	Streblin	*Streblus asper*	Tripathi *et al.* (2011)
	Religiosin	*Ficus religiosa*	Kumari *et al.* (2010)

2.5 Basic principles of cheese making

The general steps involved in cheese making is shown in Figure 2.1.

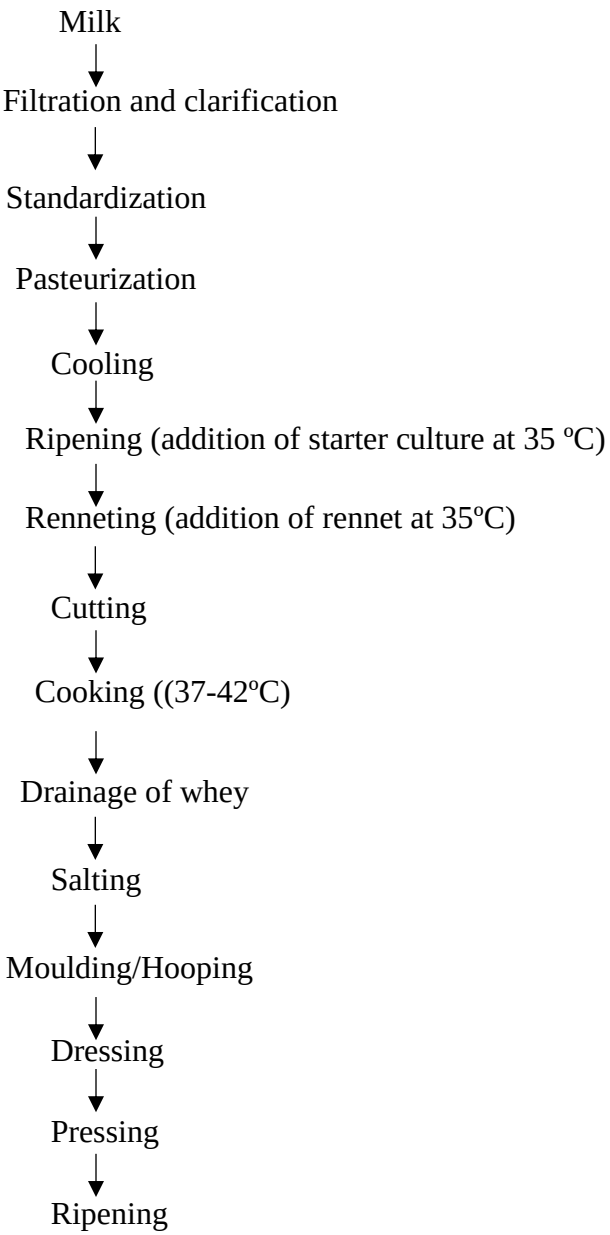

Fig. 2.1 Flowchart for cheese production

Source: Chandan and Kapoor (2011)

2.5.1 Inoculation

The starter culture is added at a rate ranging from 0.05 to 4%. The milk is left for 45 min to 1.5 h for ripening. Temperature, cheese type, and quantity of starter used are all factors that influence ripening time (Fuquay *et al.*, 2011).

2.5.2 Coagulation

The pH regulates the nature and speed of coagulum formation. Thus, rennet must so be added at the optimal pH (Ong *et al.*, 2017). The pH range used for renneting most cheeses is 6.5 to 6.35. Curd firmness is affected by the fat-to-protein ratio and the amount of whey proteins in the curd. A short, cost-effective, and well-regulated coagulation process is preferred, and the coagulation should not result in excessive curd and fat loss in whey (Scott *et al.*, 1998) . In general, one part of rennet can clot 10,000 parts (0.01% v/v) of milk (Legg *et al.*, 2017).

2.5.3 Cutting

Cutting time is very important during cheesemaking since it directly effects cheese yield and composition (Lawrence *et al.*, 2004). Temperature, curd size, agitation speed, and pH all impact cheese quality, which varies according to cheese type. Coagulum cutting time varies between 25 min and 2 h depending on the process and recipe (Scott *et al.*, 1998).

2.5.4 Cooking and whey drainage

During the manufacture of cheese, cutting is followed by cooking. To prevent excessive crushing and fat loss, the curd is gently stirred throughout cooking. When the curd's outer layer resembles a membrane, the stirring rate is increased. Cooking causes more whey to be released from the curd due to contraction and pressure exerted on the curd grains (Fagan *et al.*, 2017). Increased cooking temperature also stimulates the metabolism of the starter bacteria (present in curd), increasing the production of lactic acid and drops pH (Ong *et al.*, 2017). The reduction in lactose levels during cheesemaking has a significant impact on the growth of LAB. As a result, several techniques are imposed to adjust the lactose level in the curd (Gripon and Fox, 1993). The first approach includes production of lactic acid to decrease the pH and shrink the curd by

heat, the second way involves adding water to the whey such that an increase in osmotic pressure across the curd membrane removes lactose from the curd to the whey (Scott *et al.*, 1998). When the pH reaches 6.1-6.5, depending on the cheese variety, the whey is drained (Tunick, 2014).

2.5.5 Salting

The method of application and the type of cheese cause variations in salt content in cheese. Immersion in brine solution and dry salting are common practices. Salting regulates cheese ripening or ageing as well as proteolysis (Ong *et al.*, 2017). The procedure of salting varies according to the type of cheese. Blue veined cheese is salted on the surface of the moulded curd, Edam is brine salted, Gouda cheese is directly immersed in brine, while Cheddar and cottage cheeses are dry salted. Cheese salted with brine is held to form the curd into a compact mass of appropriate size for easy handling (Guinee and Fox, 2017).

2.5.6 Ripening

Cheese ripening is an outcome of several microbiological, biochemical, and metabolic processes usually referred to as glycolysis, lipolysis, and proteolysis (Singh *et al.*, 2003; Farkye and Fox, 2004). These are responsible for the basic flavor and textural changes (Lucey and Fox, 1993; Collins *et al.*, 2003; Smit *et al.*, 2005). The relative importance of each of the processes varies depending on the kind of cheese (Farkye and Fox, 2004).

The characteristic flavor, aroma, texture, and appearance of individual cheese varieties develop during ripening. These changes are influenced by the composition, namely moisture, pH, and salt, as well as microflora, starter, and, in particular, nonstarter microflora and adjunct starter (Singh *et al.*, 2003). The primary changes (proteolysis, lipolysis, and glycolysis) are followed and overlapped by a host of secondary catabolic change, such as deamination, decarboxylation, and desulfurylation of amino acids, oxidation of fatty acids, and some synthetic changes such as esterification (McSweeney *et al.*, 2017). The primary reactions are mainly responsible for the basic textural changes and flavor production of cheese whereas the secondary transformations are mainly responsible for the finer aspects of cheese flavor and cheese texture

modification (Singh *et al.*, 2003). The general overview of the biochemical pathways which occur during cheese ripening is shown in Fig. 2.2.

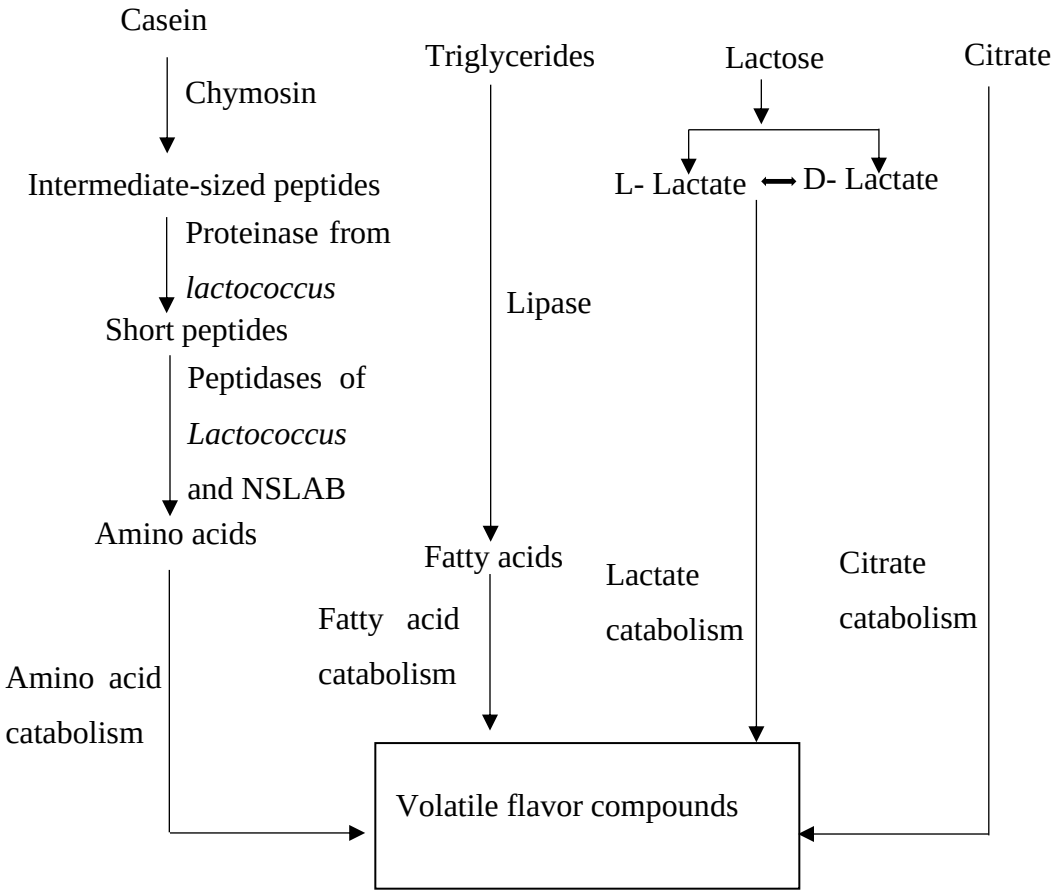

Fig. 2.2 General overview of the biochemical pathways which operate in cheese during ripening.

2.5.6.1 Catabolism of lactate and citrate

The primary glycolytic event, i.e., conversion of lactose to lactate, is normally mediated by the starter culture in the early stages of ripening. If the starter has not finished glycolysis, NSLAB may contribute. Approximately ~96 % of the lactose in milk is eliminated in the whey as lactose or, after fermentation, as lactate. However, fresh curd contains a considerable amount of lactose and the fermentation of lactose has a significant effect on the quality of cheese. Cheddar curd that has been extensively drained and milled to a pH of 5.4 or less contains 0.8 to 1.0% lactose.

As a result of less syneresis and a high pH of 6.2–6.3 at the time of molding, Gouda cheese curd has 3.0% lactose (McSweeney, 2004).

Oxidation of lactate can also occur in cheese. Lactate is converted to acetate and CO_2 during oxidation process. The size of the blocks and the oxygen permeability of the packing material both affect the availability of O_2 and the NSLAB population, which in turn affects the oxidative activity (Thomas, 1987). Acetate is present in cheese at fairly high concentration and is considered to contribute flavor to the cheese, while a high concentration may causes an off-flavor (Aston and Dulley, 1982).

Citrate is found in milk in relatively small amounts (8 mM), which masks the significance of its metabolism in some cheeses produced by mesophilic cultures. The majority of the starters used in the production of cheese, including *Lactococcus. lactis* subsp. *lactis, Lactococcus. lactis* ssp. *cremoris, Lactobacillus* spp., and *Streptococcus. thermophilus,* do not metabolize citrate. However, a minor portion of mixed-strain mesophilic starters, such as those used for Dutch-type cheeses, contain strains of *L. lactis* ssp. *lacti*s and *Leuconostoc* spp which metabolize citrate to diacetyl in the presence of a fermentable sugar during manufacture and early ripening (Fryer *et al.*, 1970).

90% of the citrate in milk is soluble and is lost in the whey, while the concentration of citrate in cheese's aqueous phase is around three times that of whey, reflecting the amount of colloidal citrate present there. Citrate is present in cheese in amounts ranging from 0.2 to 0.5% (w/w), but this amount decreases to 0.1% after six months due to the metabolism of some mesophilic lactobacilli, many of which catabolize citrate to ethanol, acetate and formate late in the ripening when the number of NSLAB has sufficiently increased (Fox *et al.*, 2017).

2.5.6.2 Lipolysis and metabolism of fatty acids

Lipids have a major effect on the flavor and texture of cheese. Lipids contribute to cheese flavor in three ways (Forss, 1969).

- As an excellent source of fatty acids, particularly short-chain fatty acids, which have distinct and potent flavors. Fatty acids are produced through the action of lipase in the

lipolysis process. Fatty acids, particularly methyl ketones and lactones, may be transformed into various sapid and aromatic compounds in some types (McSweeney, 2004).

- Fatty acids, especially polyunsaturated fatty acids, undergo oxidation with the formation of various unsaturated aldehydes which are strongly flavored, causing flavor defects referred to as oxidative rancidity. Due to the low redox potential (−250 mV) of the cheese and relatively low levels of polyunsaturated fatty acids in milkfat, lipid oxidation is very limited (Shahidi and Oh, 2020).

