HETEROLOGOUS PRODUCTION OF RECOMBINANT BOVINE CHYMOSIN FOR CHEESE PROCESSING

A thesis submitted to the University of the Punjab, Lahore,

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(Biochemistry)

Submitted by

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بسم الله الرحمن الرحيم

SO,WHICH OF THE BOUNTIES OF YOUR LORD WILL YOU DENY?

APPROVAL SHEET

Certified that the contents and form of thesis entitled **"Heterologous production of recombinant bovine chymosin for cheese processing"** submitted by **"Tasuduq Yaqoob"** Roll No. **"M. Phil-BS03F21"** has been found satisfactory for the requirements of the degree of Master of Philosophy in Biological Sciences.

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Dated: ----------

DEDICATION

I humbly dedicate my dissertation,

To my loving mother, caring father, and beautiful family,

Without whom I am nothing.

You have been the pillars of strength,

Empowering me to seize this opportunity,

Guiding me through every step,

And providing unwavering support along my academic journey.

This achievement is a testament to your belief in me,

And I am forever grateful for your love, sacrifices, and unwavering presence.

With deepest gratitude and boundless love,

I dedicate this dissertation to you,

As a token of my appreciation and enduring admiration.

DECLARATION OF ORIGINALITY

I, Tasuduq Yaqoob hereby certify that this M.Phil. thesis “Heterologous production of recombinant bovine chymosin for cheese processing” is original research. Its content was not already submitted as a whole or in parts for the requirement of any other degree and is not currently being submitted for any other degree or qualification. To the best of my knowledge, the thesis does not contain any material published or written previously by another author, except where due references were made to the source in the text of the thesis.

It is further certified that help received in developing the thesis and all resources used for the purpose, have been duly acknowledged at the appropriate places.

TASUDUQ YAQOOB

MPHIL-BS03F21

Session (2021-2023)

DECLARATION BY SUPERVISOR

It is to certify that the research work described in the MPhil thesis "Heterologous production of recombinant bovine chymosin for cheese processing" is an original work of the author. It has been carried out under my direct supervision. I have personally gone through all the data, contents, and results reported in the manuscript and certify its correctness and authenticity.

I further certify that the thesis has been compiled under my supervision and material included in the thesis has not been used partially or fully, in any manuscript already submitted or is in the process of submission in partial or complete fulfillment of the award of any other degree from any other institution. I, therefore, endorse its worth for the award of an MPhil degree by the prescribed procedure of the university.

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ABSTRACT

The production of cheese, a tradition dating back thousands of years, heavily relies on the use of chymosin, an essential enzyme responsible for milk protein coagulation. Traditionally sourced from the abomasum of young ruminant animals, chymosin extraction has been labor-intensive, expensive, and subject to supply fluctuations. Advancements in biotechnology and genetic engineering have opened new possibilities for cheese production through recombinant DNA technology. This research work explores the realm of heterologous production of recombinant bovine chymosin, aiming to provide a cost-effective and sustainable solution for the cheese industry. By employing *Escherichia coli* expression system, the chymosin gene is cloned, expressed, and purified, offering a scalable and independent source of the enzyme. This research investigates the downstream application of recombinant bovine chymosin in cheese processing, presenting a promising alternative to conventional milk coagulants for the local cheese industry in Pakistan.

KEYWORDS: *Chymosin; Genetic Engineering; Recombinant DNA Technology; Escherichia coli; Cheese*

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LIST OF ABBREVIATIONS AND SYMBOLS

β	Beta
µg	Microgram
µL	Microliter
µM	Micromolar
APS	Ammonium persulfate
BL21 DE3	B strain of *E. coli* having λDE3 lysogen
Bp	Base pairs
BSA	Bovine Serum albumin
C	Carboxyl
dNTPs	Deoxynucleotide triphosphates
ds DNA	Double-stranded Deoxyribonucleic acid
E .coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
F	Forward
g	Gram
Ibs	Inclusion bodies
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilo Daltons
L	Liter

LB	Luria Bertani
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mL	Mili liters
mM	Millimolar
NaCl	Sodium chloride
NCBI	National center for biotechnology information
^oC	Degree centigrade
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
pET	Plasmid expression vector having T7 promoter
PMSF	Phenyl methane sulfonyl fluoride
Pro	Proline
R	Reverse
RNA	Ribonucleic acid
rpm	Revolution per minute
S	Supernatant
SDS	Sodium dodecyle sulfate
Sec	Seconds
Ser	Serine

T7 p	T7 promotor
T7 t	T7 terminator
Taq	*Thermus aquaticus*
TEMED	Tetramethyl ethylenediamine
Tm	Melting temperature
Tris-Cl	Tris-chloride
UV	Ultraviolet
V	Volt
β-ME	Beta-mercaptoethanol
µ	Micro
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EtBr	Ethidium bromide
OD_{280}	Optical density at 280 nm
OD_{412}	Optical density at 412 nm
OD_{600}	Optical density at 600 nm
TAE	Tris-acetate EDTA

CHAPTER 1

INTRODUCTION AND REVIEW LITERATURE

1.1 Introduction

The process of cheese-making has been utilizing milk-clotting enzymes for over 8000 years (Andrén, 2011). These enzymes can be found in various sources such as plants, animals, or microbes (A Kumar et al., 2010). The most frequently used source is rennet extracted from the fourth stomach of a suckling calf (Mir Khan & Selamoglu, 2020). This rennet contains chymosin and pepsin and the ratio of these two enzymes is affected by the animal's age and diet (Britten & Giroux, 2022). Chymosin production is favored when the animal is young and fed with milk. On the other hand, enzymes can be produced through DNA recombinant techniques and chymosin is one of them which is highly specific and has the highest milk clotting activity (Andrén, 2021). Although the fresh extracts from the calf abomasa and dried extracts of the plants such as the *Cynara cardunculus* have been historically for cheese processing, the use of calf rennet became widespread in the 19th century when dairy farms started to produce a large quantity of milk for the production of cheese. Following the end of the 19th century, the rennet from calf was being produced on a larger scale and was being sold in the market with the standardized activity of the enzyme. However, the enzyme strength definition was based on the amount of milk clotting in a set time at a specific temperature varied between countries and producers. To improve precision, standardized milk powder was used instead of raw milk for testing. With a shortage of suckling calf abomasa, coagulants from various sources

were introduced including adult cattle abomasa extracts, microbial sources, from the stomach of the pig, and Fermentation Produced Chymosin (FPC) (Andrén, 2021).

1.2 Dairy importance of Cheese

As a solid food item, cheese is produced through the processing of milk, having a reduced amount of whey protein as well as casein in comparison to its source material milk. The composition comprises a variety of benign bacterial cultures that are capable of lactic acid production, along with substances that can modify the flavor and odor (García-Burgos et al., 2020). In addition to the aforementioned innocuous microbes, it is possible for other kinds of microorganisms that don't pose any danger to also be present, alongside appropriate enzymes such as glycosidases or proteinases (Furtado, 2022). Cheese is highly nutritious and plays a significant role in the diets of the European, American, and Asian populations. Cheese is an excellent source of many essential nutrients, including peptides, proteins, fatty acids in free form, essential amino acids as well as minerals, including calcium. In addition, cheese is packed with nutrients that are beneficial to one's health, including amino acids that are vital as well as fatty acids in free form (Al Katheeri et al., 2021). The nutritional profile of cheese can be affected by a variety of factors, such as the kind of milk that was used to make it, the length of time that it was allowed to lactate, the method that was used to ripen it, and the kind of starter that was used in the manufacturing process. It is possible to use regular milk in the production of cheese; however, the lactose levels in the finished product will be lower if the cheese is made with lactose-free milk (Picciotti et al., 2022). Certain cheeses are produced especially for people who are unable to digest lactose. This category of cheese includes, for instance, Gouda cheese. It is essential to keep in mind that consumption of cheese differs not only among individuals but also among races and ethnicities, as well as depending on

the kind of milk that is used in the production of cheese. There are many varieties of cheese, and different people have different preferences for each one (P. J. Dekker et al., 2019).

1.3 The Composition of Cheese and its Nutritional Value

Cheese is an excellent source of nutrients, including peptides, proteins, free fatty acids, essential amino acids, minerals and vitamins, and minerals. It is a very nutrient-dense diet (Ash & Wilbey, 2010). It contains several peptides and proteins that have been shown to have pharmacological properties (Galán et al., 2020). The substances are created not only during the ripening phase of cheese but also right through the process of digestion in the gastrointestinal tract of humans. Cheese ripening and digestion both result in the formation of these compounds. It has been shown that these peptides have beneficial effects on health, among which is *ACE*-inhibiting action, which assists in relaxing blood vessels and reducing blood pressure (Preedy et al., 2013). Cheese has several important peptide sources, including casein and whey proteins. The formation of free amino acids in cheese is the consequence of the activity of enzymes that are generated by both the starting culture and the rennet. However, the proportion of free amino acids in cheese might vary depending on the kind of rennet that was used and the origin of the milk that was used to make the cheese. When compared to milk proteins, the quantity of amino acids containing Sulphur is found in cheese peptides in considerably lower amounts (Yasmin et al., 2013).

1.3.1 Proteins and their Biological significance

Proteins are considered indispensable macronutrients due to their unique characteristic of being unable to remain stored in an inactive state within the human body. Fats have the ability to be stored within adipocytes, while carbohydrates can be

stored as glycogen (Ardö et al., 2017). However, proteins do not possess the capacity to be stored in a similar manner. Bovine milk, along with other varieties of milk, comprises two distinct categories of proteins, namely caseins and whey proteins. Caseins, lactoferrin, immunoglobulins, β lactoglobulin, and serum albumin are the major milk proteins that collectively exhibit diverse biological functionalities (Silva et al., 2016). During cheese production, there is a certain degree of protein loss, specifically proteins containing Sulphur, which takes place in the whey. Consequently, the nutritional value of these proteins is slightly diminished in comparison to the protein content found in milk (Preedy et al., 2013). However, the implementation of certain methodologies, such as ultrafiltration and the incorporation of whey proteins, can effectively mitigate the depletion of biologically active proteins in cheese. Bioactive peptides produced in cheese offer significant health benefits, including ACE inhibitory action, antihypertensive, antithrombosis, anticarcinogenic, opioid activity, immune stimulation, and antioxidant properties (Rafiq et al., 2021). These Bioactive peptides, containing proline and hydroxyproline, exhibit resistance to digestive enzymes and can modulate gut secretion, lower blood pressure, and act as antioxidants (Baptista & Gigante, 2021).

1.3.2 Fats and their Biological Significance

Dietary fat is an essential component in the production of cheese, playing a pivotal role in determining its characteristic flavor and texture. Cheese is known to contain various types of fats, including fatty that are saturated, and unsaturated fatty acids, and triglycerides. The lipid composition in cheese exhibits variability based on the specific animal species involved. Milk along with other dairy items and butter constitute approximately 20% of the overall fat intake (Yasmin et al., 2013). Nevertheless, the consumption of these products is associated with a notable increase

in saturated fatty acid content. This, in turn, has been strongly correlated with the development of cardiovascular diseases (CVDs), as lipoproteins have the ability to accumulate fat within the body (Ganesan et al., 2012). The correlation between the intake of cheese and its fat content and the development of cardiovascular diseases (CVDs) is primarily attributed to the influence on low-density lipoprotein (LDL) levels. However, establishing a direct causal relationship between high cheese fat intake and CVDs is challenging due to the influence of various factors like lifestyle, smoking, obesity, and other dietary choices (Ganesan et al., 2012).

1.3.3 Minerals and their Biological Significance

The mineral content in cheese is influenced by factors like cheese type, manufacturing procedures, coagulation methods, and salt quantity (Deshwal et al., 2023). The migration of minerals from the interior to the outer layer of the cheese is caused by the presence of a pH gradient. Cheese is a food product that contains a significant amount of minerals, among which are potassium, calcium, sodium chloride, phosphorus, and zinc (L. H. Dekker et al., 2019). These minerals play a crucial role in maintaining a balanced and healthy diet. Calcium, which is found in ample quantities in milk and cheese, is present in various forms including bound to casein, in colloidal as calcium phosphate, and in an ionic state (Stocco et al., 2021). Also, the incorporation of additional calcium chloride into milk increases the concentration of calcium in milk, thereby improving the cheese-making process through the reduction of clotting time and the increase in gel firmness. The bioavailability of calcium in cheese is significantly enhanced because of its complicated association with cheese peptides, thereby promoting efficient absorption within the gastrointestinal tract. The consumption of cheese has been found to contribute to the prevention of tooth caries by means of active peptides derived from

casein (Zhang et al., 2023). These peptides have the ability to inhibit bacteria and promote enhanced protection of the teeth. Whey, being a significant contributor to lactose content, is largely metabolized and eliminated, making it a valuable source of calcium for individuals with lactose intolerance. Sufficient consumption of calcium plays a crucial role in mitigating bone demineralization and osteoporosis, while potentially contributing to the reduction of blood pressure and facilitation of weight loss (Ganesan et al., 2012). The vitamin composition of cheese is subject to variability due to factors such as the vitamin content of the milk used, the microbial culture employed during production, and the conditions under which the cheese is matured. Vitamins A and D exhibit a higher degree of retention in curd, whereas other vitamins such as folate, niacin, B12, and riboflavin may be found in notable amounts, thereby exerting a significant nutritional influence. Certain vitamins may exhibit sensitivity to heat and consequently experience loss during the process of milk pasteurization. However, it is worth noting that lactic acid bacteria have the potential to contribute to the production of vitamins in cheese ("Nutritional aspects of cheese," 2007).

1.3.4 Probiotics and their Biological Significance

Probiotics are present in cheese and can provide health benefits by improving immune function, protecting the mucosa, reducing cholesterol, preventing diarrhea, relieving lactose intolerance symptoms, and exhibiting antimutagenic properties. Some probiotics naturally exist in raw milk used for cheese production, while others are added as starter cultures (Olajugbagbe et al., 2020). Lactic acid bacteria play a role in both the fermentation and maturation processes of cheese. Starter LAB (SLAB), including species like *Lactococcus lactis* and *Streptococcus thermophilus*, rapidly ferment lactose, while non-starter LAB (NSLAB), such as *Lactobacillus* species, contribute to cheese ripening. Consuming cheese with probiotics offers oral

health benefits, enhances the immune system, exhibits antimicrobial activity, and may reduce the risk of cancer (Preedy et al., 2013).

1.4 Chymosin

Chymosin with Enzyme Commission number 3.4.23.4 is a primary enzyme from class aspartyl proteinases and is found in the abomasum of newborn mammals. It has a very close resemblance to pepsin A which is the main protease found in grown-up mammals. These two proteases are produced in the stomach initially as an inactive precursor state known as prochymosin and become activated to chymosin through autocatalytic cleavage of the pro sequence when exposed to acid (A. Kumar et al., 2010). Chymosin is highly effective at coagulating milk and prolonging the retention of milk protein precipitates in the stomach allowing them to be adequately exposed to proteolytic enzymes. The main function of chymosin is to cause milk clotting by breaking the peptide bond between Phe105-Met106 in the κ-casein protein chain. This process is necessary to produce cheese which is a highly nutritious milk product also and it brings changes to the texture and enhances flavor during the cheese ripening process (Justesen et al., 2009). The current research suggests that chymosin might be employed in the manufacture of therapeutic proteins since it has the most promising features for the precise removal of fusion tags. However, as the bovine population declines and the need for cheese manufacturing rises, other animal species are being examined as potential sources of chymosin (Roller et al., 1994). Although microbial alternatives are available, they do not give the required flavors during the cheese ripening process. Nevertheless, the properties of microbial coagulants can be modified by exposure to some substances like H_2O_2 (Roseiro et al., 2003a). Plant-based proteolytic activity is also an option, but it can lead to extensive digestion of the curd which results in off flavors and impaired taste. So, these

problems impede the use of animal chymosin in the dairy industry and promote the search for alternatives. While there are various methods available for producing such substitutes such as recombinant DNA technology is usually the preferred to generate a technique to produce active chymosin that closely resembles its natural form (Akishev et al., 2023).

1.4.1 Nomenclature and History of Chymosin.

For centuries, people have used extracts from calf stomachs to clot milk in the process of cheese making. Depictions of milk processing can be observed in cave paintings from the Libyan Sahara dating back to 5500-2000 BC, as well as in Sumerian relief and stamp seals from 3500-2800 BC (Foltmann & Szecsi, 2004). Furthermore, traces of the cheese have also been discovered in clay pots originating from old Egyptian civilization, dating back to 3000-2800 BC (Foltmann, 1969). The first person to try to isolate the enzyme was Deschamps in 1840, who named it “chymosine” (derived from the Greek word chyme, meaning "(gastric) juice"). Later, Lea & Dickinson in 1890 suggested the name rennin (which comes from rennet), which was used for many years in English literature. However, this name was often confused with renin from the kidneys. Therefore, in 1970, Foltmann proposed returning to the original name, chymosin, which was subsequently adopted by the IUBMB (Foltmann, 1969). Other names for the enzyme include “pexin” (Warren, 1897) and “chymase" (Clan, 2012). Further, it was discovered that the enzyme was secreted in an inactive form, which could be activated through treatment with acids. He was the first to identify a proenzyme (Hammarsten, 1872). For a long time, it was believed that chymosin was only found in young ruminants. However, it was discovered that the enzyme like chymosin is present in many neonates. Therefore, the

enzymes like chymosin can be attributed as neonatal or fetal gastric proteinases found in mammals (Foltmann & Axelsen, 1980).

The craft of making cheese has been mentioned in ancient Greek mythology, and traces of cheese and cheesemaking have been discovered on Egyptian tomb murals that are more than 4000 years old. The accidental discovery of cheese may have resulted from storing milk in animal stomach containers (Kindstedt, 2012). The art of manufacturing cheese has evolved over time, with several cultures and geographical areas creating their own distinctive varieties. For instance, the French developed the soft and creamy Camembert cheese, while the Swiss developed the well-known Emmental cheese, recognized for its unique holes and nutty flavor (Kindstedt, 2017). Today, cheese is made all over the world, with various traditions and production methods unique to each nation and region. Making cheese is regarded as an art form in some nations, like Italy and France, where the milk is carefully chosen, and the cheese is aged to develop a distinctive flavor and texture (Licitra, 2010).

1.4.2 Physiological Significance of Chymosin

Chymosin has importance in the physiological and nutritional aspects of mammals. Chymosin, being a protease, has been the subject of research pertaining to the transmission of immunoglobulins after birth (A Kumar et al., 2010). Chymosin has undergone evolutionary changes to become a neonatal protease characterized by a significant milk clotting capability and relatively low overall proteolytic activity. This adaptation is crucial for mammals that rely on the postpartum transmission of immunoglobulins (Foltmann, 1992). The diminished overall proteolytic activity contributes to the substantial preservation of immunoglobulins, while the milk clotting activity encourages proper physiological functioning of developing stomachs.

Moreover, studies have shown that chymosin may also have other physiological functions beyond its role in milk digestion. For example, chymosin has been found to have immunomodulatory effects in vitro, suggesting a potential role in regulating the immune system. Additionally, chymosin has been found to interact with other proteins in the body, such as the enzyme elastase, indicating a possible involvement in other physiological processes (Foltmann & Axelsen, 1980).

1.4.3 Chymosin Chemistry

The aspartic protease enzyme family plays an important role in numerous metabolic processes and other aspects of human health. Calf rennet mostly consists of the enzyme chymosin. In chemical terms, it is an aspartic protease (Chitpinityol & Crabbe, 1998). Preprochymosin is the precursor form of chymosin that is synthesized in the animal stomach. It is basically a protein containing 381 amino acids in it. A sequence of 16 amino acids on the N-terminus acts as a leader signal sequence, regulating chymosin secretion across cell membranes. The pro-segments (pro sequence) that occur before the functional sections of enzymes play crucial functions in ensuring appropriate folding, targeting, and controlling zymogen activation (Koelsch et al., 1994). The sequence is made up of 42 amino acids. It has been known for a very long time that chymosin is secreted in an inactive form referred to as prochymosin (Foltmann, 1966; Foltmann, 1970; Fox et al., 2004). This form is made up of 365 amino acids, has a molecular weight of 40,777 Dalton, and has an N-terminal propeptide that prevents it from becoming active. Cleavage transforms prochymosin, which is initially inert from a catalytic standpoint, into two different active forms. The removal of the first 42 N-terminal residues at a pH of 4.2 results in the formation of one form of chymosin, which has a molecular weight of 35,600 Da, and a polypeptide chain that has 323 amino acids. This form of chymosin is an active

form of chymosin (Foltmann et al., 1979; Foltmann et al., 1977). The other active form, pseudo chymosin, contains 338 amino acids and is made by eliminating 27 amino acids at a pH of 2.0. Pseudochymosin has a molecular weight of 37,400 Da and is stable at pH 3.0 or higher; it is transformed into chymosin at pH 4.5 (BARKHOLT PEDERSEN et al., 1979). Although calf chymosin may not exhibit high levels of activity against proteins under near-neutral pH conditions commonly employed in cheese manufacturing, its specific activity targeting caseins is adequate for preserving the desired sensory attributes and textural characteristics of cheese. Both chymosin and pseudo chymosin exhibit milk clotting activity (McCaman et al., 1985).

Chymosin exists in two forms in nature: chymosin A and chymosin B (**Figure 1.1**). The second is more common than the first. These two versions differ at single position 244, with chymosin A containing an aspartate and chymosin B containing a glycine (Donnelly et al., 1986; Foltmann et al., 1977; A. Kumar et al., 2010). This discrepancy causes minor differences in electric charges, affecting the isoelectric point of these two isoforms. The specific activity of chymosin A is somewhat higher for κ-casein than chymosin B, although it is less stable as compared to chymosin B. The strong electrostatic attraction between the substrate and the chymosin A is mainly responsible for this increased activity, which increases its binding affinity to κ-casein. Chymosin C is a degraded derivative of chymosin A that lacks three D244-F246 residues (Danley & Geoghegan, 1988; Donnelly et al., 1986).