- Lipids serve as solvents for the production of sapid and aromatic chemicals, which can also be derived from proteins and lactose in addition to lipids. Lipids may also absorb compounds from the environment, which may cause off-flavor (Fox, 2002).

Of the various possible contributions of lipids to cheese flavor, lipolysis and modification of the resultant fatty acids are the most significant. Depending upon the types of cheese, the degree of lipolysis in cheese varies widely between varieties, from ~6 meq/100 g free fatty acids in Gouda to 45 meq/100 g fat in Danish Blue. However, extensive lipolysis is essential and desirable as part of overall flavor development in certain cheeses, such as hard Italian cheeses, Blue and Feta cheese (Forde and Fitzgerald, 2000; Alewijn *et al.*, 2005; Perotti *et al.*, 2005).

Cheese contains lipases that come from starter, adjunct starter, non-starter bacteria, milk lipase, chymosin paste, and exogenous lipase. Triglycerides, diglycerides, monoglycerides, fatty acids, and glycerol are hydrolyzed by lipases. The principal biochemical transformation of fat during ripening is the hydrolysis of triglycerides, which account for more than 98% of cheese fat and produce free fatty acid (FFA), diglycerides, monoglycerides, and possibly glycerol. It has a significant role in the flavor and aroma development in cheese during ripening (Adda *et al.*, 1982). Low quantities of FFA are produced by the lipolytic activity of lactic acid bacteria, which can contribute to the background flavor of cheese (Olson, 1990). The pathways for the production of flavor compounds from fatty acids during cheese ripening is shown in Fig.2.3.

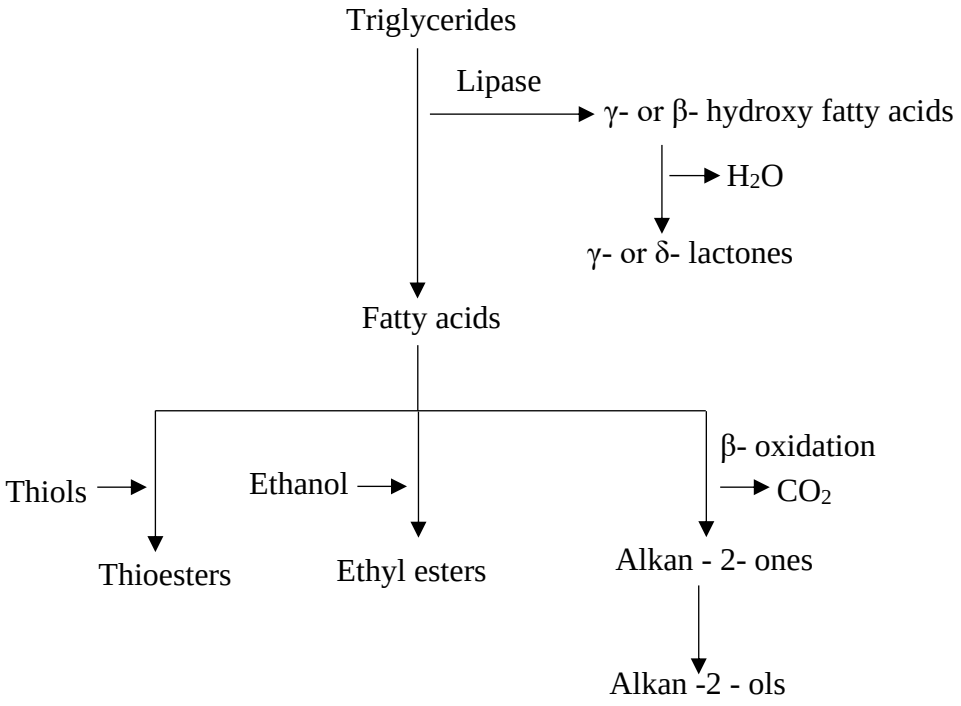

Fig. 2.3 Pathways for the production of flavor compounds from fatty acids during cheese ripening

2.5.6.3 Catabolism of amino acids

The flavoring molecules amines, aldehydes, alcohols, and ammonia are formed when amino acids are degraded into volatile aroma compounds. The compounds produced by the catabolism of free amino acids directly contribute to the taste and aroma of cheese. The total amount and composition of the amino acid mixture in cheese has long been used as an index of cheese ripening (Wallace and Fox, 1997). The primary pathway for degradation results in a transamination process that leads to the formation of keto acids, which are then broken down into other aroma compounds. LAB and NSLAB cooperate in aroma formation in cheese. Lactobacilli initiate the conversion of amino acids into keto- and hydroxyl acids, while *Lactococcus* strains further convert these products into carboxylic acids. This cooperation between LAB and NSLAB enhanced the flavor of the cheese.

The first step in the degradation of amino acid is transamination in case of lactococci, leading to the formation of α-keto acids (Gao *et al.*, 1997). Aromatic aminotransferase enzymes from *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. lactis initiate the degradation of Valine (Val), Leucine (Leu), Phenylalanine (Phe), Tyrosine (Tyr), Tryptophan (Trp), and Methionine (Met), all of which are known precursors of cheese flavor compounds. It has been demonstrated the aroma formation during cheese ripening is decreased by inactivation of aminotransferase enzymes involved in the breakdown of amino acids (Rijnen *et al.*, 1999). Phenyl pyruvic acid formed from Phe by transamination is further degraded to the flavor compounds phenyl lactate and phenyl acetate by lactococcal cells (Yvon *et al.*, 1997).

2.5.6.4 Proteolysis

Proteolysis is the most complex and most important primary event which occur during cheese ripening and plays a vital role in the development of texture and flavor. Proteolysis is primarily responsible for textural changes, e.g., in hardness, elasticity, cohesiveness, fracturability, stretchability, meltability, adhesiveness and emulsifying properties and makes a major contribution to cheese flavor and flavor perception (Murtaza *et al.*, 2014). In the majority of cheese types, proteolysis is crucial during maturing. The extent of proteolysis varies from very limited (e.g., Mozzarella) to very extensive (e.g., Blue varieties) and the products range in size from large polypeptides only slightly smaller than the intact caseins, through a range of medium and small peptides to amino acids. Small peptides and amino acids play a direct role in cheese flavor. Amino acids can be broken down into a variety of sapid and aromatic compounds, such as amines, acids, carbonyls, and sulfur-containing compounds, which are all significant cheese flavor contributors (Fox, 1989).

Proteolysis contributes to the development of cheese texture via hydrolysis of the protein matrix of cheese and a decrease in water activity (a_w) through uptake of water molecules and increase of water binding capacity by the new ionized carboxylic acid and amino groups liberated as a result of hydrolysis of peptide bonds. Proteolysis during ripening is influenced by coagulating proteases, plasmin, and microbial proteases (from starter and non-starter bacteria). The principal indigenous milk proteinase, plasmin, is thought to be mainly responsible for the relatively limited proteolysis of α-casein in cheese (Visser, 1993). Despite being thought to

contribute little to primary proteolysis in cheese, *Lactococcus lactis* has a very comprehensive proteolytic system that is principally responsible for the production of short peptides and free amino acids (O'Keeffe, 1984).

Cheese proteolysis is mostly controlled by the final pH, moisture content, salt in moisture (S/M), temperature, and duration of ripening. The key stage in the manufacture of cheese occurs when the whey is drained since it affects the cheese's mineral content, the proportion of residual chymosin in the cheese, the final pH, and the moisture-to-casein ratio (Lawrence *et al.*, 1983). The initial level of chymosin and the pH during whey drainage determine how much chymosin is incorporated into the cheese curd. Chymosin and plasmin are responsible for the primary proteolysis (Forde and Fitzgerald, 2000). The summary of the proteolysis and amino acid catabolism in cheese during ripening is shown in Fig.2.4.

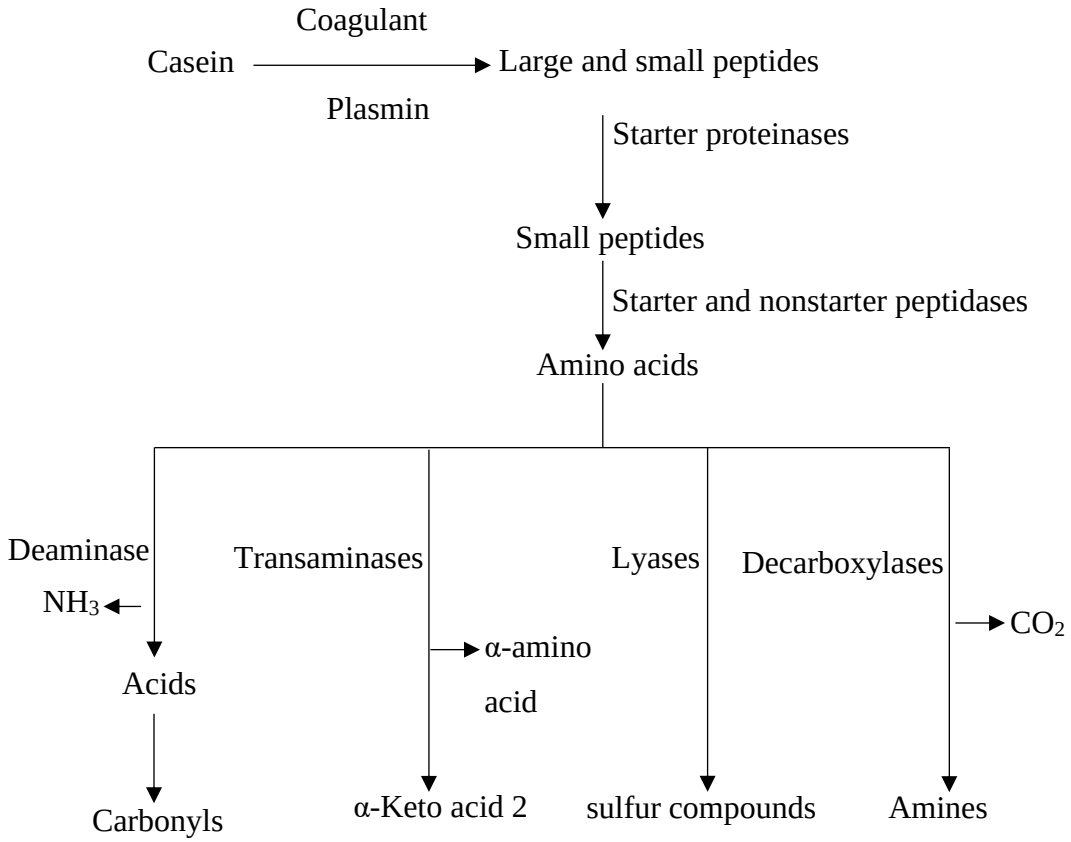

Fig.2.4 Summary of proteolysis and amino acid catabolism in cheese during ripening

2.5.6.4.1 Assessment of proteolysis

Proteolysis is routinely monitored on cheese ripening and is a useful index of cheese maturity and quality. Various analytical techniques have been developed to estimate proteolysis in cheese. These approaches are divided into two categories: specified and non-specified. The non-specific techniques involve determination of nitrogen soluble in various solvents or precipitants e.g., water, pH 4.6 buffers, NaCl, ethanol, trichloroacetic acid (TCA), phosphotungstic acid (PTA) and quantified by Kjeldahl method. The specific techniques involve the use of chromatography and/or electrophoresis. (Christensen et al., 1991; Fox and McSweeney, 1997; Ardö, 1999). The N compounds used in cheese fractions is shown in the Table 2.5.

Table 2.5 Groups of N compounds used in cheese fractions

Fraction	N compounds in fraction
Water soluble nitrogen (WSN)	Whey proteins, protease-peptones, peptides, amino acids, smaller N compounds and casein components at high pH or salt content
Trichloroacetic acid soluble nitrogen (TCA-SN)	Medium-sized small peptides, amino acids, and smaller N compounds, such as amines, urea and ammonium
Phosphotungstic acid soluble nitrogen (PTA-SN)	Very small peptides, amino acids and smaller N compounds except ammonia

Source: Ardö (1999)

2.5.6.5 Ripening agents in cheese

The ripening of cheese is catalyzed by the metabolic activity of living organisms and enzymes from these organisms or from other sources.

- **Coagulant**

Coagulant usually contributes chymosin or other suitable proteinase, however some Italian varieties which employ rennet paste contains both chymosin and lipase, pregastric esterase (PGE). The high temperature required to prepare curd denatures the coagulant enzymes found in high-cooked cheeses such as Emmental and Parmesan, as well as pasta filata varieties such as Mozzarella (McSweeney 2004).

- **Milk**

Milk contains around 60 natural enzymes and only few are important in cheese ripening. Proteinases, particularly plasmin, lipoprotein lipase (present in raw milk cheese), acid phosphatase, and xanthine oxidase, play important roles in cheese ripening. Most naturally occurring enzymes are highly heat stable and can withstand pasteurization totally or partially. Furthermore, because they are either linked to the casein micelles or present in the fat globule membrane, they are more likely to be integrated into the cheese curd rather than lost in the whey. Enzymes present in the serum phase are generally lost in the whey and hence have minimal role in cheese ripening (Fox *et al.*, 2015).