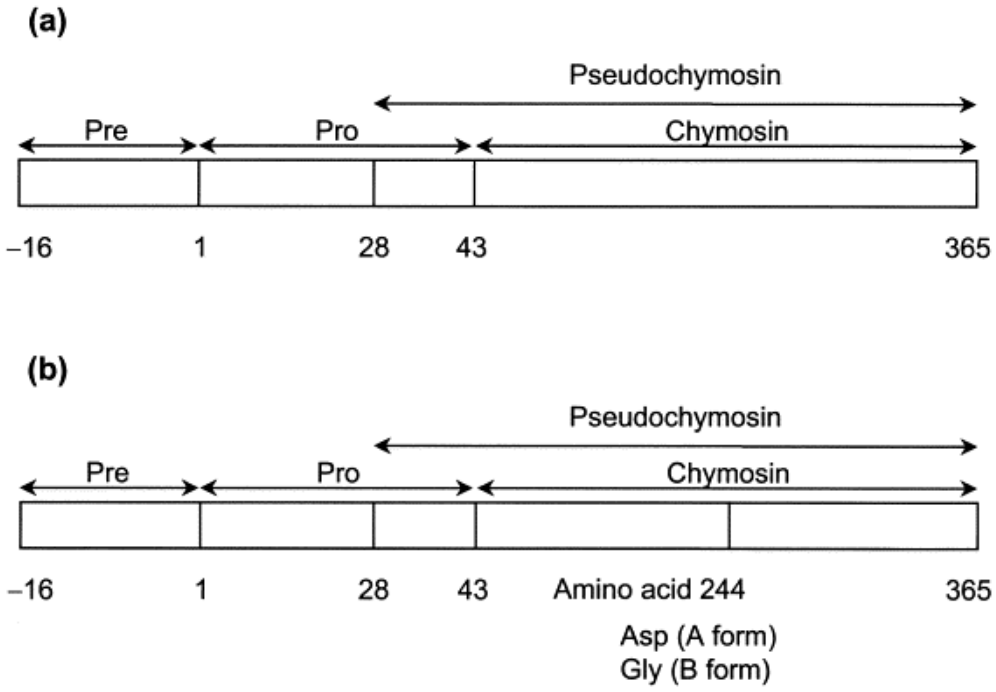

Figure 1.1 Isoform A & B of Chymosin

1.4.4 Chymosin Structure

Chymosin enzyme has kidney shaped bilobed structure with a long deep pocket inside its structure (**Figure 1.2**). There is a C-terminal domain and an N-terminal domain which are responsible for the similar shape and geometry of these folds and are stabilized by a unique network of hydrogen bonding (Tang et al., 1978). Aspartic acids 32 and 215 are the main catalytic residues and are separated by an extended deep cleft. This structure is responsible for the cleavage of peptides. The residues like serine 35, threonine (32,216,218), amino glycine 34, and 217 are the main catalytic residues that are responsible for the stabilization of the domains through hydrogen bonding. This structure is solely responsible for determining the state of ionization of the catalytic site of the enzyme (Pearl & Blundell, 1984). The examination of the structural properties of the family members was conducted using x-ray crystallography and Insilco graphics modeling. The findings revealed that the

aspartic acids at positions 32 and 215 come together through hydrogen bonding, creating the catalytic center that exhibits a localized two-fold symmetry. Aspartic acid present at position 303 interacts with the oxygen of carbonyl carbon of peptide bond in threonine 216 resulting in providing stability and maintaining the correct orientation of aspartic acid residues of the active site (Mantafounis & Pitts, 1990).

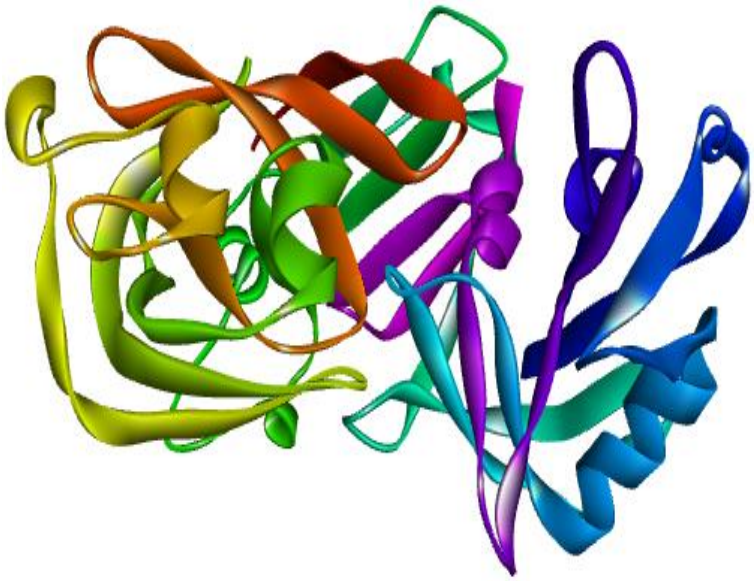

Figure 1.2 PDB Structure of Bovine Chymosin

Chymosin's secondary structure is chiefly composed of β-pleated sheets along with a very small proportion of α-helical structures. These β-strands are antiparallel, and they are arranged in three sheets, N-terminal domain contains sheets 1,2,3 and on the other hand, the C-terminal domain is composed of 7/8, 4, and 6 antiparallel beta strands respectively (Gilliland et al., 1990). The β-strands (N and C-terminal) of sheet two are present below the N and C-terminal β-strands of sheet one while on the other hand, the β-strands of sheet three are present under the active site pocket and involved in its base formation. There is the formation of two-fold symmetry is because of 4 helix structures which are present in inter-domain and an intra-domain manner, while

on the other hand, the β-strands in the N-terminal domain and a large portion C-terminal domain are formed by the 5 and 6 α-Helix structures (Newman et al., 1991). The two aspartic acid residues 32 and 215 are aligned towards each other at the catalytic site forming an interlobar pseudo-axis diad with a complex network of hydrogen bonding. The two amino acid residues set (31,32,33,34,35) and (214,215,216,217,218) along with the middle water molecule are central players in the formation of this extensive hydrogen bonding network. This active site is mainly involved in the recognition amino acid sequence of histidine 98 to lysine 111 present in milk protein (k-casein) (Gustchina et al., 1996). This long active site pocket consists of domains that are composed of amino acid residues ranging from 1-175 and 176-232. Both domains contain antiparallel and parallel beta strands. An amino acids envelope spanning from 75-85 surrounds the aforementioned domains, so this area contributes binding selectivity to the substrate. Chymosin can exist in two distinct structural states: an active one where the S1 and S3 binding pockets are available for binding to the substrate, while the other one is in a self-inhibited state where the binding pockets are blocked by the protein's native tyrosine 77 amino acid residue (Andreeva et al., 1992). Gilland and his colleagues found that the amino acid cluster of histidine and proline is involved in the conversion of this self-inhibitory form of chymosin into active chymosin (Kaye & Jollès, 1978). Newman stated the 2.3 angstrom and 2.2-angstrom resolution structures of recombinant chymosin, which showed three disulfide bridges are present between amino acid residue 45-50, 206-210, and 249-282. Along with disulfide linkages, there are also ion pairs present between arginine 59 and aspartate 57, arginine 157 and isoleucine 326, arginine 307 and aspartic acid 11, arginine 315 and aspartic acid 138 respectively (Gilliland et al., 1990; Newman et al., 1991).

1.4.5 Mechanism of action of chymosin

Milk is one of the most important, nutritious, and very complex biological liquids. It is composed of proteins, water, citrate, lactose, vitamins, and minerals like calcium phosphate, and iron. Milk from bovine and from other species like buffalo, camel, and goat contains proteins that are categorized into different protein groups such as caseins, non-casein (whey), and the other proteins that are associated with lipids. Casein belongs to the class of phosphoproteins that contributes 80% of nitrogen present in the milk of bovine and this protein is precipitate out in the form of micelles that are associated with calcium from skim milk at 20 °C and 4.6 pH. Casein is classified into four different proteins, α-S1, α-S2, β (beta), and κ (kappa) which present in 40:10:35:12 respectively (Fox et al., 2004). All four types are phosphorylated in nature, and they differ from each other based on slight differences in their binding affinity to calcium due to differences in the presence of different levels of phosphate contents in these proteins. The caseins from the class alpha (α-S1, α-S2) and beta (β) contain high levels of phosphate contents, and they are hydrophobic which is why they very quickly precipitate out by calcium. While on the other hand, κ-casein contains only a single phosphate molecule which is why it interacts with the calcium weakly, and it does not precipitate out in the presence of high levels of calcium. κ-casein can associate with the other three types of casein (α-S1, α-S2, β) molecules hydrophobically, which is why it can stand up to 10 times more concentration of calcium as compared with other types of casein (Fox, 1988). In milk, about 95% of caseins exist in soluble form because of the interaction of α and β-casein with κ-casein that keeps the major portion of milk protein in soluble form and prevents their aggregation into micelles which are naturally transferred from milk to curd (A Kumar et al., 2010).

The process of milk coagulation is a two-step process (**Figure 1.3**); the first phase which is called the proteolytic stage (primary phase) involves the production of insoluble para-κ-casein through enzymatic reactions. Chymosin is the main enzyme which involves in the conversion of soluble κ-casein into para-κ-casein which is insoluble and along with smaller soluble TCA peptides also called macro-peptides. The second phase is called the coagulation stage in which the coagulation of para-κ-casein occurs in the presence of calcium ions and high temperature up to 20 °C (P. Fox et al., 2017).

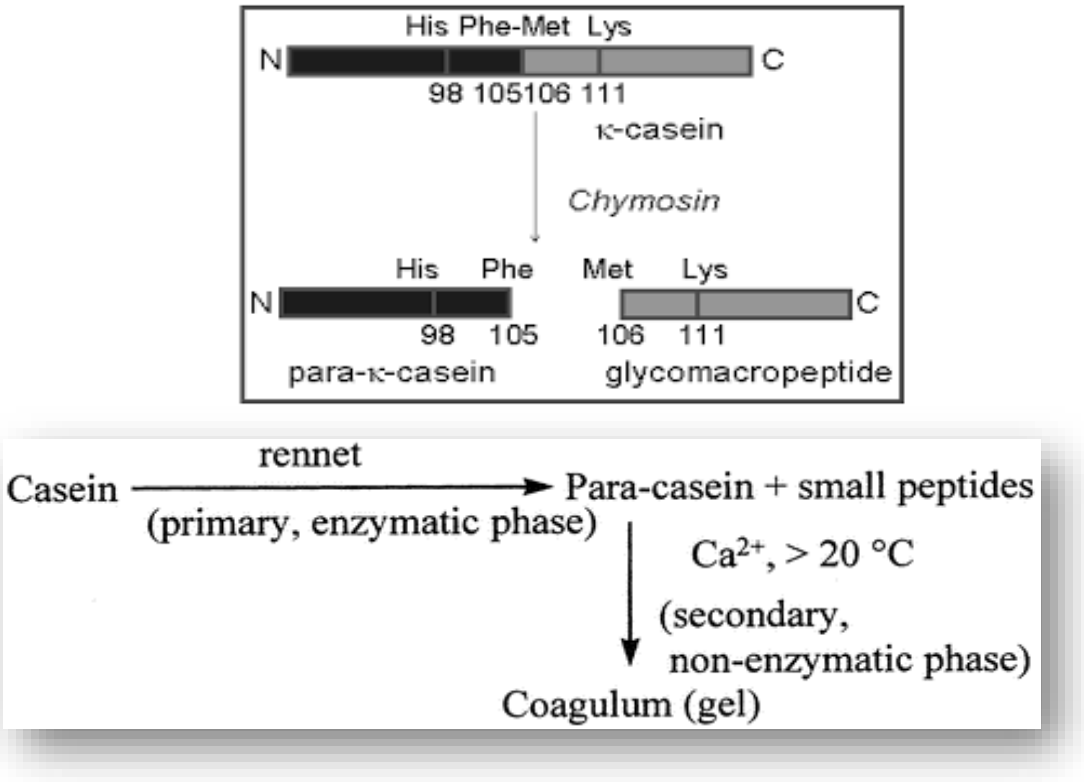

Figure 1.3 Mechanism of Action of Chymosin

If calcium is absent and then the insoluble para-κ-casein produced during the proteolytic stage interacts with other casein to prevent precipitation and vice versa (A Kumar et al., 2010; Mathur & Dutta, 1983). Several studies have revealed that the primary substrate for the chymosin enzyme is κ-casein, although it can also break

down the α and β-casein proteolytically (Herman et al., 2023; Vega-Hernández et al., 2004). It was also proposed that the amino acid residues that lie in the vicinity of the bond to be cleaved are also involved in this hydrolytic reaction. The enzymatic hydrolysis of κ-casein with chymosin enzyme occurs at pH 6.7 in the absence of calcium, so the rennin action is independent of calcium (Kato et al., 1980; Lucey & Fox, 1993). Chymosin attack on peptide bond present between phenylalanine 105 and methionine 106 to initiate milk clotting. This Phe-Met peptide is preferably attacked by these milk clotting acid proteinases than any other bond and this is quite interesting. The length of the peptide and the amino acid residues around this susceptible bond are also very important in the action of the enzyme because a dipeptide consisting of phenylalanine-methionine and even a tri- or tetrapeptides with Phe-Met bond are not hydrolyzed by chymosin enzyme (Fox, 1988; Salesse & Garnier, 1976). The kinetic studies performed on synthetic peptides have revealed that two amino acid residues on each side of this susceptible bond are also important in this reaction. The process of milk coagulation is affected by several variables, including pH, temperature, concentration of ions, enzyme content, and minerals. The coagulation duration, as well as curd firmness, are both decreased with an elevated pH (6.6-6.7), while the low-level pH (3-4) affects curd production due to increased hydrolysis. Initially, clotting rates can be affected by factors such as the chymosin and milk source, the composition of the milk, and the preliminary treatment method. Concentrations of Sr^{+2}, Mg^{+2}, and Ba^{+2} ions can also aid in the clotting process, like how calcium ions promote the milk clotting process (P. F. Fox et al., 2017; Okigbo et al., 1985).