- **Starter culture**

Although live starter cells are assumed to have a minor role in cheese ripening, they do contain a variety of intracellular enzymes that are released during cell death and lysis. These starting enzymes greatly enhance the ripening process (Fox *et al.*, 1998).

- **Secondary microflora**

Several cheeses have a secondary (non-starter) microflora that has secondary role other than acid production; in many cases, the secondary culture's metabolic activity dominates flavor development. Yeasts, molds, coryneform bacteria, and propionic acid bacteria are among the microorganisms. Cheeses also include NSLAB, which are derived from milk or the environment and whose growth is normal and encouraged. Because of the selective nature of the cheese's interior, its adventitious microflora is predominantly composed of mesophilic lactobacilli and, to a lesser extent, pediococci (Beresford *et al.*, 2001).

- **Exogenous enzymes**

Exogenous enzymes, usually proteinases and maybe peptidases and lipases, have been added to cheese curd to accelerate ripening (Law, 2001).

2.5.6.6 Microbiological changes during ripening

Microorganisms, including bacteria, yeast, and molds, are present in cheese throughout ripening. The majority actively contribute to cheese maturation, either directly through their metabolic activity or indirectly by the release of enzymes into the matrix of the cheese during autolysis (Beresford and Williams, 2004). The cheesemaker promotes the development of these microbes. The starter LAB and the secondary microbiota are two groups that may be linked to cheese ripening. Starter bacteria are primarily responsible for acid production during manufacture; thus, they must be able to produce enough acid to quickly lower milk's pH. As a general guideline, milk should reach a pH of 5.3 in 6 h at 30-37°C. Although secondary microbiota plays no active part in the production of cheese, it is involved with starter bacteria in the ripening process. *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* are considered as starter bacteria (Bintsis, 2018) The secondary microbiota may be divided into a number of primary groups, including (1) NSLAB consisting of nonstarter lactobacilli, *Pediococcus*, *Enterococcus*, and *Leuconostoc* (2) propionic acid bacteria (PAB), (3) molds, and (4) bacteria and yeast, which grow on the surface of smear-ripened cheeses (Cotter and Beresford, 2017).

- **Sources of microorganisms in cheese**

Microorganisms enter to the cheese either by direct addition of starter culture, or from direct contact with cheesemaking and ripening equipment. As a result, the production process plays a key role in determining the cheese biota's biodiversity. The speed and intensity of milk cooling after milking has a major effect on the microbial biota. Mesophilic bacteria, particularly *Lactococcus* and *Enterobacter* spp., predominate in milk chilled to 15–21°C (Bramley, 1990). Growth of the microorganisms will be retarded when milk is cooled to 4°C, however

psychrotrophic bacteria like *Pseudomonas* and *Acinetobacter* will continue to grow slowly and become dominant (von Neubeck *et al.*, 2015).

Pasteurization considerably reduces psychrotrophic and mesophilic populations but there is now some evidence that some of these microorganisms enter a viable but nonculturable state, resulting in their levels being underestimated when traditional culture-based approaches are used. Regardless, it is clear that spores, predominantly from the genera *Bacillus* and *Clostridium*, and a variety of other thermoduric organisms can survive pasteurization and gain entry to the cheese, along with other microorganisms that are introduced through post pasteurization contamination. The starter bacteria are the most prevalent microorganisms in cheese, especially during the early stages of ripening. Rennet and salt are other ingredients that are used in the production of cheese (Quigley *et al.*, 2013).

Salt may be added either (1) directly to the milled cheese curd, (2) rubbed to the surface of molded curds as a dry salt or salt slurry, or (3) by immersion of molded cheese in a brine solution containing15%–23% NaCl (Cotter and Beresford, 2017). Although rubbing dry salt to the surface of the cheese helps transport microorganisms from the environment and the cheesemaker's hands to the surface of the cheese, it is unlikely that salt is directly responsible for the addition of any biota. Industrial brines are rarely pasteurized but are commonly utilized. Although the relatively high salt content of brine inhibits the growth of many microorganisms, leaching of proteins and other nitrogenous compounds from the cheese into the brine may enhance survival of microorganisms that enter to the brine (Marino *et al.*, 2015). According to several studies, the microbiota of commercial brines include LAB, such as *Lactobacillus paracasei, Lactobacillus casei*, and *Lactobacillus plantarum* (Bintsis *et al.*, 2000; Bokulich and Mills, 2013*)*, as well as yeast, such as *Debaromyces hansenii* and *Candida versatilis* (Seiler and Busse, 1990; Bokulich and Mills, 2013) The presence of these bacteria in the brine subsequently influences the surface microbiota of cheese (Mounier *et al.*, 2006).

During ripening process, complex microbial communities made up of bacteria, yeast, and mold form on the surface of smear- and mold-ripened cheeses. The smear microorganisms might develop as a result of natural contamination of the milk or cheese, including microorganisms

that come from the facility's equipment and other areas. Cheese may also get contaminated with bacteria by the deliberate inoculation of the surface (Cotter and Beresford, 2017)

- **Factors that influence the growth of microorganism in cheese**

The final environmental conditions of a cheese are significantly influenced by the production process. The extremely selective environment of cheese has a significant influence on the development and survival of microorganisms during processing and ripening. In order to make cheese, moisture must be removed while casein and milk fat are concentrated. This is accomplished by coagulating casein to form a gel and then, removal of the moisture from the gel by the process called synersis. Coagulation is achieved either through heating, acid production, or by enzymes, while the drivers of syneresis are acid production, heat, and mechanical stress. Manufacture of most cheese varieties involves coagulation at a temperature of 30–37°C followed by cooking at 37–54°C. This heat treatment is considered to play an important role in controlling the growth of starters and undesirable microorganisms (Steffen *et al.*, 1993). Changes in these temperatures also have an effect on the final flavor profile (Kocaoglu-Vurma *et al.*, 2008). The manufacturing process also influences the gross composition of the cheese, which is best defined by the four parameters of salt-in-moisture, moisture-in-nonfat substance, fat-in-dry-matter, and pH (Gilles and Lawrence, 1973). These parameters, in turn, influence the environment in which the microorganisms proliferate. Water and salt content, pH, the presence of organic acids and nitrate, redox potential, and ripening temperature are the primary environmental factor controlling the growth of microorganisms (Beresford *et al.*, 2001).

2.6 Sensory evaluation of cheese

Sensory evaluation refers to a collection of techniques for accurately measuring human response to foods. Sensory assessment is described as a scientific process for measuring, analyzing, and interpreting responses to product as perceived via the senses of sight, smell, touch, taste, and hearing (Stone and Sidel, 2004). The dairy industry recognizes the significance of sensory quality as a crucial aspect in sales and marketing and has been at the forefront of sensory development, using cheese grading and judging to evaluate sensory quality before modern-day

sensory analysis techniques were developed. The purpose of any sensory quality program should be to meet the consumer requirements. Sensory evaluation is commonly used in the cheese industry to analyze cheese quality, characterize cheeses during development, and test customer acceptance (Singh *et al.*, 2003; Drake, 2007) .

2.6.1 Sensory characteristics of cheese

Sensory characteristics of cheeses are human responses to perceptions of stimuli that are experienced with the cheese and may be broadly characterized under the areas of appearance, flavor, and texture. Sensory characteristics are the consequence of interactions between the human sensory modalities of vision, touch, olfaction, gustation, and mouthfeel and stimuli caused by the cheese's rheological, structural, and chemical components. Consumers experience sensory characteristics when they view, manipulate, smell, and take cheese into their mouths for consumption, and these perceptions are then expressed as a behavioral response via actions or descriptive terms. Sensory perception varies between individual due to physiological, psychological, social, and cultural differences (Delahunty and Drake, 2004).

- **Cheese appearance**

Human vision is the perception of shape and texture, size and distance, brightness, color, and movement. Appearance characteristics of cheese are evaluated visually, generally before consuming or when preparing the cheese for consumption by cutting or spreading. Color, the presence or lack of eyes or holes (or openness), mold, rind, and visual texture are all appearance characteristics of cheese. Furthermore, appearance includes a cheese's market image (e.g., size, shape, packaging) because most cheese are marketed in this form (Murray and Delahunty, 2000).

- **Cheese texture**

Texture is a cheese attribute that results from a combination of physical properties, such as size, shape, number, nature, and confirmation of the constituent structural elements, and is perceived by a combination of the senses of touch (tactile texture), vision (visual texture), and hearing (auditory texture). For example, the softness of a cream cheese may be assessed visually when the cheese is cut, by proprioceptive feelings when the cheese is spread, and lastly by tactile

sensations in the mouth during consumption. Texture perception occurs during mastication and consumption in the surface structures of the mouth, around the roots of the teeth, and in the muscles and tendons. Firmness, rubberiness, crumbliness, graininess, cohesion, and adhesiveness are common cheese texture characteristics. Texture perception incorporates physical, physiological, and psychophysical factors, as do other types of sensory perception (Foegeding and Drake, 2007; Foegeding *et al.*, 2011).

- **Cheese flavor**

Flavor is commonly characterized as the combined perception of olfactory, gustatory, and chemesthetic (or trigeminal) sensations. Flavor perception begins before consumption when a consumer smells a cheese, but it is finally perceived during consumption when compounds that stimulate the olfactory system in the nose, the taste system in the mouth, and the trigeminal system in the mouth and nose are released from the cheese and become available to receptors. (Delahunty and Drake, 2004).

Aroma is typically the first aspect of flavor that a customer encounters. Smell stimuli are airborne compounds of volatile substances that allow them to move from their source to the olfactory receptors, where perceptions endowed with individual odors are generated. Volatile stimuli are emitted into the air from the cheese and can be transmitted to the nose orthonasally, typically consciously, by smelling (for example, while opening a cheese packaging or removing a trier from the cheese for assessment). Hundreds of different volatile chemicals have been found in cheese, each with a distinct olfactory character and they make the greatest contribution to the variety of cheese flavors. Fatty acids, methyl, ethyl, and higher esters, methyl ketones, aliphatic and aromatic hydrocarbons, short- and long-chain alcohols, aromatic alcohols, aldehydes, amines, amides, phenols, and sulfur compounds have all been found in cheese (Maarse *et al.*, 1994).

Taste is another aspect of flavor. Tasting occurs in the oral cavity, mostly on the tongue, but sometimes on the soft palate. Non-volatile compounds are the principal taste stimulus, and they must come into touch with the taster receptors. This interaction produces sensations that impart four unique taste qualities: sweet, salty, sour, and bitter. A fifth flavor (umami) has lately gained acceptance, especially in Japan and other countries where it is most known and easily

recognized. Lactic acid (sour), sodium chloride (salty), mineral salts of potassium, calcium, and magnesium (salty), and free amino acids and peptides of various sorts (sweet, bitter, umami) are all compounds that contribute directly to cheese flavor (Warmke *et al.*, 1996; Engel *et al.*, 2000; Drake, 2007)

2.6.2 Methods of sensory evaluation of cheese

Sensory evaluation can be used to determine whether cheeses have defects or other undesirable characteristics, whether a difference in overall sensory character can be detected between two or more cheeses, and whether specific differences in sensory characteristics can be perceived. Sensory evaluation can also be used to quantify the intensity of one or more sensory characteristics, the onset, maximum intensity, and decline of a sensory characteristic, and to determine whether consumer preferences can be met. Quality scoring, discrimination testing, descriptive testing, time-intensity testing, and consumer acceptance testing are most commonly used sensory evaluation methods (Meilgaard *et al.*, 1999; Lawless and Heymann, 2010).

- **Quality scoring**

Quality scoring is conventional and still most extensively used technique of formal sensory evaluation in the cheese industry. Cheese grading is done to classify a cheese's ability to acquire a satisfactory flavor throughout aging and to retain quality at the moment of sale. Grading standards often include a scoring system in which the highest grade is assigned a maximum score and points are deducted when defects are found. For example, the IDF provides standard scorecards for cheese and defines a scale ranging from 5 (best possible quality) to 0 (lowest possible quality) (IDF, 1997).

- **Discrimination tests**

Sensory discrimination tests involve direct comparisons of cheeses to determine whether there is either an overall difference between them, or whether they differ for a specific characteristic. The paired comparison test, duo-trio test, triangle test, and ranking tests are the most commonly used discrimination tests. In the paired comparison test, two cheeses are compared and assessors are asked whether they vary; normally, a difference for one specific sensory attribute is

examined (ISO, 2005). In the duo- trio test, assessors are asked which of two products is most similar to a third reference product, allowing a common reference to be used again (ISO,1991). In the triangle test, assessors are provided with three cheeses and asked to select the one that differs the most from the other two (ISO, 1983). The ranking test normally compares four to six cheeses for a single defined attribute, and the accessor is asked to rank them in order of increasing intensity of that attribute (ISO,1988).