1.5 Chymosin Physicochemical Characteristics

1.5.1 Molecular Weight

Chymosin's molecular weight is found to range between 23 to 49 kDa. In 1952, centrifugation methods were used to quantify the molecular weight of chymosin for the first time. Nevertheless, more research was done to provide a more accurate estimate of the molecular weight. Foltmann calculated chymosin's MW through amino acid analysis and found it to be approximately 31 kDa (Foltmann, 1964). Different forms of chymosin have been reported to have varied molecular weights by other researchers. According to the literature, the molecular weight of buffalo chymosin is 36.5 kDa (Mohanty et al., 1999), while calf chymosin is between 36.3 and 36.5 kDa (Kleinert et al., 1988). Molecular weights of 36 and 37.5 kDa have been reported for the chymosin found in lamb, while those of 36 and 44 kDa have been recorded in children (Moschopoulou et al., 2006).

1.5.2 Isoelectric Point (pI)

The coagulating enzymes of milk of different species have been analyzed by researchers to identify their isoelectric point (pI). Foltmann and Righetti found that calf chymosin had an isoelectric point (pI) of 4.6 (Foltmann, 1970), while Martin and Kleinert found that it was 4.5 and 5.0 respectively (Kleinert et al., 1988; MARTIN et al., 1982), and Bines and his colleagues found that it was 4.55 and 5.0 (Bines et al., 1989). Amourache and Vijayalakshmi reported an isoelectric point of 6.0 for juvenile chymosin (Amourache & Vijayalakshmi, 1984), while Moschopoulou found a range from 4.6-5.1 of isoelectric point for chymosin (A Kumar et al., 2010; Moschopoulou et al., 2006).

1.5.3 Optimum pH

The findings have revealed that chymosin works most efficiently at low pH levels. It was reported that pH 5.8 is optimal for the proteolysis process of the casein protein by chymosin enzyme (Foltmann, 1969). Proteolysis yields distinct compounds between pH 4.6 and 6.4. Bovine serum albumin (BSA) was found to have optimal pH values of 3.4, whereas α-casein, β-casein, and κ-casein exhibited optimal pH values of 4.5, 5.5, and 5.5 for each one respectively (A Kumar et al., 2010).

1.5.4 Optimum Temperature and Heat stability

The chymosin enzyme activity is found between 20 and 50 °C, while it is optimal between 30 and 50 °C (Hayaloglu et al., 2014). It was first reported that purified chymosin shows the highest activity between 30 and 40 °C (Foltmann, 1969). Foltmann further studied that at 2 °C chymosin was more stable than at 25 °C. Another study revealed that under temperatures of 50 °C or less, chymosin was found to be relatively stable. Further, it was found that chymosin activity quickly decreased as the temperature increased from 45°C to 55°C (Kawaguchi et al., 1987). Various group researchers proposed that the oxidation of His by photolysis alteration in the ε-amino group of Lys can drastically reduce the chymosin activity (Türkmen & Güler, 2022).

1.6 Sources and Localization of Chymosin

The cheese was originally made using enzymes taken from the abomasum of newborn calves. Although buffalo, goats, sheep, and rabbits have all been speculated about as potential rennet sources, but no major research has been conducted on these animals (Olempska-Beer et al., 2006). However, there have been extensive studies into microbial sources as potential substitutes for cattle chymosin. Several different types of bacteria and fungi have been proposed as possible alternatives to rennet.

Mucor pussillus, Endothica parasitica, Aspergillus oryzae, and *Irpexlactis* are only a few of the fungi used to cultivate the microbial rennet that is now widely used in the dairy sector (da Silva, 2017; Punt et al., 2002). More than half of all cheese is made with this microbial rennet instead of calf chymosin. Despite this, the demand for animal chymosin continues to rise since it gives native cheeses a flavor that is hard to achieve with microbial rennet. Calf chymosin has a 1.5-fold higher ratio of milk clotting activity to proteolytic activity than any microbial rennet, while the proteolytic activity of microbial rennet can cause problems including the inability to stiffen the curd and a large loss of fat and protein in the whey (JACOB et al., 2011). As a result, cheese production is inefficient, and the product can have unpleasant flavors or even taste harsh. Commercial microbial rennet substitutes have been used to produce some varieties of cheese that are satisfactory thanks to advances in cheesemaking technology or the use of mixtures of these preparations with other proteases. However, these microbial preparations have not been able to completely replace animal rennet or overcome its deficiencies (Oštarić et al., 2022).

Prochymosin and pepsinogen are present in the chief cells of stomach tissue. When a calf is in the milk-feeding stage, it keeps on producing prochymosin, but as the calf becomes mature the lower cells lose their prochymosin immunoreactivity and stop its production, but the upper base of the gland retains its prochymosin immunoreactivity (A Kumar et al., 2010). The synthesis of prochymosin initiates during the 10^{th} week of gestation and attains its peak level a few weeks before delivery. A substantial output of prochymosin persists during breastfeeding until at least 6 months of age (Belenkaya et al., 2020). In the case of a conventional mixed diet, a reduction in production happens after three months, though small amounts of prochymosin continue to be manufactured even in adulthood. As for pigs,

prochymosin creation starts approximately at ten weeks of gestation and climaxes at birth. A swift decline following the initial week of life, and no prochymosin generation is detected after 50 days of age. So, during the milk feeding stage, the production of chymosin is high but as the calf turns into mature bovine the chymosin production declines sharply. On the other hand, Pepsinogen is present in each of these cells both before and after weaning. This suggests that dietary elements, especially exposure to milk components, have an impact on the differentiation of these cells in the gastrointestinal mucosa (Acharya et al., 2023; Kurabayashi et al., 1991).

1.6.1 Chymosin Production

To coagulate the milk used to make cheese, traditionally, enzymes isolated from the abomasal tissues of calves have been used. Milk clotting enzymes have been extracted from a wide variety of juvenile animal species. Chymosin was purified from commercial rennet using diethyl aminoethyl DEAE cellulose column chromatography (Mohanty et al., 2003). Bovine chymosin was stable up to 50 °C, but beyond that, its capacity to coagulate milk dropped by about 55%. Cat chymosin, based on findings is most active as a general proteolytic enzyme around a pH of 2.5. Cat prochymosin and chymosin have an amino acid makeup that was very comparable to that of their bovine counterparts (Jensen et al., 1982). In a study by Baudys and his colleagues, they looked at the differences between chymosin from calves and found that both enzymes had a single, distinct band at 36 kDa after chromatographic purification (Baudys et al., 1988). Chymosin enzyme found in the abomasal tissue of buffalo was studied and found structural similarities but not exact matches with bovine enzymes. When tested against hemoglobin, buffalo chymosin showed a higher pH optimum than bovine chymosin, indicating some small functional differences. Using DEAE cellulose ion exchange column chromatography, Kumar and his colleagues separated

three primary activity peaks in goat chymosin (**Figure 1.4**). The purified enzyme preparation displayed greater thermostability than that of bovine chymosin (Kumar et al., 2006).

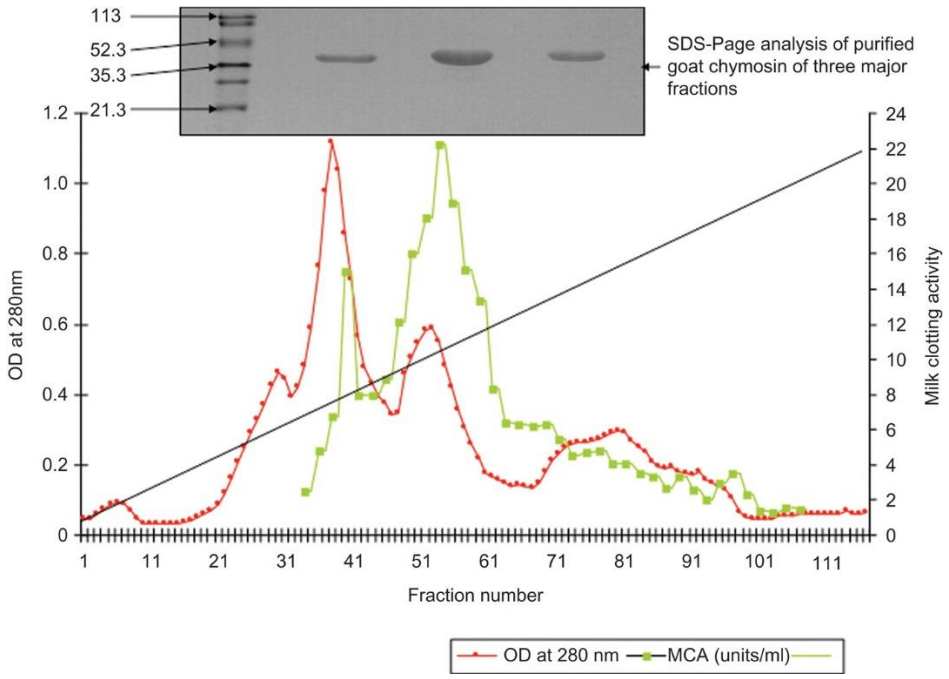

Figure 1.4 Elution of goat chymosin by DEAE cellulose ion exchange chromatography (Kumar, 2010)

1.6.2 Plant Milk Coagulants

There are many limitations from ethical and religious point of view on the use animal derived chymosin and other enzyme found in rennet. Additionally, animal rennet has been in short supply and high demand prompting researchers to look elsewhere for the enzyme. Vegetable extracts extracted and purified from various plant parts, such as seeds, latex, and fruits, have been studied for their potential as coagulants in cheese manufacturing (Folgado & Abranches, 2020). Researchers have found that the latex of some Ficus species helps the milk to thicken. Bromelain from pineapple, however, has been unsuitable as a rennet alternative due to its proteolytic character. From plant *Cyanara cardunculus L* researchers have identified two novel proteinases called cardosins A and B. Structural and kinetic examination of cardosins

points to the possibility that they are the end products of many, independently evolved genes (Mazorra-Manzano et al., 2013). However, the high proteolytic activity of most plant proteases causes problems including too much acidity, a harsh taste, texture flaws, and lower yields of cheese. This is the main reason why the plant bases proteases are not being employed in the cheese industry (Roseiro et al., 2003b).