- **Descriptive analysis**

Descriptive sensory analysis refers to a group of techniques that aim not only to distinguish between the sensory characteristics of a variety of cheeses, but also to determine a quantitative description of all the sensory distinctions that can be identified. It also provides a basis for determining which characteristics are affected by changes in cheesemaking technique or composition, as well as which attributes are vital for customer acceptance. The most commonly used descriptive methods include the flavor profile method (Cairncross and Sjostrom, 2004), texture profile method (Brandt *et al.*, 1963), quantitative descriptive analysis (Stone *et al.*, 2008) and the spectrum method (Meilgaard *et al.*, 1999). Each descriptive test consists of three stages: (a) selecting a panel, (b) establishing the language or vocabulary to describe the cheese, and (c) quantifying the sensory results (Delahunty and Drake, 2004).

- **Time-intensity sensory analysis**

Time-intensity methods are useful for studying new cheese types, such as low-fat cheeses, because the reduction in fat content influences not only sensory character development, but also the breakdown of the cheese in the mouth during consumption and the rate of release of flavor-contributing compounds. In a study of Cheddar cheese flavor, for example, the time needed to attain maximum intensity for sharpness, bitterness, and astringency was consistently longer in reduced-fat Cheddar than in full-fat Cheddar, and, more critically, the rate of flavor release was greater in reduced-fat Cheddar (Shamil *et al.*, 1991). Delahunty *et al.* (1996) also showed that by correlating time-intensity sensory data with dynamic volatile compound release data, an improved relationships between volatile composition and perceived sensory characteristics may be achieved.

- **Consumer acceptability testing**

Understanding consumer perception of cheese flavor crucial for effective marketing and product development. Typically, 8-12 participants are to describe the cheese. Such sessions are undertaken to obtain information on preference, motivation, and product attributes. Effective studies measure consumer response and give information on consumer likes and dislikes (hedonic response). Consumer are presented with cheese and asked specifically to rate their like on nine-point hedonic scale (Drake, 2007). Rating scales are used to measure relative likes and dislikes based on preference rather than difference. These tests should be performed with unskilled consumers or subjective assessors. At least 50 personals are required, the more the assessors, the greater the reliability of result (Delahunty *et al.*, 2004; Delahunty and Murray, 1997).

2.7 Kiwi fruit (*Actinidia deliciosa*) protease

Kiwifruit (*Actinidia deliciosa*) is indigenous to Southern China. It was originally known as "Yang Tao" in China and "Chinese Gooseberry" in rest of the world. Its cultivation extended from China to New Zealand in the early 20th century, when Isabel Fraser introduced seeds to New Zealand (Dadlani *et al.*, 1971). It is known to contain highly active proteolytic enzymes and dominant one is actinidin (Kaur *et al.*, 2010).

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

Actinidin is a single polypeptide chain globular protein with a sequence of 220 amino acid residues, a molecular weight of 24.0 kDa (Carne and Moore, 1978). The polypeptide is folded into a two-domain structure. The L-domain (domain I) consists of residues 19-115 and 214-220 and the R-domain (domain II) residues 1-18 and 116-213 (Boland, 2013). It has the ability to form milk clots so that the enzyme is fully compatible with conditions used in cheese

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

2.7.1 Major soluble protein in kiwifruit

There are four major soluble protein in kiwifruit.

- Actinidin: Actinidin is the predominant enzyme in kiwifruit which accounts for 30-60% of soluble protein in kiwifruit with molecular mass of 24 kDa (Paul *et al.*, 1995).This proteolytic enzyme belongs to the cysteine proteinase family (EC 3.4.22.14) which is concentrated in kiwifruit flesh (Préstamo, 1995).
- Kiwellin: This makes up 20% to 30% of total soluble protein in kiwifruit which contain 189 amino acid residues with a molecular mass of 28 kDa. (Tamburrini *et al.*, 2005).
- Thaumatin: It is another soluble protein found in kiwifruit with molecular mass of 24 kDa. (Gavrović-Jankulović *et al.*, 2002).
- Kirola: It has recently been isolated and sequenced protein with molecular mass of 17 kDa. (D'avino *et al.*, 2011).

2.7.2 Application of actinidin in food industry

The applications of the proteolytic activity of actinidin have been widely studied. Actinidin's ability to degrade cream milk proteins into fragments < 29 kDa suggests its use as a preliminary treatment for producing milk-derived products with reduced undesired protein content (Puglisi *et al.*, 2012). Purified actinidin can totally degrade α-lactalbumin and 65.3% of β-lactoglobulin (Vázquez-Lara *et al.*, 2003). Majdinasab *et al.* (2010) found that actinidin raised the nitrogen solubility index of soymilk in a dose-dependent manner. This suggests that actinidin might enhance soymilk functioning by enhancing protein solubility. Actinidin is widely used in the

food industry for meat tenderization. Lewis and Luh (1988) investigated actinidin's potential to tenderize meat. The results demonstrated that actinidin greatly tenderizes beef muscles. Actinidin has been shown to tenderize pig muscles by affecting both myofibrils and connective tissue. Actinidin has been shown to improve protein solubility and water retention capacity in sausages (Aminlari *et al.*, 2009). Actinidin has been studied as a potential coagulant enzyme for cheese production (Lo Piero *et al.*, 2011).

2.8 Various methods for the purification of protein

Various extraction techniques, such as Soxhlet or solvent extraction (SE), pressurized liquid extraction (PLE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), and enzyme-assisted extraction (EAE), have been developed to isolate and produce bioactive compounds from natural matrices (Joana Gil-Chávez *et al.*, 2013). Furthermore, many advanced purification techniques, such as ultrafiltration (UF), nanofiltration (NF), ion exchange chromatography (IEC), gel filtration chromatography (GFC), and affinity chromatography (AC), have been used to further purify the desired molecules (Akin *et al.*, 2012). However, these described approaches are frequently expensive and time-consuming, involving vast volumes of organic solvents and exposing the extract to the danger of degradation or alteration of some of the contents using multistep processes. In addition, many existing procedures require equipment with high investment and maintenance costs. In light of these considerations, there is an increasing demand in the scientific community for a rapid, green, efficient, adaptable, and cost-effective approach for recovering and purifying large amounts of bioactive compounds.Tan and Lovrien (1972) for the first time, proposed the use of three phase partitioning (TPP) as a promising alternative strategy to traditional extraction and separation techniques.

2.8.1 Three phase partitioning

TPP is a simple, quick, and efficient method for separating and purifying proteins from complicated mixtures. Tan and Lovrien (1972) initially defined it, and it has since been extensively utilized to purify several target macromolecules. It has now become a versatile and common bio separation process with a wide range of applications. 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 basic principle behind this new technology is to combine crude protein extract with solid salt mainly ammonium sulfate and an organic solvent, commonly tert-butanol, to develop three phases. After centrifugation at 4°C, the desired enzymes or proteins are preferentially partitioned to one phase, while other impurities such as pigments and lipids are partitioned to the other. An interfacial protein precipitate separates the upper organic phase, which contains non polar compounds (pigments, lipids), from the lower aqueous phase, which contains polar compounds (proteins, carbohydrates) (Dennison and Lovrein, 1997). The schematic representation of TPP is shown in the fig. 2.5.

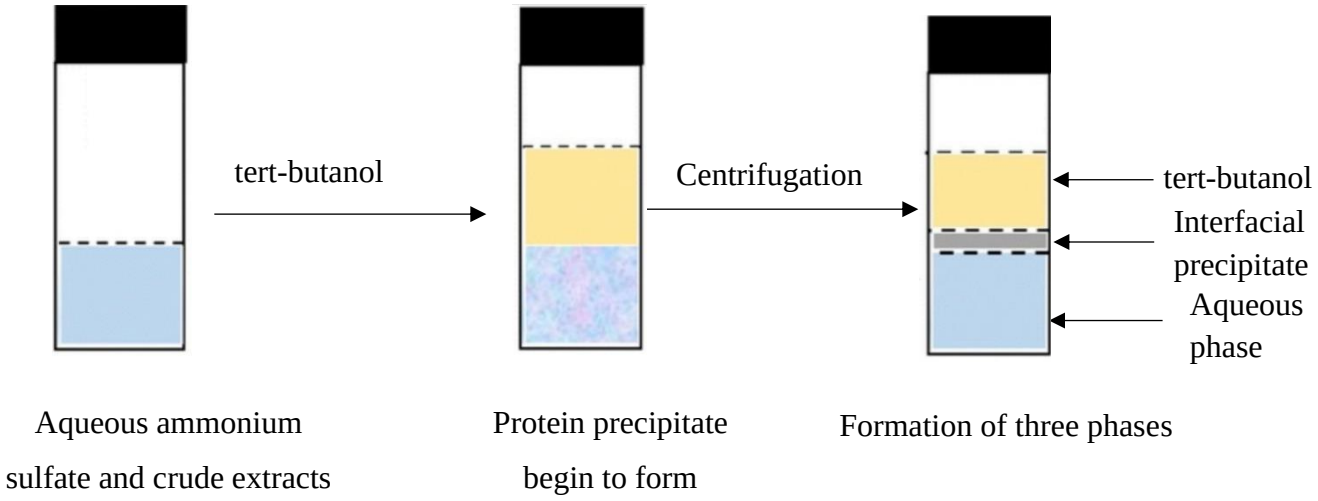

Fig. 2.5 Schematic diagram of Three phase partitioning experiment

Source: Yan *et al.* (2018)

Several enzymes have been purified using TPP including invertase, pectinase, α-galactosidase, trypsin inhibitor, laccase and catalase. Numerous proteases were purified using TPP technique which is shown in the Table 2.6

Table 2.6 Some proteolytic enzymes purified using TPP

Enzyme	Source	Reference
Actinidin	Kiwifruit	Maskey and Karki (2023)
Ficin	*Ficus carica L.*latex	Gagaoua *et al.* (2014)
Zingibain	Zinger rhizome	Gagaoua *et.al* (2015)
Papain	Papaya latex	Hafid *et al.* (2020)
Calotropin	*Calotropis procera*	Rawdkuen *et al.* (2010)
Bromelain	Pineapple crown	Gul *et al.* (2022)

Part III

Materials and methods

3.1 Materials

3.1.1 Sample

Fresh cow milk was purchased from the local market of Dharan. The kiwifruit (*Actinidia deliciosa*) was collected from the supermarket of Dharan. The freeze dried thermophillic culture (*Lactobacillus helveticus* and S*treptococcus thermophilus*) was obtained from Trishul Trade links, Kathmandu.

3.1.2 Chemicals and apparatus

All the chemicals, glass wares and equipment required were used from Central Department of Food Technology laboratory (CDFT), Dharan**.**

3.2 Methods

3.2.1 Preparation of crude dialyzed kiwifruit extract

Extraction of crude kiwifruit enzyme was carried out according to Gagaoua *et al.* (2015)) with slight modification. The kiwifruit was peeled and cut into small pieces. It was then mixed with 50 mM sodium phosphate buffer containing 10 mM cysteine and 2.5mM ethylenediamine tetra acetic acid (EDTA) in the ratio 1:0.5 (w/v) and homogenized in a blender (Model Havel's grind 14000). The slurry was then stirred for 45 min at 4°C before being filtered through cheesecloth and centrifuged at 7000 rpm (Model DLAB D3024R) for 10 min at 4°C to separate impurities. After centrifugation, the supernatant was saturated with 30% ammonium sufate and again centrifuged for 10 min at 4ºC and 5000 rpm. The supernatant obtained was further saturated with 65% ammonium sulfate which is followed by centrifugation at 15000 rpm and 4ºC for 10 min.

A kiwifruit weighing 66 g was used for enzyme extraction, yielding 29 ml of crude enzyme. The crude extract was then dialyzed overnight using dialysis membrane of molecular weight cut-off (MWCO) 12 kDa against three changes of the same buffer. The crude extract was taken for purification.

3.2.2 Three phase partitioning of crude dialyzed kiwifruit extract

Purification of the crude dialyzed extracts was carried out by TPP as described by Maskey and Karki (2023). In brief, the crude extract was treated with 40% ammonium sulfate (w/v) saturation at pH 6, followed by 1.0:0.75 tert-butanol (v/v). The mixture was vortexed and centrifuged at 5000 rpm for 5 min at 4°C to separate the three phases. Using a Pasteur pipette, the top layer of tert-butanol and lower aqueous phase were removed, and the interfacial layer was dialyzed overnight through membrane (MWCO:12 KDa) with three changes of buffer (pH 7.0) at 4ºC. After TPP purification of 29 ml crude dialyzed kiwifruit extract, 15 ml purified of purified enzyme was obtained.

3.2.3 Milk clotting activity

The milk clotting activity (MCA) was determined following the procedure described by IDF (1992). 60 g of skimmed milk powder was reconstituted in 500 ml of 0.01 mol/L $CaCl_2$ (pH 6.5). The enzyme was added at a proportion of 200 µl per 2.0 ml of milk. The clotting point was estimated during the manual shaking of the test tube. At very short time intervals (5-10 s), the coagulation time was noted. One milk clotting unit was defined as the amount of enzyme that clots 10 ml of substrate within 40 min at 37°C (Berridge, 1952). It can be calculated as:

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

Where T = time necessary for the curd formation (s), V_S = volume of the milk (ml), V_E = volume of the enzyme (ml).