1.6.3 Milk Coagulants from Microorganisms

Alternatives to the rennet of milk coagulants can be found in microorganisms such as moulds and bacteria. The most common bacterial species which is readily used in milk clotting enzyme production for cheese processing are *Bacillus mesentricus, Bacillus cereus, Micrococcus caseolyticus, Bacillus subtilis, Streptococcus liquifaciens, Bacillus polymyxa* and *Bacillus coagulans* (Feijoo-Siota et al., 2014). Mold-derived milk clotting enzymes have been shown to be an effective curdling process, and patents have been issued for the manufacture of milk coagulants derived from the species like *Rhizomucor miehe, Rhizomucor pusillus* and *Cryphonecteria prasitica* (Mandujano-González et al., 2016). Areces found that an acid protease from *Mucor bacilliformis* exhibited milk clotting activity and shared a comparable amino acid makeup to other fungal proteinases. Due to its heat lability and high clotting/proteolytic activity ratio, the author has proposed it as a possible replacement for bovine chymosin. Further studies also revealed that the old culture obtained from *Penicillium oxalicum* showed a very promising potential to be a rennin producer, and this strain was shown to be optimal for producing milk-clotting enzymes (A Kumar et al., 2010).

1.7 Recombinant Chymosin

Clotting enzymes in milk can be obtained from a wide variety of plant, animal, and microbial sources. Alternatives to calf rennet can be found in the rennet like

proteases produced by many bacteria and plants. Rennin is in low supply all over the world because of its crucial role in cheese manufacturing. Due to the high demand, other alternatives have been developed, including plant rennet, microbial rennet, and recombinant rennet (Beppu, 1983). The rennet derived from plants and microorganisms is characterized because of the non-specific hydrolytic breakdown of casein, which results in undesirable flavor and taste and a lower cheese yield (A Kumar et al., 2010). Animal rennet enzyme (chymosin) is a preferable choice for manufacturing high-quality cheese. Dairy chymosin, especially bovine chymosin, is a key ingredient in making cheese. However, the conventional method of preparing bovine chymosin calls for the brutal killing of a huge number of unweaned calves, which has a significant financial impact on the country as a whole. It takes a long time to extract and purify, and it's highly vulnerable to degradation during processing. To meet the demand for the enzyme in the cheese industry, biologists have resorted to recombinant production of mammalian chymosin. One-third of all cheese produced now is made with recombinant chymosin (Myagkonosov et al., 2022).

Due to improved understanding of the genetics of eukaryotes has resulted in the generation of animal chymosin using genetic engineering techniques in various microorganisms. Efforts are ongoing to enhance the expression of chymosin protein by utilizing strong promoters in vectors and by employing new protein expression and purification techniques. Most of the genes which are in the chromosomes of higher eukaryotes contain an intronic sequence. These intronic sequences are responsible for disruption in exonic sequences which encode mRNA for protein translation. In eukaryotic cells introns are excised from the mRNA transcript through a splicing mechanism, leading to the production of mature protein correct amino acid sequence (Beppu, 1983). However, this is not the same with prokaryotic (bacterial) cells which

lack splicing machinery, making it impossible to accurately express genes derived from eukaryotic chromosomes in a bacterial expression system. So, there is a need for a standard approach for introducing these genes through cloning which involves the synthesis of cDNA from mRNA through reverse transcription from which the intron has been removed (Guarente et al., 1980). Alternatively, the eukaryotic gene can be artificially synthesized without introns and incorporated into specialized bacterial, yeast, or mammalian vectors to produce recombinant chymosin enzymes (Luo et al., 2016). The chymosin gene has been successfully expressed using a number of plasmids and modified vectors into the host species like *Saccharomyces cerevisiae, Bacillus subtilis, Escherichia coli, Kluyveromyces lactis,* baby hamster kidney cells, and *Aspergillus species.* Chymosin, perprochymosin, prochymosin, and met-prochymosin have been successfully produced through these methods (Wei et al., 2016). The gram-negative bacteria *E. coli* is one of the most popular systems for generating heterologous proteins because of its fast growth under high density on cheap growth media, well-defined genetic makeup, plenty of cloning vectors, and the presence of mutant host strains. In bacteria, the chymosin gene from a calf was one of the first mammalian genes to be cloned. It was selected because it has a high degree of specificity and consistent proteolytic activity, neither of which causes problems for milk proteins. Mildly acidic conditions are ideal for maximizing activity, which in turn produces the best flavor and curd production in cheeses and results in the highest yields (Kawaguchi et al., 1987). The proenzyme is secreted by *Escherichia coli* as an insoluble protein, requiring further solubilization and renaturation procedures for separation from inclusion bodies. One benefit of purifying and activating a proenzyme is that it is insoluble in water. The lower pH needed for prochymosin activation agrees with the precipitation of most *E. coli* proteins in acidic circumstances. It's worth

noting that the enzymatic properties of recombinant chymosin are identical to those of native chymosin (Aboulnaga, 2019).

1.8 Prokaryotic Expression System

Gram-negative bacteria like *Escherichia coli* are among the most often employed prokaryotic cells in genetic engineering. Due to its quick growth, low cost, simple dietary needs, high-level expression capabilities, and efficient transformation process, *E. coli* is an important organism for recombinant DNA cloning and the manufacture of heterologous proteins. However, this bacterial expression strategy has drawbacks, such as heterologous protein aggregation and misfolding, the production of lipopolysaccharide, a lack of posttranslational modifications, and protease-mediated protein degradation (Porowińska et al., 2013).

There are a number of reasons why *E. coli* is regarded as a good host organism. First, its growth kinetics are unsurpassed; under ideal circumstances in a glucose salt medium, its doubling time is just around 20 minutes (Sandomenico et al., 2020). Additionally, *E. coli* permits the development of high cell-density cultures. Approximately 1×10^{13} live bacteria ml^{-1} is the theoretical maximum density that may be achieved in liquid cultures of *E. coli*. In complex media, however, exponential growth often stops far short of this maximal density, reaching only around 1×10^{10} cells ml^{-1} under standard laboratory conditions. High cell-density culture approaches have been developed to circumvent this barrier and improve recombinant protein synthesis. The effectiveness of these methods may be attributed, in part, to the deep understanding of the physiology of *E. coli*. Using *E. coli* as a host also allows for the easy preparation of rich complex media using affordable and easily accessible components. In addition, *E. coli* facilitates rapid and easy transformation using

exogenous DNA, with plasmid transformation requiring just around five minutes (Cole et al., 2020).

The expression of heterologous proteins in *E. coli* has several restrictions, though. Codon bias, which can hinder gene expression and protein synthesis in *E. coli* in comparison to eukaryotic organisms, is one such factor. Other limitations include its inability to perform eukaryotic-like posttranslational modifications due to its prokaryotic nature, its limited capacity for extensive disulfide bond formation which results in the production of proteins in insoluble forms as inclusion bodies due to misfolding and aggregation, and its inadequate expression (Bhatwa et al., 2021).

1.9 Tailoring of chymosin Activity and Production

Tailoring of chymosin through directed evolution involves optimizing its activity, specificity, and stability to meet specific process requirements. Naturally occurring enzymes often fall short in these aspects, which is where directed evolution comes in. This technique allows for the deliberate introduction of designed or random changes into the gene through methods like site-directed mutagenesis or DNA shuffling. The goal is to create customized proteins with novel and desirable properties. To successfully tailor an enzyme through protein engineering, two key prerequisites are essential: identification of the gene and knowledge of its three-dimensional structure. Understanding the 3D structure helps predict the functional roles of specific amino acid residues in catalysis, substrate specificity, thermostability, and the overall structure-function relationship of the enzyme (Arnold & Moore, 2007).

In the prokaryotic expression system, various challenges can arise in the process of protein secretion, including issues related to signal peptides, promoter

efficiency, codon usage, folding within the endoplasmic reticulum and Golgi apparatus, environmental factors, and proteolysis. To overcome these hurdles, optimizing codon usage in the target protein to align with the codon bias of the host strain has shown significant potential for enhancing protein expression. This optimization often leads to substantial improvements, ranging from 10 to 50-fold increases (Burgess-Brown et al., 2008). Additionally, the secondary structure of ribosomal binding sites plays a dynamic role in translational initiation within prokaryotes. For instance, alterations in the translation initiation region, in conjunction with plasmids containing a trp promoter, resulted in the hyperexpression of prochymosin, accounting for 39% of the total cellular protein (Bloom & Arnold, 2009). Overall, tailoring chymosin through directed evolution allows for the optimization of its properties, while overcoming challenges associated with protein secretion and expression. This approach leverages genetic manipulation and knowledge of protein structure to create tailored enzymes with improved characteristics for various applications (Santos & Stephanopoulos, 2008).

1.10 Need of Chymosin in Pakistan

Pakistan is one of the world's leading producers of milk and has a significant dairy industry. In 2020-2021, the Pakistan Bureau of Statistics estimated that the nation produced approximately 61.75 billion liters of milk. Also, in 2023, the cheese market will generate US $1.83 billion in revenue. The market is anticipated to expand at 5.43% yearly from 2023 to 2028 ("Cheese - Pakistan | Statista Market Forecast,"). This high milk production provides a solid foundation for the expansion and development of Pakistan's cheese industry. Changing lifestyles, urbanization, and the influence of Western cuisine have all contributed to Pakistan's consistently growing cheese consumption over the years. Not only is the demand for cheese increasing in

main cities, but also in rural areas. The consumption of cheese in Pakistan has increased by 38% over the past five years, according to a report by the Pakistan Dairy Development Company (PDDC). To satisfy the rising demand for cheese, the dairy industry in Pakistan is anticipated to expand significantly in the coming years. According to the PDDC report, the cheese market could reach approximately 125,000 metric tonnes by 2025. This indicates a significant opportunity for the establishment of cheese manufacturing and processing facilities in the nation ("Pakistan's Cheese Market Report 2023 - Prices, Size, Forecast, and Companies," 2023).

Chymosin is essential to cheese production because it aids in the coagulation of milk and the formation of curds, which are then processed into various varieties of cheese. The increasing demand for cheese in Pakistan will increase the demand for chymosin, a crucial enzyme in the cheese-making process. The availability and application of chymosin in the dairy industry will be required to satisfy the rising demand for cheese production.

1.11 AIMS AND OBJECTIVES

The primary aim of this study is to achieve lab-scale production of Chymosin utilizing a straightforward expression system in gram-negative bacteria, specifically *E. coli*.

The following are the key objectives of this proposed research:

i. Cloning the heterologous chymosin gene into engineered strains of *E. coli*, employing a synthetic gene construct designed for this purpose. This step will enable the introduction of the desired chymosin gene into the bacterial host.
ii. Optimizing the expression of recombinant chymosin in suitable strains of *E. coli.* Through various techniques, the expression levels of chymosin will be enhanced to achieve higher yields of the recombinant protein.
iii. Small-scale production of the recombinant chymosin protein will be done by the cultivation of the engineered bacterial strains and the extraction of the produced protein for further analysis and characterization.
iv. The recombinant chymosin obtained protein will undergo purification procedures to isolate and obtain a high-purity form of chymosin suitable for subsequent experimentation.
v. To investigate the potential application of the purified recombinant chymosin protein in cheese production and depending on the duration of the project, the testing of chymosin's activity and its impact on cheese production will be explored.

CHAPTER 2

MATERIALS AND METHODS

2.1 Vendors List for Chemicals and Reagents

The following is the list of vendors for laboratory chemicals and reagents.