3.3 Experimental design for optimization of milk pH and temperature for cheesemaking

The experimental design, data analysis, as well as model building were carried out using Design Expert software (version 13, Stat- Ease Inc., USA). The influence of pH and temperature of milk in cheesemaking was analyzed using response surface methodology (RSM) with central composite randomized design (CCRD). The response variable was milk clotting activity of TPP purified protease. The different constraints for optimization of actinidin is shown in the Table 3.1

Table 3.1 Range of factors for RSM/CCRD

Factors	-1	0	+1
Milk pH	6	6.25	6.5
Milk temperature (ºC)	45	50	55

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

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

The polynomial coefficient was represented by β_0 (constant), β_1, β_2, (linear effects), β_{12}, (interaction effects), β_{11}, β_{22}, (quadratic effects), and ε (random error). Multiple regression analysis was used to model the data and analysis of variance (ANOVA) was used to determine the term's statistical significance.

3.4 Preparation of cheese

A general procedure for cheese preparation as reported by Chandan and Kapoor (2011) with slightly modification is shown in the Fig.3.1. Two batches of cheese using actinidin and chymosin was prepared. 3 L Milk having 3% fat and 8% SNF was heated to 80ºC and then left for cooling. When the milk temperature decreased to 45ºC, thermophillic culture containing *Streptococcus thermophilus* and *Lactobacillus helveticus* was added at the rate of 1%. Then the

milk was incubated at 45ºC for 1h and calcium chloride at the rate of 0.02% was also added. In one batch of milk, actinidin was added at the rate of 1% when the pH of the milk was 6.25 at 55ºC and in another batch chymosin at the rate 2.5 g/ 100 L of milk was added when the milk pH was 6.1 at 45ºC. Then both batches of cheese milk were stirred gently and allowed to coagulate for 40 min. The curd was then cut vertically and horizontally into 5 cm3 by stainless steel knife and it was further cooked upto 50ºC for 40 min until the curd becomes firm. Whey was drained using cheese cloth and the curd was pressed overnight with 10kg mass. The cheeses blocks were then immersed in 15% brine solution overnight. Then the cheese blocks were vacuum packed in low density polyethylene (LDPE) and ripened at 10ºC and relative humidity of 75-80 % for 60 days.

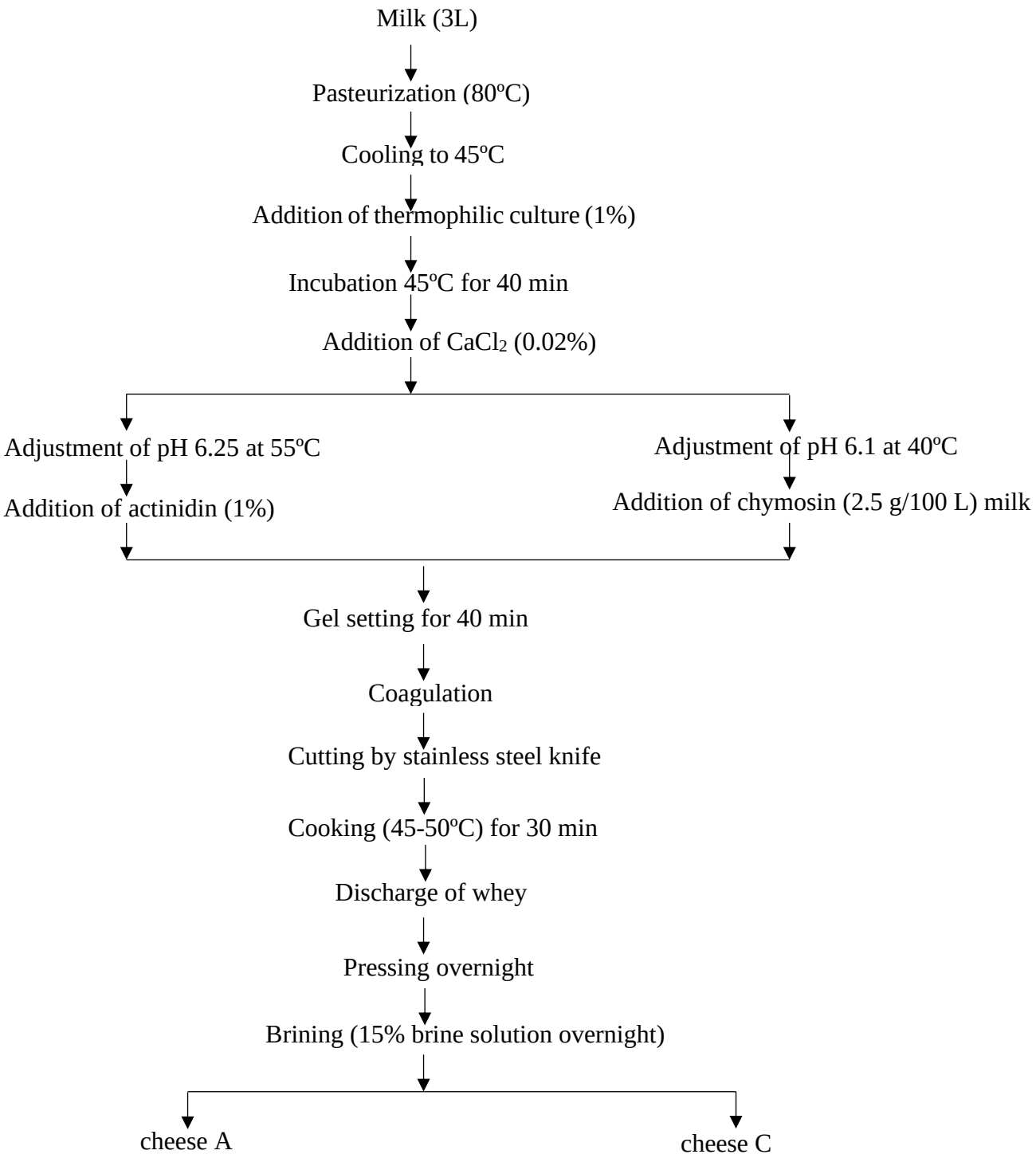

Fig. 3.1 Preparation steps of cheeses

3.5 Physiochemical analysis of cheeses during ripening

Cow milk was analyzed for fat, protein, solid not fat, ash, acidity, calcium and pH. Cheeses A and C were analyzed for physiochemical composition at interval of 15 days.

- Moisture: The moisture content of cheese A and B was estimated by oven- drying method as described in AOAC (2005).
- pH: The pH of milk and cheeses was determined by the method mentioned in AOAC (2005).
- Protein: The protein content of milk and cheese samples was estimated by Kjeldahl method as mentioned in AOAC (2005).
- Fat: Fat content of milk and cheese samples was estimated by the method in AOAC (2005).
- Salt: Salt content of cheese samples was determined by the method mentioned in Ranganna (1986).
- Ash: Ash content of cheese samples was estimated by dry ashing method as mentioned in AOAC (2005).
- Yield: The yield of cheese samples was calculated using the equation as per Mahdian Dehkordi *et al.* (2022).

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

3.6 Determination of proteolytic parameters of cheeses during maturation

The total nitrogen (TN) content of cheese A and B was determined by the Kjeldahl method AOAC (2005). The water soluble nitrogen (WSN), 12% trichloroacetic acid soluble nitrogen (TCA-SN) and 5% (w/w) phosphotungstic acid soluble nitrogen (PTA-SN) were determined according to the method reported by Bütikofer *et al.* (1993). The nitrogen content of each fraction was expressed as % of the TN of cheese.

3.7 Determination of total free amino acid of cheeses during ripening

The level of total free amino acid (FAA) was determined by the method of Folkertsma and Fox (1992). A 100 µl of WSN was diluted with H_2O into 1 ml in which 2ml of Cd-ninhydrin reagent. The reagent was prepared by dissolving 0.8 g ninhydrin in a mixture of 80 ml ethanol and 10 ml glacial acetic acid followed by the addition of 1 g cadmium chloride ($CdCl_2$) dissolved in 1 ml of distilled water. Then the tubes were vortexed and heated at 84°C for 5 min and cooled in ice water. The absorbance was determined at 507 nm. Thus, obtained results was expressed as mg leucine per g cheese by reference to a standard curve which was first be prepared using leucine at various concentrations (0.05 – 0.50 mg leu/ ml).

3.8 Determination of lipolysis of cheeses during ripening

Lipolysis was measured as acid degree value (ADV) according to the procedure of Nunez *et al.* (1986) and Hayaloglu (2007). A 10 g sample of cheese was macerated in a mortar with 6 g of anhydrous sodium sulfate (Na_2SO_4), and then 60 ml of diethyl ether was added to the mixture in a 100 ml screw-capped bottle. After 2 h of stirring, the mixture was filtered using Whatman No. 1 paper. Following three consecutive times of re-suspending the precipitate in a bottle with 20 ml diethyl ether, filtration was performed. Then, diethyl ether was removed from the filtrate at 50°C using a rotary evaporator. After complete removal of the diethyl ether, the liquid fat was transferred into a flask and weighed. A 5 ml of ethanol and diethyl ether (1:1) were added to the flask. The total mixture was titrated with 0.05 N alcoholic potassium hydroxide (KOH) solution. The result was expressed as mg KOH per g of cheese.

3.9 Microbiological analysis of cheeses during maturation

The microbiological analysis of cheeses was done as per the method described by AOAC (2005). The total viable count was determined by plate count agar (PCA), yeast and molds by potato dextrose agar (PDA), *Lactobacillus* by MRS agar and *Streptococcus* by M17 agar. Coliform bacterial count was determined using violet red bile agar (VRBA).

3.10 Sensory analysis of cheeses during ripening

The prepared cheeses were evaluated organoleptically by 10 semi-trained panelists following the recommendations of ISO (2009) and Puglisi *et.al* (2014). The training session was first conducted to define the sensory terminologies and then, the cheese samples were presented in properly ventilated and well-lit laboratory. The panelists evaluated each sample for sensory attributes (texture, appearance, flavor, odor, aftertaste and overall acceptability) by using a five-point hedonic scale, with 1 being poor and 5 being excellent quality.

3.11 Statistical method

The obtained data were statistically analyzed using paired t-test in Microsoft Excel 2019 at 5% level of significance. The graph was constructed in Microsoft Excel (2019). The data pertaining to optimization study (RSM/CCRD) were analyzed in Design Expert software using analysis of variance (ANOVA) and fit statistics for the significant model and R^2.

Part IV

Results and discussion

In this study, the effect of milk pH and temperature on the milk clotting activity (MCA) was studied using response surface methodology (RSM). The cheeses, prepared from actinidin (A) and chymosin (C) were analyzed for physicochemical (moisture, pH, protein, fat, salt, ash and yield), proteolysis, free amino acids and lipolysis during ripening for 60 days at interval of 15 days. Microbiological (total plate count, lactobacilli, streptococci, coliform and yeast and mold count) properties and various sensory attributes (texture, appearance, flavor, odor, aftertaste and overall acceptability) were analyzed during ripening for 60 days at interval of 15 days.

4.1 Numerical optimization of milk pH and temperature for milk clotting activity of actinidin.

The determined values of MCA for actinidin ranged from 198.347 to 827.143 units respectively as shown in Table 4.1. The experimental design plan using RSM/CCRD is shown in Table 4.1. Table A.1 and Table A.2 (Appendix A) showed the model and other statistical characteristics of MCA.

Table 4.1 Experimental design plan using RSM/CCRD

Run	Factor 1	Factor 2	Response
	A: pH	B: Temperature (ºC)	MCA (U/ml)
1	6.25	50	480
2	6.25	57	705.882
3	6.50	45	214.286
4	6.50	55	260.87
5	6.25	50	521.739
6	6.00	55	827.143
7	6.25	50	500
8	6.25	50	571.428
9	6.00	45	452.83
10	6.60	50	198.347
11	6.25	50	545.455
12	6.25	43	296.296
13	5.90	50	666.667

The coded equation for actinidin is given as,

$$MCA=523.72 - 187.14\ A + 128.77\ B - 89.43\ AB - 50.74\ A^2 - 16.45\ B^2 \quad (4.1)$$

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

The quadratic equation 4.1 showed that pH of milk (A) had significant ($P<0.05$) negative effect but temperature of milk (B) had significant ($P<0.05$) positive effect on MCA at 95% level of confidence. The interaction term of A and B (AB) had significant ($P<0.05$) negative effect on MCA. Likewise, A^2 had significant ($P<0.05$) negative effect and B^2 had non- significant ($P>0.05$) negative effect on MCA as shown in Table A.2 (Appendix A).

The ANOVA Table A.2 in appendix A indicated that model is significant (P<0.0001) and the lack of fit is insignificant, indicating that the regression model can be fitted well. This was supported by the value of R^2 (0.9751) and adjusted R^2 (0.9573).