Table 2.1 List of Vendors

Supplier	Web Link
Thermo Fischer scientific	http://www.thermofisher.com
Invitrogen Life Technologies	http://www.invitrogen.com
GE Health Care Life Sciences	http://www.gelifesciences.com/
Sigma-Aldrich chemical company Ltd.	https://www.sigmaaldrich.com/
Qiagen	http://www.qiagen.com/
MERCK	http://www.merck.com/
Fermentas	https://www.fermentas.com/
Genescript	https://www.genscript.com/

2.2 Chemicals and Enzymes List

Detailed information on the chemicals and enzymes used in this study is presented in **Table 2.2.**

Table 2.2 Chemicals and Enzymes List

Chemical	Company	Catalog/ Article Product number/CAS no
10 Kb GeneRuler DNA Ladder Mix	Thermo Scientific	Cat no. SM0331
TriTrack DNA Loading Dye (6X)	Thermo Scientific	Catalog no. R1161
Agar	ThermoFischer Scientific	Cat no. 22700025
Agarose	HydraGene	Cat no. R9012LE
Coomassie Brilliant Blue G250	Sigma Aldrich	Cat no. 27815
Acrylamide	Invitrogen	Cat no. 15512-023
Absolute Ethanol	Riedel-de Haën, Germany	Cat no. 32221
Tryptone	Biobasic	Cat no. TG217(G211)
Yeast Extract	Biobasic	Cat no. G096
Sodium Chloride (NaCl)	Sigma Aldrich	S3014
Chloramphenicol	Sigma Aldrich	EC no. 200-287-4
Ampicillin	Sigma Aldrich	Cat no. A9393
Glucose	Sigma Aldrich	Cat no. G8270
Glycerol	Sigma Aldrich	EC no. 200-289-5
Calcium Chloride Dihydrate (CaCl2. 2H2O)	Sigma Aldrich	Cas no. 10035-04-8
Orange Buffer O	ThermoFisher Scientific	Cat no. BO5
Trizma Base	Merck Sigma Aldrich	EC no. 77-86-1

Chemical	Company	Catalog/ Article Product number/CAS no
Glycine	AMRESCO, USA	97061-128
Sodium dodecyl sulfate SDS	Thermo Scientific	AM9022
Hydrochloric acid HCl	Daejung, South Korea	4090-4100
Sodium Hydroxide NaOH	Strem Products	1310-73-2
Isopropyl β-D-1-thiogalactopyranoside IPTG	Fischer Scientific	BP1755-10
Tetramethylethylene diamine (TEMED)	Gibco	15524-010
Lysozyme	Roth, Germany	3899.4
Potassium Phosphate	ThermoScientific	447370010
Ethidium Bromide	Research Organics	3011E
Ammonium persulfate (APS)	Roth, Germany	9592.1
Bisacrylamide	Biobasic Canada	BB0025
Beta mercaptoethanol (BME)	Roth, Germany	4227.1
Desthiobiotin	IBA-Lifesciences	2-1000-002
Dithiothreitol (DTT)	ThermoFisher Scientific	D-1532
Phenylmethylsulfonyl fluoride (PMSF)	ThermoFisher Scientific	36978
HindIII	New England BioLabs	R0111L
EcoRI	New England BioLabs	R0146L
Taq Polymerase	ThermoFisher Scientific	EP0402
Magnesium Chloride (MgCl2)	ThermoFisher Scientific	R0971
dNTP Mix	ThermoFisher Scientific	R0191
Yeast extract	BioBasic	G0961

PageRuler Pre-stained ladder (10 to 180 kDa)	ThermoFisher Scientific	26616
PageRuler™ Unstained Protein Ladder (10 to 200 kDa)	ThermoFisher Scientific	26614

2.3 List of Bacterial Strains

The bacterial strains utilized in the research project are listed in **Table 2.3**.

Table 2.3 ***List of Bacterial Strains***

Bacterial strains/ plasmids	Genotype/Description	Source
E. coli DH5$^{\alpha}$	*F^- φ80lacZΔM15 Δ(lacZYAargF)U169 recA1 endA1 hsdR17(r_K^-, m_K^+) phoA supE44 λ^-thi-1 gyrA96 relA1*	SBS Stocks
E. coli BL21 (DE3)	*F^- ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm (DE3)*	SBS Stocks

2.4 Solutions and Reagents Preparation

Recipes of various solutions and reagents used in the present study are taken from Sambrook and Russell (2001) given below:

2.4.1 LB medium, (*Luria Bertani* medium)

For the growth of bacterial strains, the LB broth, and LB agar medium was used, the composition of the media has been mentioned in **Table 2.4 & 2.5**. With the distilled water the required volume of the media was made up and the pH 7 was maintained by using a 5 N sodium hydroxide solution. The prepared media was autoclaved at 15 psi for 30 minutes and at 121 ºC. The respective antibiotics were added to the sterilized media when the temperature dropped to 45 ºC.

The LB agar plates with and without antibiotics were poured into the laminar airflow safety cabinet, with about 25 mL media in each Petri plate (**Figure 2.1**). The agar plates were solidified at room temperature and then they were sealed with the parafilm and stored at 4 ºC.

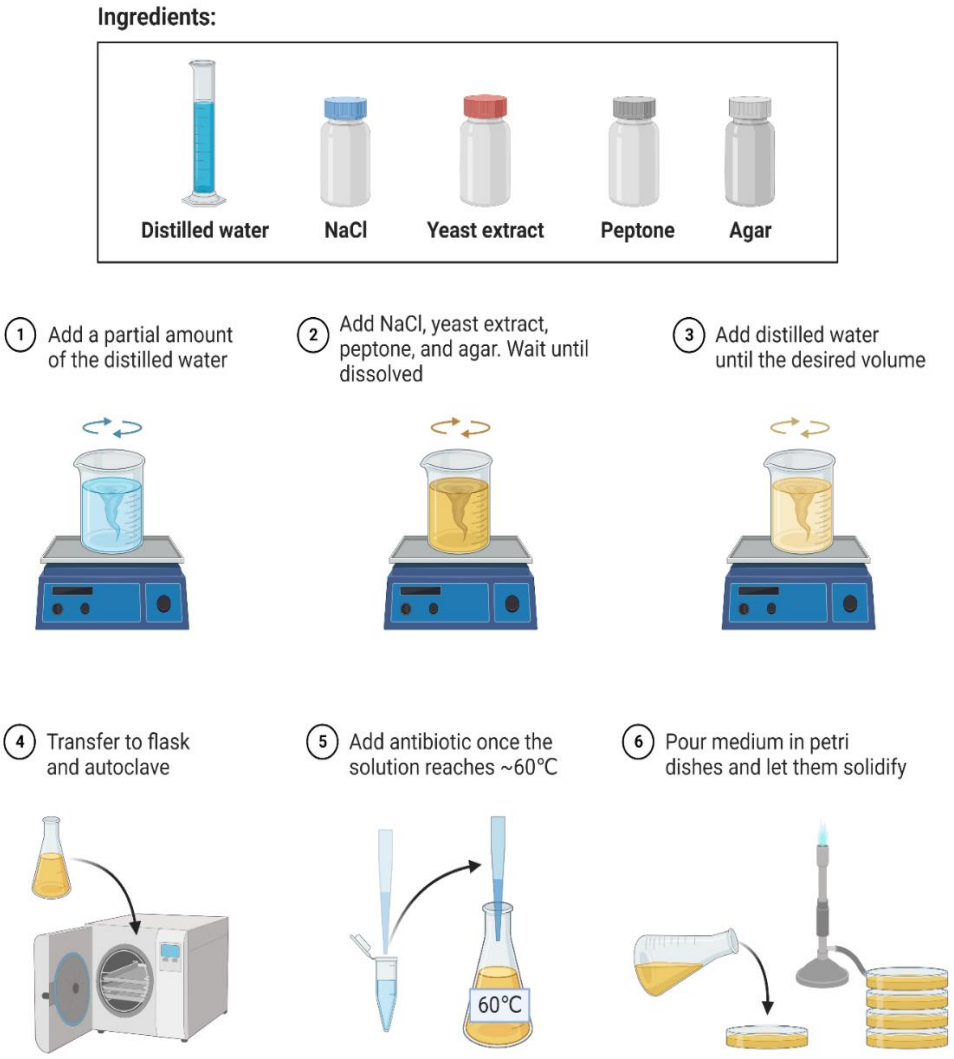

Figure 2.1 LB medium, (Luria Bertani medium) preparation

Table 2.4 ***LB medium recipe (1 L)***

Components	Amount per volume (g/L)
Tryptone	10 g
NaCl	5 g
Yeast Extract	5 g
Distilled water	Up to 1000 Ml
pH at 25 ºC	7.0 ±0.2

Table 2.5 LB Agar recipe (1 L)

Components	Amount per volume (g/L)
Tryptone	10 g
NaCl	5 g
Yeast Extract	5 g
Agar	10 g
Distilled water	Up to 1000 mL
pH at 25 ºC	7.0 ±0.2

2.4.2 Antibiotic Stock Preparation

Stock solutions of ampicillin (100 mg/mL) and chloramphenicol (50 mg/mL) were prepared in Milli-Q water and 95% ethanol, respectively, as mentioned in **Table 2.6.** Afterward, the antibiotic solutions were filter sterilized by using a 0.2 µm filter. 1 ml aliquots were stored at -20 °C. The working concentration of ampicillin was adjusted to 100 µg/ml and for chloramphenicol 60 µg/ml in LB agar media for *E. coli*.

Table 2.6 Antibiotic Stock Preparation Recipe

Antibiotic	Stock Concentration (mg/mL)	Working Concentration (µg/mL)	Solvent
Ampicillin	100 mg/Ml	100 µg/mL	Milli-Q Water
Chloramphenicol	100 mg/mL	60 µg/mL	95% Ethanol

2.4.3 X-gal Stock Solution

A stock solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at a concentration of 20 mg/mL was prepared by combining 200 mg of X-gal with 10 mL of N, N-dimethylformamide (DMF). The solution was then filter sterilized and stored at a temperature of -20 °C. Due to X-gal's sensitivity to light, each aliquot of the stock solution was covered with aluminum foil for protection.

2.4.4 IPTG Stock Solution

A stock solution of IPTG (isopropyl-β-D-thiogalactopyranoside) at a concentration of 100 mM was prepared using distilled water (0.238 g / 10 mL). The IPTG solution was then subjected to sterilization through filtration using a 0.22 µm membrane and subsequently stored at a temperature of -20 °C for preservation.

2.4.5 LB Agar Plates

In the oven, 100 ml of sterile LB agar was melted. In a laminar cabinet, 25-30 ml of the medium was poured into sterilized Petri plates and solidified at room temperature. The plates were wrapped in parafilm and kept at 4 °C.

2.4.6 LB Agar Ampicillin (100 µg/ml) Plates

In 100 ml melted sterilized LB agar, 100 µl ampicillin (100 mg/ml) was added. 25-30 ml of the media was poured into sterilized Petri plates in a laminar cabinet and solidified at room temperature. Parafilm paper was used for sealing the plates and then plates were stored at 4 °C.

2.4.7 LB Agar Chloramphenicol (100 µg/ml) Plates

In 100 ml melted Sterilized LB agar, 60 µl chloramphenicol (100 mg/ml) was added. 25-30 ml of the media was poured into sterilized Petri plates in a laminar cabinet and solidified at room temperature. The plates were sealed for storage at 4 °C.

2.4.8 LB Agar Ampicillin X-Gal/IPTG Plates

The LB agar ampicillin plates were prepared as mentioned in section 2.4.5 and left undisturbed to solidify. To dry the plates, they were opened and placed at room temperature under UV light for 30 minutes. After that 40 µL of X-gal stock solution (20 mg/mL) and 40 µL of IPTG 100 mM were added to the plates and evenly spread using a sterile spatula. The plates were sealed for storage at 4 °C.

2.5 Primer Designing for Chymosin

I was provided with a chymosin construct by my supervisor that was originally a *Bos taurus* gene taken from NCBI accession number (*NM_180994.2*). It was codon optimized for *Bacillus subtilis* and contained an additional signal peptide at the start of the gene for soluble expression. This was synthesized into the *pUC57* cloning vector. However, my present work is the production of chymosin without signal peptide in the *Escherichia coli* expression system. So, to deal with this first, I synthesized primers with suitable restriction sites to amplify the chymosin gene from *pUC57*. For the design of these primers, I utilized SnapGene7.0 software (**Appendix**

I). To ensure efficient cloning of the chymosin gene into the expression vector, I introduced the EcoRI site in the forward primer and the Hind III restriction site in the reverse primer shown in **Table 2.7.** The primers were synthesized from **macrogen** Korea.

Table 2.7 List of primers designed for chymosin gene amplification.

Primer Name	Primer sequence	Melting Temperature Tm
Chymosin-F	5′-CCGGAATTCATGGCGGAAATTACAAGAATC-3′	63 ºC
Chymosin-R	5′-CCCAAGCTTTTAAATCGCTTTCGCTTTCGCCAGTC-3′	65 ºC

2.6 PCR Amplification of Chymosin from pUC57

The PCR amplification of chymosin was carried out using the designed primers containing Hind III and EcoRI restriction sites. PCR was carried out in Applied Biosystem, 2720 Thermocycler. The primers were designed with Hind III and EcoRI restriction sites to facilitate efficient cloning of the chymosin gene into the desired expression vector. The PCR reaction mixture was prepared by combining the DNA template, primers, dNTPs, Taq DNA polymerase, PCR buffer, and MgCl as mentioned in **Table 2.8.** All the components were added into the microfuge tube and the template was added at the end and after a short spin, the tubes were put into the PCR machine. The thermal cycler was programmed with PCR cycling conditions as mentioned in **Figure 2.2,** including initial denaturation (5 minutes) at 95 ºC and final denaturation (30 seconds) carried out at 94 ºC, annealing was carried out at 63 ºC for 40 seconds, initial extension (90 seconds) and final extension (7 minutes) both were carried out at 72 ºC, total cycles were 30.

Table 2.8 Reaction mixture composition for PCR amplification of Chymosin from pUC57

Components	Stock Concentration	Required Concentration	Required Volume (µl)
10X Taq buffer	10X	1X	5
Magnesium chloride ($MgCl_2$)	25Mm	2mM	4
dNTP's	2.5mM	0.2mM	4
Forward primer	10 µM	0.1 µM	0.5
Reverse primer	10 µM	0.1 µM	0.5
Taq Polymerase	5U/ µl	0.15U/ µl	1
Template DNA			1
ddH_2O			34
Total Volume			50

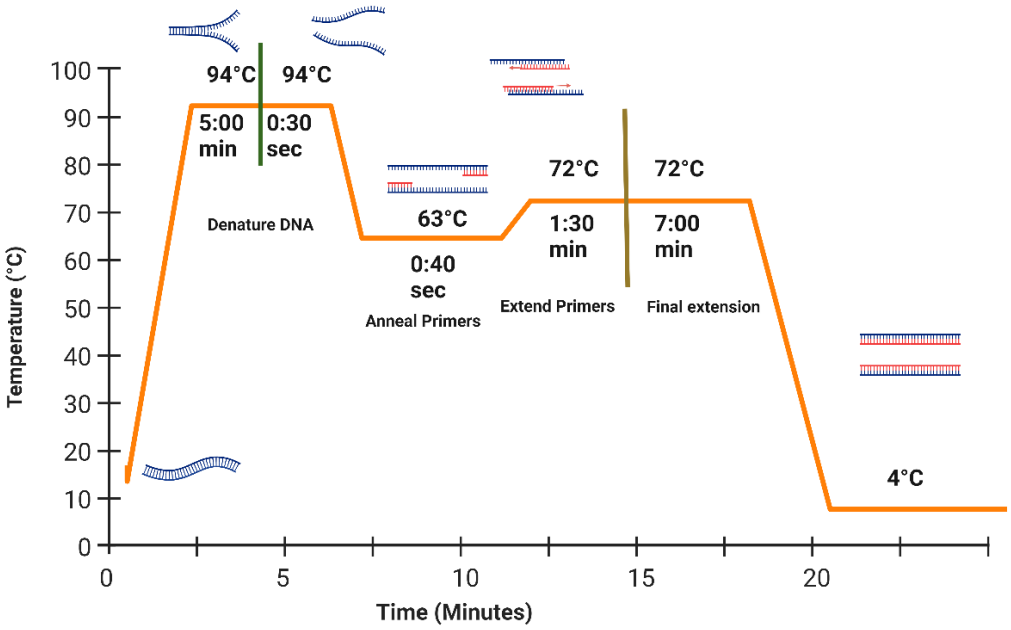

Figure 2.2 PCR Cycle Design

2.7 Agarose Gel Electrophoresis of PCR product (Chymosin)

Agarose gel electrophoresis is a widely used technique for separating nucleic acid molecules based on their size. The process involves the movement of negatively charged nucleic acid molecules through an agarose gel matrix from the negative electrode (Anode) to the positive electrode (Cathode), resulting in the separation of molecules according to their molecular weight (Yılmaz, 2012 #131).

To analyze the PCR product of chymosin, an apparatus from Biocomdirect was utilized. A 1% agarose gel was prepared by dissolving 0.8 g of agarose in 80 mL of 1x Tris-acetate EDTA (TAE) buffer. The TAE buffer was prepared by diluting a 50x TAE stock solution according to the recipe in **Table 2.9**. The agarose solution was heated in a microwave for 100 seconds until it was completely dissolved, resulting in a transparent solution. Subsequently, the solution was allowed to cool down to a temperature between 40-60 ℃. To visualize the DNA, the agarose solution was supplemented with 3µL of ethidium bromide from a 10 mg/mL stock solution, achieving a final concentration of 0.4 µg/mL. The solution was thoroughly mixed and poured into a gel casting tray with the desired comb. The gel was then allowed to solidify at room temperature for approximately 15-20 minutes. The comb was carefully removed, and the solidified gel was transferred to a horizontal electrophoresis tank already filled with 1x TAE buffer. For DNA sample loading, a 6x bromophenol blue DNA loading dye was prepared according to the recipe mentioned in **Table 2.10**. The 50 µl DNA sample was mixed with 10 µL of the loading dye, and the mixture was loaded into the respective wells of the gel. Electrophoresis was conducted at 100 V in 1x TAE buffer. Finally, the gel was visualized under UV light using a transilluminator.

Table 2.9 Composition of 50x TAE Buffer (1 L)

Components	Final concentration	Amount required
Trizma base	24.4% w/v	242 g
Glacial Acetic acid	5.71% v/v	57.1 g
EDTA (0.5 M) pH 8.0	10% v/v	100 mL
Distilled water	-	843 mL

Table 2.10 Recipe of 6x Bromophenol Blue DNA loading dye (10 mL)

Components	Amount required
Bromophenol Blue	0.005 g
Glycerol	4 mL
Distilled water	6 mL

2.8 Gene Purification of PCR product (Chymosin)

The amplified DNA was purified using the Fermentas GeneJET^{TM} Gel Extraction Kit (#K0692). This kit consisted of a binding buffer, wash buffer, elution buffer, and GeneJET^{TM} purification columns with collection tubes. The following procedure was employed to purify the amplified product from the agarose gel:

i. The bands containing the amplified product were excised from the gel using a surgical blade and placed in a pre-weighed Eppendorf tube. The weight of the gel slice was determined by subtracting the weight of the empty Eppendorf.

ii. An equal volume of binding buffer was added to the gel slice and incubated at 50-60 °C for approximately 10 minutes, gently inverting the tube multiple times until the gel slice completely dissolved, resulting in a transparent mixture.

iii. The transparent mixture was then transferred to the GeneJET^{TM} purification column and centrifuged at 10,000 g for 1 minute at 4 °C.

iv. The column, now containing the bound DNA, was washed twice with 700 µl of wash buffer containing ethanol, followed by centrifugation at 10,000 g for 1 minute at 4 °C after each wash.

v. To remove any residual ethanol that could potentially contaminate the purified DNA, the empty column was centrifuged for an additional 1 minute.

vi. Finally, the column was transferred to a clean 1.5 ml Eppendorf tube, and 50 µl of elution buffer was added. After a 2-minute incubation at room temperature, the column was centrifuged at 4 °C for 1 minute at 10,000 g to elute the purified DNA.

vii. This elution process was repeated twice, and the purified product was then analyzed on a 1% agarose gel as mentioned in **Section 2.7.**

The whole process of gel purification is summarized in the following **Figure 2.3.**

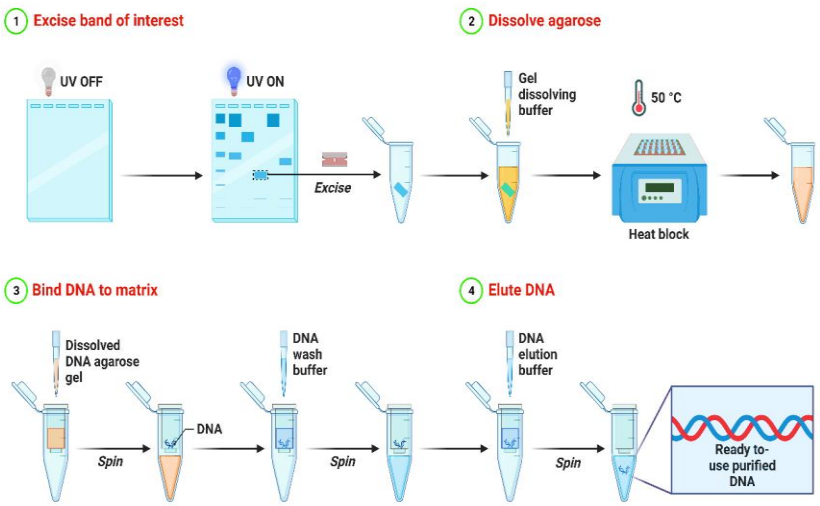

Figure 2.3 Process of Gel Purification

2.9 Streaking of *E. coli DH5α* and *BL21 (DE3)-RIL* Strain

For sterilization, the steel loop was red hot in a flame and allowed to cool by touching it on sterilized agar. 5-10 µl of *DH5α* from glycerol stock was streaked on the agar plate while 5-10 µl of *BL21(DE3)-RIL* from glycerol stock was streaked on the chloramphenicol agar plate. The plates were incubated at 37 °C overnight to obtain proper growth. The plates having isolated bacterial colonies were stored at 4 °C for further use.

2.10 Preparation of Glycerol Stocks

To create glycerol stocks of *E. coli* strains at concentrations of 20% and 40%, a single colony of each strain was inoculated into 20 mL of LB broth. The cultures were then incubated overnight at 37 ºC and 150 rpm in a shaker. In parallel, 80% glycerol was prepared and autoclaved. For the preparation of 40% glycerol stocks, 500 µL of the overnight culture was combined with 500 µL of the autoclaved 80% glycerol solution. The resulting mixture was stored at -20 ºC. To create 20% glycerol stocks, 750 µL of the overnight culture was combined with 250 µL of the autoclaved 80% glycerol solution. These stocks were stored at either -40 ºC or -70 ºC, ensuring their viability for periodic revival of the required cells.

2.11 Preparation of Bacterial Competent Cells

For transforming the cells with the desired genome (plasmid), first of all, the cells are made competent, due to which allows the extracellular genome to enter into the cells (Yang, 2022). There are two methods by which the cells can be made competent: The physical method and Chemical method, Physical method involves electroporation and the chemical method involves the use of $CaCl_2$ or $MgCl_2$ followed

by a heat shock step for the transformation of bacteria with plasmids (Zhou et al., 2018; Grosser & Richardson, 2014).