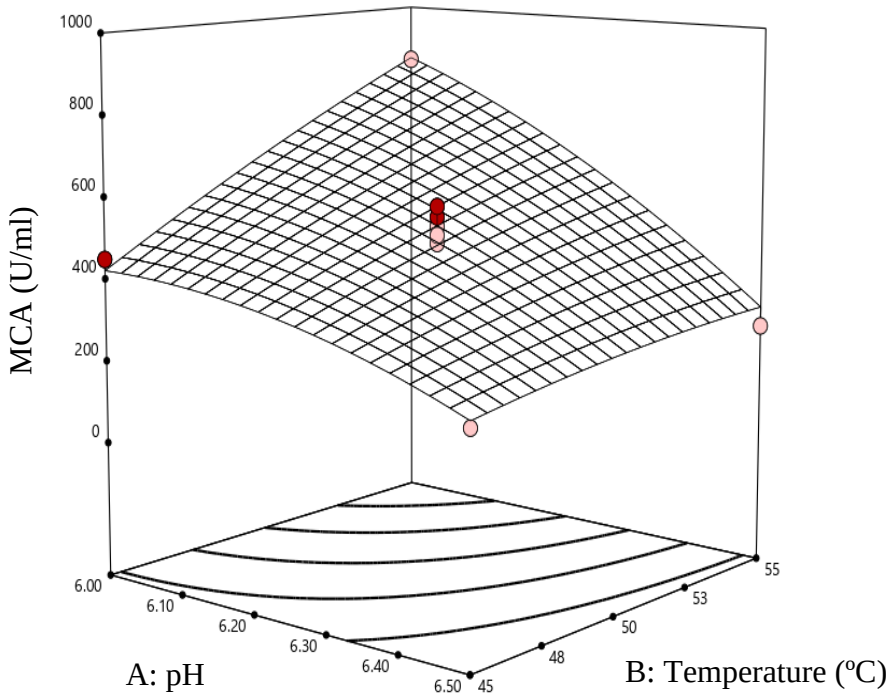

Fig. 4.1 Response surface plot of MCA of actinidin as a function of temperature and pH of milk

Fig. 4.1 shows that MCA increased with the increase in milk temperature and decreased with the increase in pH of milk. Similar findings of increasing MCA of partially purified kiwifruit and ginger rhizome protease with increase in temperature and decrease in pH were reported by Maskey and Karki (2022) and Maskey *et al.,* (2023), respectively.

4.2 Optimization of actinidin

Numerical optimization method was used for determination of optimum combination of pH and temperature of milk for maximum MCA, which is shown in the Table 4.2.

Table 4.2 Different parameters for optimization of actinidin

Parameters	Goal	Lower limit	Upper limit
pH	Target=6.25	6	6.5
Temperature (ºC)	In the range	45	55
MCA (U/ml)	Maximized	198.347	827.143

Under the assumptions by Design Expert (version 13), the optimum operating conditions for maximum MCA of actinidin was found to be 6.25 pH and 55°C of milk temperature with the desirability of 0.815 as shown in Table B.1 (Appendix B).

4.3 Verification of model

Within the scope of the variables investigated in Central Composite Rotatable Design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. Table 4.3 represents the outcomes of the confirmatory experiments.

Table 4.3 Predicted and observed value of responses

Response	condition		Predicted value	Mean observed value	Deviation (%)
	pH of milk	Temperature of milk			
MCA	6.25	55	636.045	623.65	1.987

The observed value of MCA was 623.65 U/ml which was supported by a deviation of 1.987 percentage.

4.4 Physicochemical properties of milk

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

Table 4.4 Proximate composition of raw cow milk

Parameters	Cow milk
Fat (%)	3.4 ± 0.1
Protein (%)	2.98 ± 0.05
SNF (%)	8.42 ± 0.12
Ash (%)	0.78 ± 0.01
Acidity (% lactic acid)	0.14 ± 0.01
Calcium (mg/100 ml)	121.42 ± 6.13
pH	6.7 ± 0.05

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

The values for fat, protein, solids not fat, ash, acidity, calcium and pH are similar to those reported by Walstra *et al.* (2006) and any variation may be due to cow breed, milking condition and milking time.

4.5 Physicochemical changes in cheeses during ripening

4.5.1. Chemical changes

The chemical composition of cheeses A and C throughout 60 days of ripening are shown in the Table 4.5.

Table 4.5 Chemical changes occurring in actinidin and chymosin cheeses during ripening

Parameters	Ripening days	Cheese A	Cheese C
Moisture	1	50.57 ± 0.15^{a}	51.15 ± 0.20^{b}
	15	49.45 ± 0.08^{a}	49.95 ± 0.05^{b}
	30	46.80 ± 0.18^{a}	47.03 ± 0.05^{a}
	45	45.60 ± 0.18^{a}	46.04 ± 0.07^{b}
	60	43.97 ± 0.20^{a}	45.19 ± 0.17^{b}
Protein	1	39.29 ± 0.21^{a}	38.65 ± 0.29^{b}
	15	40.35 ± 0.22^{a}	39.85 ± 0.37^{b}
	30	41.62 ± 0.08^{a}	43.32 ± 0.29^{b}
	45	44.45 ± 0.09^{a}	45.03 ± 0.29^{b}
	60	44.56 ± 0.20^{a}	49.003 ± 0.33^{b}
Fat	1	44.42 ± 0.23^{a}	49.18 ± 0.11^{b}
	15	47.85 ± 0.11^{a}	43.44 ± 0.23^{b}
	30	49.04 ± 0.18^{a}	51.82 ± 0.28^{b}
	45	50.75 ± 0.21^{a}	51.86 ± 0.18^{b}
	60	50.69 ± 0.10^{a}	52.96 ± 0.21^{b}

Salt	1	3.73 ± 0.11^{a}	3.29 ± 0.10^{b}
	15	3.97 ± 0.12^{a}	3.79 ± 0.09^{a}
	30	4.15 ± 0.02^{a}	4.52 ± 0.05^{b}
	45	4.47 ± 0.03^{a}	4.57 ± 0.09^{a}
	60	5.17 ± 0.08^{a}	5.05 ± 0.11^{a}
Ash	1	7.30 ± 0.21^{a}	7.18 ± 0.07^{a}
	15	7.37 ± 0.13^{a}	7.27 ± 0.16^{b}
	30	7.40 ± 0.05^{a}	7.36 ± 0.09^{a}
	45	7.52 ± 0.17^{a}	7.35 ± 0.03^{a}
	60	7.54 ± 0.10^{a}	7.43 ± 0.04^{a}
pH	1	5.40 ± 0.04^{a}	5.37 ± 0.005^{a}
	15	4.87 ± 0.01^{a}	4.96 ± 0.02^{b}
	30	5.03 ± 0.015^{a}	4.91 ± 0.01^{b}
	45	5.15 ± 0.01^{a}	5.12 ± 0.005^{a}
	60	5.11 ± 0.01^{a}	5.04 ± 0.01^{b}

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance.

Moisture is the major component of cheese which acts as a lubricant or plasticizer in the protein matrix thereby making it less elastic and more susceptible to fracture upon compression (Fox and McSweeney, 2017). Throughout the ripening days, cheese A and C showed a decreasing trend which is attributed to both the inherent propensity to dewhey and the effect of the drying environmental condition (Sanjuán *et al.*, 2002). A similar trend was also reported by Sousa and Malcata (1997) for ovine cheese, Sanjuán *et al.* (2002) for Los pedroches cheese, Roa *et al.* (1999) for La Serra cheese manufactured with either *Cynara cardunculus* or animal rennet. Cheese A had significantly lower moisture content ($P<0.05$) than that of cheese C throughout the ripening period of 60 days. Sanjuán *et al.* (2002) described that the lower moisture content in cheese made using plant rennet (*Cynara cardunculus)* might be due to the development of structure by vegetable rennet with milk component that allowed for less liquid retention.

Fat and protein in the cheese are major component that influence not only its physical properties, but mainly the development of the flavor (Fox and McSweeney, 2017). The mean total protein concentrations tended to increase during the ripening period. The obtained values of protein for cheeses A and C were higher than the ones found by Gummalla and Broadbent (1999) and Roa *et al.* (1999) in La Serena cheese manufactured with *C. cardunculus* and animal rennet during 3 months of ripening. The mean total protein concentration of cheese A was found to be significantly ($P<0.05$) higher than that of cheese B. Sanjuán *et al.* (2002) and Galán *et al.* (2008) both mentioned an increased protein level throughout the ripening time.

As ripening proceeds, the fat content of cheeses A and C showed an increasing trend. However, Sousa and Malcata (1997) reported decreased fat content for cheese made with *C. cardunculus* and animal rennet throughout ripening days. There was a significant difference ($P<0.05$) between cheeses A and C during the maturation period. Given that both cheeses were made from the same milk, there appears to be a higher ability for the animal rennet to capture fatty components in the curd.

Salt has three primary function in a cheese: preservation, nutrition and flavor (Guinee and Fox, 2017). The salt content of cheeses A and C increased throughout the 60 days of ripening, with a significant difference ($P<0.05$) on the initial day and 30^{th} day of ripening. The absorption of NaCl into the cheese, the impact of environmental factors, the pH of the cheese, the size of the grain when cut, and the initial moisture in the curd may all contribute to this rising tendency (Guinee and O'Kennedy, 2007). Similar observation was reported by Galán *et al.* (2008) who studied the influence of amount of vegetable coagulant and animal rennet on proteolysis of cheese made with sheep milk.

The ash in cheese is made up of salt, which is usually added to the curd, and the mineral substances produced from the sodium, potassium, calcium, and magnesium chlorides, phosphates, and citrates that make up the milk salts (Guinee and Fox, 2017). Both the cheese A and B had an increasing tendency in terms of ash content with a significant difference ($P<0.05$) on 15^{th} day of ripening. The increase in salt content in the cheese manufactured throughout the ripening process can be used to explain the rising level of ash as the cheese ages.

The pH value of cheese A decreased until 15th day of maturation then rose till 45th day and from then onwards it fell until the last day, but for cheese C, the pH dropped till 30th day of aging before showing the same trend as the previous one. Despite the fact that throughout the cheesemaking process, 98% of the lactose is eliminated as lactose or lactate in the whey (Huffman, 1985), the curd still retains 0.8% to 1.0% lactose (Turner and Thomas, 1980) , which is utilized by starter culture and produce lactic acid and CO_2 (McSweeney *et al.*, 2017) . Thus, the declined in the pH content during the initial days of ripening of cheeses can be explained by production of lactic acid due to microbial fermentation of residual lactose present in the curd. The starter and non-starter microbiota present in curd metabolized lactic acid and contribute to proteolysis through their proteinase activity or peptidolytic potential, which raises pH by releasing ammonia as a result of amino acid catabolism (Curtin and McSweeney, 2004; McSweeney *et al.*, 2017). Significant difference ($P<0.05$) was observed in both cheeses with the value lower for cheese C which might be due to higher whey retention in chymosin cheese allowed more lactose to be available for microbial fermentation.

4.5.2 Yield of cheeses

The yields of the cheese prepared using actinidin and chymosin were found to be 382.21 g (12.74%) and 393.90 g (13.13%), respectively. The higher moisture content of chymosin cheese may be responsible to higher percentage of yield. Cheese produced with plant protease have less yield due to rapid proteolysis, leading to the loss of large amount of protein, fat, and moisture during synersis. These differences in yield can be related to process of cheesemaking and the proteolytic activity of enzyme. The yield of cheese A and B is shown in the Table 4.6.

Table 4.6 % yield of cheeses prepared from actinidin and chymosin

Yield %		Reference
Actinidin cheese	Chymosin cheese	
8.93	10.47	Karki and Ojha (2018)
13.83	17.51	Shrestha (2023)
12.17	13.58	Maskey and Karki (2022)
19	23.16	Sharma and Vaidya (2018)
17.8	20.2	Mazorra-Manzano *et al.* (2013)
12.74	13.13	Present study

Similar observation in cheese yield was reported by Maskey and Karki (2022). However, higher yield was observed in Mazorra-Manzano *et al.* (2013) and Sharma and Vaidya (2018) which might be due to difference in milk composition and processing techniques.

4.6 Proteolysis of cheese during ripening

The contribution of actinidin, chymosin and starter bacteria to proteolysis was calculated statistically using the three ripening indicators (WSN, TCA-SN and PTA-SN). The changes of these indicators as cheeses matured is depicted in Table 4.7.

Table 4.7 Changes in nitrogen fraction in actinidin and chymosin cheeses during ripening

Parameters	Ripening days	Cheese A	Cheese C
TN	1	6.16 ± 0.03^{a}	6.06 ± 0.04^{b}
	15	6.32 ± 0.03^{a}	6.25 ± 0.05^{a}
	30	6.52 ± 0.01^{a}	6.79 ± 0.04^{b}
	45	6.97 ± 0.01^{a}	7.06 ± 0.04^{b}
	60	7.80 ± 0.03^{a}	7.68 ± 0.05^{b}
WSN (%TN)	1	6.10 ± 0.96^{a}	4.31 ± 0.57^{a}
	15	9.83 ± 0.25^{a}	7.01 ± 0.76^{b}
	30	12.10 ± 0.39^{a}	11.34 ± 0.34^{b}
	45	13.64 ± 1.35^{a}	12.57 ± 0.51^{b}
	60	14.91 ± 0.68^{a}	13.06 ± 0.83^{b}
TCA-SN (%TN)	1	1.54 ± 0.45^{a}	1.41 ± 0.52^{a}
	15	5.52 ± 2.66^{a}	3.43 ± 1.62^{b}
	30	6.41 ± 2.66^{a}	4.38 ± 1.70^{b}
	45	8.40 ± 1.22^{a}	6.50 ± 0.38^{b}
	60	9.33 ± 0.50^{a}	8.45 ± 0.50^{b}
PTA-SN (%TN)	1	0.82 ± 0.74^{a}	0.92 ± 0.50^{a}
	15	3.39 ± 0.22^{a}	2.42 ± 1.41^{b}
	30	3.79 ± 0.82^{a}	3.16 ± 0.57^{b}
	45	4.04 ± 1.64^{a}	3.25 ± 0.5^{b}
	60	4.16 ± 0.88^{a}	3.71 ± 0.20^{b}

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance.

The amount of WSN (%TN) has been used as a ripening index for cheese as it measures the degree of casein hydrolysis caused by the action of coagulant and milk proteases present at the start of ripening (Şengül *et al.*, 2014). The WSN (%TN) of cheeses A and C increased as the cheese ripened with a value significantly ($P<0.05$) higher for actinidin after 15 days of ripening.

Visser (1977) mentioned that soluble nitrogen is mainly produced by the action of rennet, starter bacteria and plasmin. Since same pasteurized milk and starter microflora were used to prepare both cheeses, it appears that the greater levels of WSN (%TN) in cheese A are solely attributable to actinidin's potent proteolytic effect. The results were consistent with those found in La Serena cheese made using *cynara cardunculus* as a coagulant as reported by Del Pozo *et al.* (1988) and Nuñez *et al.* (1991). Further, Sousa and Malcata (1997) showed significantly higher WSN values for plant coagulated ovine cheese when comparing the proteolysis of animal rennet.

TCA-SN (%TN) has been used as a measure of ripening depth (Şengül *et al.*, 2014). This fraction, which has peptides with 2 to 20 amino acid residues, is used to assess how lactic acid bacteria contribute to the synthesis of soluble nitrogen compounds in cheese (Furtado and Partridge, 1988). Cheeses A and C showed an upward trend throughout the course of 60 days of ripening with significant difference ($P<0.05$) between them after 15 days onwards. The increase in TCA-SN (%TN) was more pronounced at the beginning and was higher in cheese A. Starter and non-starter proteinases and peptidases play a major role in the synthesis of short peptides and free amino acids (O'Keeffe, 1984; Singh *et al.*, 1995). The increased proteolytic activity of actinidin results in higher SN content in cheese prepared with it. This suggest that actinidin has more access to substrates i.e., casein polypeptides, allowing it to produce more low molecular weight nitrogen than animal rennet. The result obtained was in agreement with the results reported by Nuñez *et al.* (1991) and Fernández-Salguero and Sanjuán (1999) for cheeses prepared with *cynara cardunculus.*

PTA-SN (%TN), which comprises low molecular weight and free amino acid (Çakır and Çakmakçı, 2018), increased over the 60 days ripening period and a significant difference ($P<0.05$) was observed in cheese A and C after 15 days of ripening. Since residual rennet had little effect on peptides with a small number of amino acid residues, the enzymes from starter culture are responsible for the production of nitrogen compounds soluble in 5% PTA (Pereira *et al.*, 2008). Given that the same starter culture was employed for both cheeses, the actinidin cheese's greater value for PTA-SN (%TN) throughout the ripening period may have resulted from the availability of more substrate for microflora, as seen by the higher WSN value from Table 4.7.

4.7 Changes in free amino acid during ripening

Free amino acids are associated with the volatile fraction of cheese because they are the precursors of many volatile aroma compounds (amines, carboxylic acids, thiols, esters, alcohols, aldehydes, and thioesters) (Mei *et al.*, 2015). Certain FAAs play crucial roles in flavor development. Arginine is related to bitterness, while proline, serine are related to sweetness (Izco and Torre, 2000). Fig. 4.2 shows the level of free amino acid produced in cheese A and C throughout the 60 days of ripening period. An increasing trend as ripening proceeds was observed for both cheeses with a value significantly ($P<0.05$) higher for cheese A after 15 days of ripening. For both cheeses, the value increased rapidly till 15 days of maturation while after that a slow increase in FAA was observed till the end of maturation day. This result can be corelated with PTA-SN (%TN) in the Table 4.7. A similar trend was observed by Francisco-José *et al.* (2010) in Spanish soft cheese prepared with raw ewe milk and vegetable rennet.

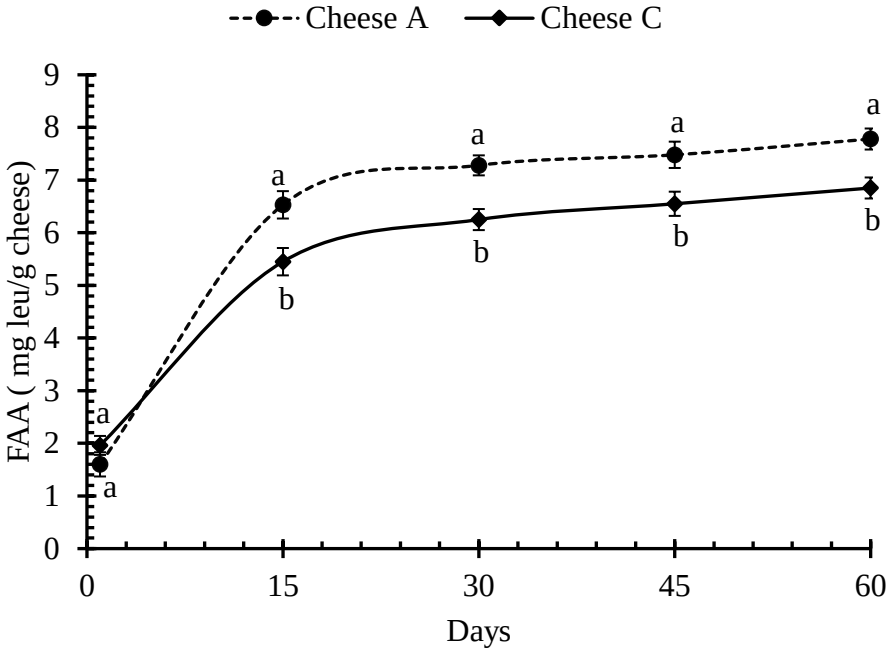

Fig. 4.2 Free amino acid of actinidin and chymosin cheeses throughout 60 days of ripening. Data represents mean ± standard deviation different superscript are significant different at 5% level of significance

4.8 Changes in free fatty acid during ripening

The lipolytic agents in cheese are lipolytic enzymes found naturally in milk (milk lipase), chymosin (pregastric esterases) and microflora. Fat degradation and oxidation during cheese ripening significantly impact quality. Endogenous milk enzymes and starters hydrolyze triglycerides in milk into FFAs (Collins *et al.*, 2003). The total FFAs of cheeses A and C increased during the ripening period with significant difference ($P<0.05$) between two cheeses after 30 days. Similar observation was reported by Yasar and Guzeler (2011) for Kashar cheese using chymosin and *Cryphonectrica parasitica* protease as coagulants. Acid degree value (ADV) is used to characterize the degree of lipolysis in cheese (Litopoulou-Tzanetaki and Tzanetakis, 2011). The higher values of cheese C throughout the ripening period might be due to the use of chymosin as coagulant which have high lipolytic activity due to their contents of pregastric esterases (PEG). The significant proteolytic activity of actinidin could be responsible for for the partial inactivation of native milk lipase, resulting in less fat hydrolysis. The result obtained was in agreement with the results reported by Nuñez *et al.* (1991) for cheese prepared with *Cynara cardunculus* flower extract. The changes in free fatty acid of actinidin and chymosin cheese during the maturation is shown in the Fig. 4.3.

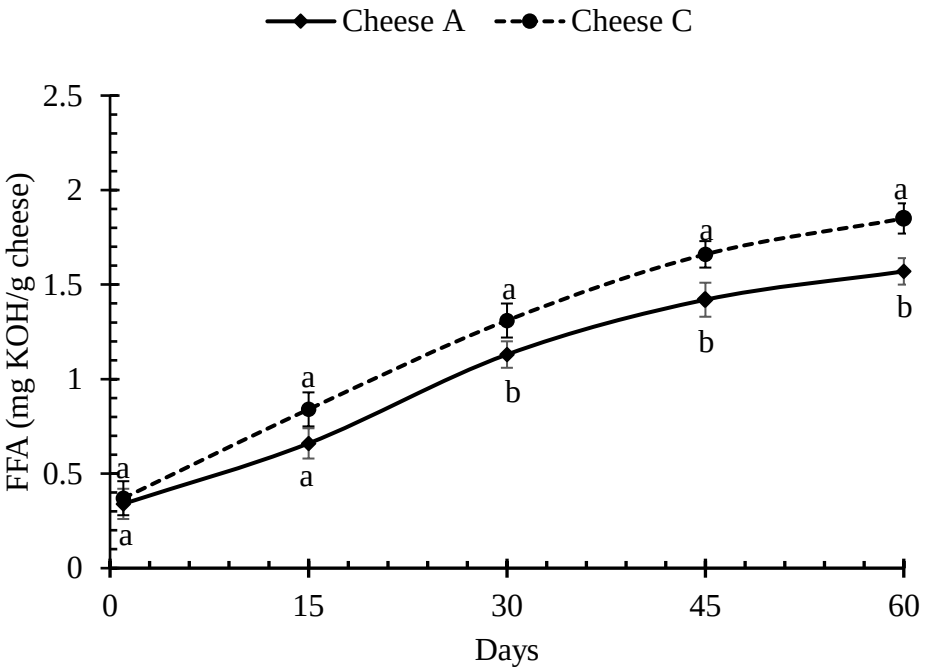

Fig. 4.3 Free fatty acid acid of actinidin and chymosin cheeses throughout 60 days of ripening. Data represents mean ± standard deviation and different superscript are significant different at 5% level of significance

4.9 Microbiological changes during ripening

Cheese has a complex microbiological ecology that is made up of bacteria, yeasts, and molds which is influenced by cheesemaking and ripening techniques, milk quality, water activity, salting techniques, and the overall chemical makeup of finished cheese (McSweeney *et al.*, 2017). The average numbers log cfu/g of total viable counts on PCA, yeasts and molds on PDA, *Lactobacillus* on MRS and *Streptococcus* on M17 agar for cheeses A and C are tabulated in Table 4.8.

Table 4.8 Log count per gram cheese sample of different microbial groups during ripening

Microflora	Ripening days	Cheese A	Cheese C
Total viable count	1	7.68 ± 0.02^{a}	6.78 ± 0.05^{b}
(Log CFU/g)	15	7.84 ± 0.04^{a}	6.95 ± 0.04^{b}
	30	9.43 ± 0.23^{a}	8.04 ± 0.07^{b}
	45	8.78 ± 0.20^{a}	7.91 ± 0.04^{b}
	60	8.61 ± 0.09^{a}	7.81 ± 0.05^{b}
Yeast and mold	1	4.30 ± 0.14^{a}	4.51 ± 0.08^{a}
(Log CFU/g)	15	5.57 ± 0.09^{a}	5.86 ± 0.05^{b}
	30	6.65 ± 0.05^{a}	6.62 ± 0.09^{a}
	45	6.68 ± 0.14^{a}	6.65 ± 0.12^{a}
	60	6.69 ± 0.04^{a}	6.75 ± 0.20^{a}
Lactobacillus	1	5.95 ± 0.05^{a}	5.34 ± 0.05^{b}
(Log CFU/g)	15	6.17 ± 0.19^{a}	5.96 ± 0.02^{a}
	30	7.99 ± 0.01^{a}	7.21 ± 0.02^{b}
	45	8.92 ± 0.02^{a}	8.24 ± 0.10^{b}
	60	8.56 ± 0.02^{a}	7.88 ± 0.01^{b}
Streptococcus	1	4.22 ± 0.03^{a}	4.68 ± 0.03^{b}
(Log CFU/g)	15	5.32 ± 0.02^{a}	5.97 ± 0.02^{b}
	30	7.05 ± 0.08^{a}	7.32 ± 0.02^{b}
	45	6.93 ± 0.04^{a}	6.96 ± 0.03^{a}
	60	5.75 ± 0.03^{a}	4.43 ± 0.13^{b}

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance

Cheese A had almost 1 log unit significantly ($P<0.05$) higher total viable count than cheese C throughout the ripening days, with the greatest value at 30^{th} days of maturation for both after which a decline in viable count was noticed till the end. The decrease might be caused by the

low moisture content and high salt content of the ripened cheese, which would account for the death of certain microbial groups. Moreover, higher temperature (55˚C) for coagulation of actinidin than chymosin may have favored the microbial growth initially. Nuñez *et al.* (1991) also noted considerably higher microbiological counts in La Serena and Los Pedroches cheese made with C. *cardunculus* in comparison to those prepared with chymosin, which was in agreement with this findings. Fernández-Salguero and Sanjuán (1999) also mention higher total viable count in cheese manufactured with cardoon aqueous extract. Coliform were not present in the cheeses indicating high sanitary quality of the prepared cheese.

Cheese A had higher yeast and mold counts than cheese C throughout the maturation period with a significant difference ($P<0.05$) on 15^{th} day. Utilization of the organic acid generated by lactic acid bacteria, which in turn caused an elevation in pH could be the cause of the growth in yeast and mold (De Llano *et al.*, 1992).

Cheese A had significantly ($P<0.05$) higher *lactobacilli* count than cheese C throughout the ripening period. The highest count of *lactobacilli* was encountered during 45 days of ripening after which a slightly decrease in count was noticed. According to Gobbetti *et al.* (2007), the fact that lactobacilli can survive in a variety of environments, including pH, low moisture, salt, and a variety of temperatures could be the reason for increase in viable count of *lactobacillus* in both types of cheeses. Cheese C had significantly ($P<0.05$) higher *Streptococcus* count than cheese A during maturation period.

4.10 Sensory analysis of cheeses during ripening

The sensory evaluation of cheese made with actinidin and chymosin throughout 60 days of ripening is shown in the Table 4.9.

Table 4.9 Changes of sensorial properties in actinidin and chymosin cheeses during ripening

Parameters	Days	Cheeses	
		A	C
Texture	1	4.1 ± 0.54^{a}	4.7 ± 0.46^{b}
	15	3.14 ± 0.35^{a}	4.57 ± 0.49^{b}
	30	3 ± 0.76^{a}	4.42 ± 0.49^{b}
	45	2.85 ± 0.35^{a}	4.14 ± 0.64^{b}
	60	2.57 ± 0.49^{a}	4 ± 0.75^{b}
Appearance	1	4.2 ± 0.4^{a}	4.6 ± 0.49^{a}
	15	4.14 ± 0.64^{a}	4.43 ± 0.49^{a}
	30	3.71 ± 0.45^{a}	4.28 ± 0.45^{b}
	45	3.28 ± 0.45^{a}	4 ± 0.53^{b}
	60	3 ± 0.53^{a}	3.86 ± 0.64^{b}
Flavor	1	4.3 ± 0.64^{a}	4.5 ± 0.5^{a}
	15	4.14 ± 0.35^{a}	4.42 ± 0.49^{a}
	30	3.28 ± 0.45^{a}	4.28 ± 0.88^{b}
	45	2.28 ± 0.45^{a}	4 ± 0.53^{b}
	60	1.85 ± 0.35^{a}	3.85 ± 0.84^{b}
Odor	1	4.4 ± 0.49^{a}	4.5 ± 0.5^{a}
	15	4.28 ± 0.45^{a}	4.43 ± 0.49^{a}
	30	3.71 ± 0.45^{a}	4.27 ± 0.45^{b}
	45	3 ± 0.76^{a}	4 ± 0.53^{b}
	60	2.85 ± 0.64^{a}	3.86 ± 0.64^{b}
Aftertaste	1	4.3 ± 0.46^{a}	4.6 ± 0.49^{a}
	15	4.14 ± 0.64^{a}	4.43 ± 0.49^{a}
	30	3.28 ± 0.45^{a}	4.27 ± 0.45^{b}
	45	2.43 ± 0.49^{a}	4.14 ± 0.84^{b}
	60	1.86 ± 0.35^{a}	4 ± 0.76^{b}
Overall	1	4.3 ± 0.46^{a}	4.6 ± 0.49^{a}
	15	4 ± 0.53^{a}	4.42 ± 0.48^{a}

30	3.14 ± 0.83^{a}	4.28 ± 0.45^{b}
45	2.57 ± 0.49^{a}	4.14 ± 0.35^{b}
60	1.86 ± 083^{a}	4 ± 0.53^{b}

Note. Values are means ± standard deviation of three determinations. Values in rows having different superscript are significant different at 5% level of significance

The texture, appearance, flavor, odor, aftertaste and overall acceptance of cheeses A and C were affected by the ripening period. The mean score for texture, appearance, flavor, texture and aftertaste decreased during ripening period. Similar observation was reported by Yasar and Guzeler (2011) who studied the effects of calf rennet, protease from *Rhizomucor miehei* and *Cryphonectria parasitica* on the organoleptic properties of Kashar cheese made from cow milk. As ripening proceeds, the residual coagulant and microflora present in cheese act upon cheese component (protein, fat) and causes their hydrolysis thereby contributing to cheese flavor, texture and appearance. Proteolytic activity is higher in vegetable coagulant than chymosin (Pino *et al.*, 2009), that might be the reason for significant difference ($P<0.05$) between cheeses A and C.

The texture score of cheeses A and C decreased during ripening and a significant difference ($P< 0.05$) was observed in both cheeses throughout the ripening period. In relation to attributes hardness, cheese A was softer than cheese C. The higher proteolytic activity in the breakdown of caseins and the first degradations products in cheese made with vegetable coagulant led to a softer texture (Tejada, 2001). Similar observation was reported by who studied the effect of *Cyanara cardunculus* L. and animal rennet on sensory characteristics of La Serena cheese made from Merino ewe's milk.

The mean score for appearance attribute of cheeses A and C decreased during ripening with significant difference($P<0.05$) after 30 days. A slight increase in yellowish color was seen in cheese A as ripening proceeds which was probably due to the higher proteolysis produced in the vegetable coagulant (Tejada, 2001).

The mean score for flavor attribute of cheese A and C decreased during ripening and a significant difference ($P<0.05$) was observed after 30 days. Similar observation was reported by Topçu and Saldamli (2006) who studied the sensorial changes during ripening of Turkish White Cheese made of pasteurized cow milk. An increase in a salty taste was observed during the ripening of goat's milk cheese (Gaborit *et al.*, 2001). A bitter taste was observed in actinidin cheese after 30 days of ageing which might be due to bitter peptides that contain more hydrophobic amino acid residues (Singh *et al.*, 2003).

In terms of odor, cheeses A and C was milky in odor but as the ripening proceed a slight increase in acidic odor was observed. The mean score for odor attribute of both cheeses decreased during ripening with significant difference ($P<0.05$) after 30 days.

Aftertaste is very important attribute since it determines consumer preference. The mean score for aftertaste of cheese A and B decreased during ripening with a significant difference ($P<0.05$) after 30 days. A bitter aftertaste was observed in actinidin cheese after 30 days.

The mean sensory scores for overall acceptability of cheeses A and C decreased as ripening proceed to 60 days and a significant difference ($P<0.05$) was observed between two cheeses after 30 days. The mean sensory score was found to be highest for chymosin cheese and lowest for actinidin cheese throughout the ripening days. Nuñez *et al.* (1991) concluded that using vegetable rennet (*Cyanara cardunculus* L) performed significantly better than animal rennet in the manufacture and ripening of Merino ewes milk cheese whereas our findings were not in line with this statement. Since, the mean sensory score for overall acceptability of actinidin cheese dropped to less than 3 after 30 days which indicated poor quality. Also, a bitter flavor and aftertaste was developed after 30 days. Hence, actinidin cheese should be consumed within 30 days after its production.

PART V

Conclusions and recommendations

5.1 Conclusions

On the basis of this research, the followings can be drawn:

1. The optimum condition for maximum milk clotting activity (MCA) for three phase partitioning (TPP) purified kiwifruit protease was 636.045 U/ml at pH 6.25 and temperature 55ºC.
2. The chemical parameters (protein, fat, salt and ash) increased whereas the moisture content and pH of both cheeses decreased throughout the ripening period of 60 days.
3. The yield of chymosin cheese was higher than that of actinidin cheese.
4. The content of water souble nitrogen, 12% trichloroacetic acid-soluble nitrogen and 5% phosphotungstic acid-soluble nitrogen increased during the ripening period of both cheeses with the value significantly ($P<0.05$) higher for actinidin cheese.
5. The free amino acid increases with increase in ripening period with significantly ($P<0.05$) higher value for actinidin cheese after 15 days.
6. The total free fatty acid of both cheeses increased with lipolytic activity significantly ($P<0.05$) lower for actinidin cheese.
7. The total viable count and *Lactobacillus* count of actinidin cheese were significantly ($P<0.05$) higher than chymosin cheese.
8. The sensory analysis of both cheeses were significantly ($P<0.05$) different in appearance, flavor, odor, aftertaste and overall acceptability after 30 days of ripening.

5.2 Recommendations

Based on the current study the following recommendations can be made

1. Actinidin cheese should be consumed within 30 days after production.
2. The kiwifruit protease may be purified using chromatographic techniques and its effect on properties of cheese during ripening could be studied.

Part VI

Summary

The primary objective of this research work is to study the ripening characteristics of the cheese prepared from three phase partitioning (TPP) purified kiwifruit (*Actinidia deliciosa*) protease and chymosin. The optimized condition of the actinidin protease for maximum milk clotting activity (MCA) was determined using response surface methodology. The prepared cheeses were ripened at 8-10ºC and relative humidity of 80-85 % for 60 days. The ripened cheeses were analyzed for physiochemical, proteolysis, free amino acid, lipolysis, microbiological and sensorial characteristics with the interval of 15 days.

The present work revealed that the TPP purified kiwifruit protease showed optimum temperature at 55ºC and pH 6.25 which gave maximum MCA (636.045 U/ml). Actinidin cheese had lower moisture content and higher pH than chymosin cheese throughout the ripening period. The fat and protein content of both cheeses showed an increasing trends as ripening proceeds with protein content higher for actinidin cheese. However, the fat content was higher for chymosin cheese. Both cheeses had an increasing tendency in terms of salt and ash content with the value higher for actinidin cheese. Actinidin cheese had significantly ($P<0.05$) higher proteolytical activity. Cheese prepared with actinidin had significantly ($P<0.05$) higher free amino acid after 15 days than that prepared with Chymosin. Actinidin cheese had significantly ($P<0.05$) lower lipolytic activity than chymosin cheese. The total viable counts and *Lactobacillus* counts were significantly ($P<0.05$) higher in actinidin cheese. Coliforms were not detected in both cheeses.

Significant difference ($P<0.05$) in sensory attributes including appearance, flavor, odor, aftertaste and overall acceptability were observed between actinidin and chymosin cheese after 30 days of ripening. However, texture showed significant difference ($P<0.05$) from the initial day of ripening. This study showed that a bitter flavor and aftertaste was developed rapidly in actinidin cheese after 30 days of ripening. Hence, it is best to consume the actinidin cheese within a month.

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Appendices

Appendix A

Table A.1 Model summary statistics for milk clotting activity (MCA)

Source	Std.dev	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	79.01	0.8686	0.8424	0.7400	123600	
2FI	58.16	0.9360	0.9146	0.8310	80332.06	
Quadratic	**41.14**	**0.9751**	**0.9573**	**0.8837**	**55248.77**	**suggested**
Cubic	34.84	0.9872	0.9694	0.8695	62041.34	Aliased

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

Source	Sum of square	df	Mean square	F-value	P-value	
Model		5	92680.81	54.76	< 0.0001	significant
A	463400	1	282200	165.54	< 0.0001	
B	280200	1	132600	78.37	< 0.0001	
AB	31992.44	1	31992.44	18.90	0.0034	
A^2	17908.30	1	1790.38	10.58	0.0140	
B^2	1881.66	1	1881.66	1.11	0.3267	
Residual	11847.45	7	1692.49			
Lack of fit	6620.96	3	2206.99	1.69	0.3058	not significant
Pure error	5226.49	4	1306.62			
Core total	475300	12				

Appendix B

Table B.1 Solutions of optimization results

Number	pH	Temperature (ºC)	MCA (U/ml)	Desirability	
1	**6.250**	**55.000**	**636.045**	**0.815**	**Selected**
2	6.242	55.000	644.700	0.810	
3	6.211	55.000	678.410	0.783	

Appendix C

Sensory Evaluation Card

Date _____

Name_____

Product: Cheese

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

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

Samples	Parameters					
	Texture	Appearance	Flavor	Odor	Aftertaste	Overall
A						
B						

Comments (if any) _____

Table C.1 T-test: Two-sample assuming equal variances for texture

	Variable 1	Variable 2
Mean	4.1	4.7
Variance	0.322222	0.233333
Observations	10	10
Pooled Variance	0.277778	
Hypothesized Mean Difference	0	
Df	18	
t Stat	-2.54558	
P(T<=t) one-tail	0.010143	
t Critical one-tail	1.734064	
P(T<=t) two-tail	0.020286	
t Critical two-tail	2.100922	

Color Plates

P.1 Cutting cheese coagulum

P.2 Actinidin cheese

P.3 Protein determination by Kjeldahl

P.4 Sensory analysis of cheese