First of all, 50 mM $CaCl_2$ solution was made according to the recipe mentioned in **Table 2.11.**

Table 2.11 50 mM $CaCl_2$ Solution Recipe (1L)

Solutions	Molecular weight (g/ mole)	Final Concentration	Amount per volume
$CaCl_2$. $2H_2O$	147.01 g	50 mM	7.3505 g/L

After that, it was sterilized by autoclaving. For all the strains, a standard protocol was followed to make the *E. coli* cells competent. The cells were streaked on the LB agar medium, freshly. On the next day, 2 to 3 colonies were inoculated into the LB broth media and incubated in the shaking incubator at 150 rpm, 37 ºC, overnight, it is known as primary culture. On the next day, from the primary culture, the inoculum was given into the secondary culture LB broth flask, the final concentration of the inoculum was 2%, so 1 mL of primary culture was added into the 50 mL LB Broth, was incubated in a shaker until the OD600 reached to 0.6. Afterward, the culture was poured into the prechilled conical falcon tube. The cells were centrifuged for 15 minutes at 6000 rpm at 4 ºC. After centrifugation supernatant was discarded and the cell pellet was resuspended in the 20 mL of prechilled 50 mM calcium chloride solution and 40 minutes of incubation were given to cells in ice, afterward, the cells were centrifuged for 15 minutes at 6000 rpm, 4 ºC. The supernatant was discarded and then the cell pellet was re-suspended into the 2 mL of 50 mM calcium

chloride, cells were stored at 4 ºC and were used on the next day because the efficiency of the competent cells is high after 24 hours.

2.12 Storage of Competent Cells

To store *E. coli* competent cells for long periods, two options are available: glycerol stocks or DMSO stocks. For glycerol stocks, 500 µl of 99% sterilized/autoclaved glycerol was added to 5 ml of competent cell suspension. Precooled 1 ml vials were then prepared and frozen using liquid nitrogen, after which they were stored at -80 °C. As an alternative, competent cells can also be stored in DMSO (Dimethyl sulfoxide). For every 4 ml of cell suspension, 150 µl of DMSO was required. Thus, 37.5 µl of DMSO was added to 1 ml of competent cells in microfuge tubes. The tubes were sealed with parafilm and stored at -80 °C.

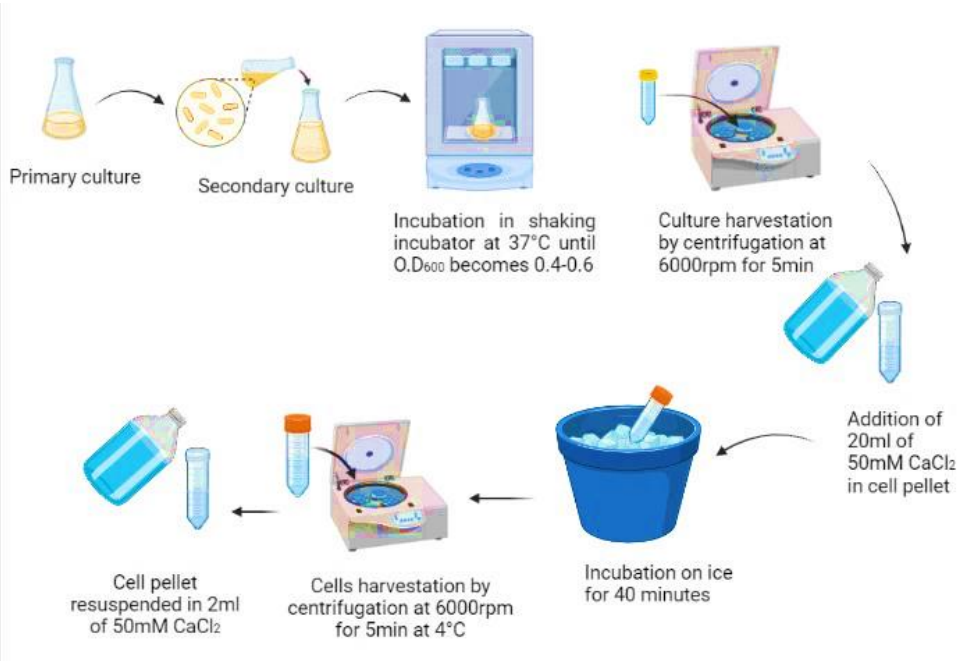

Figure 2.4 Basic steps to form the *E. coli* competent cells.

2.13 Ligation of Chymosin PCR Product to *pTZ57R/T* Cloning Vector

Due to the unique property of Tag polymerase to add poly A tails at the ends of PCR products, the chymosin gene amplified from *pUC57* contained poly A tails at

both ends. Unfortunately, these poly A tails hindered the efficient ligation of the gene into the desired expression vector, *pET28a*. To address this issue, we employed a TA cloning strategy by ligating our PCR product into *pTZ 57 R/T* vector (**Appendix I**). The purified PCR product, measuring 1098 bp and weighing 0.133µg, was combined with *pTZ57R/T* vector (0.180µg) in a ligation mixture consisting of 1X ligation buffer and 5 units of T4 DNA ligase (1µl) while in control there was no PCR product (**Table 2.12**). This 30µl ligation mixture was then incubated at 22°C for approximately 12-16 hours to allow for the ligation process to occur. After the ligation was complete, the resulting product was transformed into *DH5α* cells to facilitate the replication and propagation of the recombinant DNA.

Table 2.12 Composition of Ligation Reaction

Components	**Ligation Reaction (µL)**	**Control Reaction (µL)**
pTZ57R/T cloning vector	3	-
Chymosin PCR product	5	5
T4 ligase buffer	3	3
T4 ligase	1	1
Water	18	21
Total volume	30	30

2.14 Transformation of Ligation Mixture to *DH5a* Competent Cells

The transformation of *E. coli DH5α* cells was conducted according to the recommended protocol outlined by Sambrook J., 2001. To begin, 20 µl of the ligation

mixture was combined with 200 µl of competent cells, thoroughly mixed, and placed on ice for a duration of 40 minutes. Subsequently, the mixture was subjected to incubation at 42 °C for 90 seconds, followed by another round of cooling on ice for 5 minutes. To initiate cell recovery, 800 µl of LB broth was added, and the cells were incubated at 37 °C for 2 hours. As a control, only cells without plasmid were subjected to the same procedure, and for negative control, only the vector was transformed. After the incubation period, 200µl of the transformed cell mixture was spread onto LB-ampicillin X-Gal/IPTG plates. The remaining 800µl of the mixture was centrifuged at room temperature at 12,000 g, and 500 µl of the supernatant was discarded. The resulting cell pellet was resuspended in the remaining 300 µl of supernatant and spread onto separate LB-ampicillin X-Gal/IPTG plates (**Figure 2.5**). All the plates were inverted and incubated at 37 °C for approximately 14-16 hours, allowing colonies to grow for subsequent isolation of *Chymosin/pTZ57 R/T*. The results of the transformation were expected to yield colonies displaying either a blue or white phenotype. The white colonies indicated successful insertion of our gene into the vector, as successful ligation would disrupt the β-galactosidase gene responsible for the blue coloration in colonies where only a self-ligated vector is present.

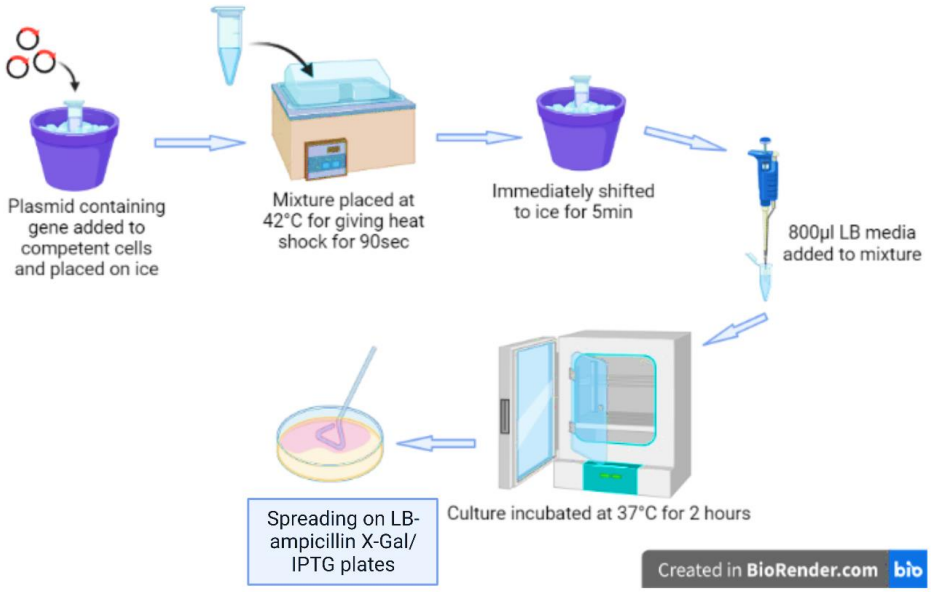

Figure 2.5 Basic Steps Involved in the Transformation

2.14.1 Restreaking of Transformed colonies of *Chymosin / pTZ57 R/T* in *DH5α*

The positive (white) colonies were further restreaked on LB-ampicillin plates to increase the number of single colonies for future use.

2.14.2 Colony PCR

Colony PCR was conducted to confirm the successful transformation of *Chymosin/pTZ57R/T* plasmid in *DH5a* cells. A single restreaked bacterial colony was carefully selected using a sterile loop and transferred into a sterile microcentrifuge tube containing a suitable volume of sterile distilled water. The tube was appropriately labeled for identification purposes. Subsequently, the tube was incubated in the water bath at a temperature ranging between 95-100 °C for 10-15 minutes. This "boiling" or "lysis" step facilitated the release of DNA from the bacterial cells. Following the boiling step, the tube was briefly centrifuged to collect the liquid at the bottom, ensuring that any remaining bacterial debris remained undisturbed. A small volume

(1-2 µl) of the supernatant containing the released DNA was carefully transferred to a new sterile PCR tube, taking precautions to prevent cross-contamination between samples. The PCR reaction mixture mentioned in **Table 2.13** was prepared by adding the necessary components, including PCR buffer, dNTPs, forward and reverse primer, Taq polymerase, and sterile distilled water.

Table 2.13 Reaction mixture composition for colony PCR

Components	Stock Concentration	Required Concentration	Required Volume (µl)
10X Taq buffer	10X	1X	2
Magnesium chloride ($MgCl_2$)	25Mm	2mM	1.5
dNTP's	2.5mM	0.2mM	1.5
Forward primer	10 µM	0.1 µM	0.5
Reverse primer	10 µM	0.1 µM	0.5
Taq Polymerase	5U/ µl	0.15U/ µl	1
Bacterial colony			
ddH_2O			13
Total Volume			20

The PCR tubes were then placed into the thermal cycler and programmed with the PCR cycling conditions as described in **Section 2.6**. Upon completion of the PCR, the PCR tubes were carefully removed from the thermal cycler and stored appropriately. The PCR products were subsequently analyzed through electrophoresis on a 1% agarose gel, employing standard protocols for gel preparation, sample loading, and running the gel at a suitable voltage and duration as in **Section 2.7.**

2.15 Plasmid (*Chymosin/pTZ57R/T*) isolation from *DH5a*

To isolate the plasmid DNA from transformed bacterial colonies containing *Chymosin/pTZ57 R/T*, the following procedure was carried out using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Cat. #. K0502):

i. Firstly, 2 to 3 transformed bacterial colonies were selected and inoculated into 5mL of LB broth supplemented with 100 µg/mL ampicillin. The culture was then incubated in a shaking incubator (Labwit, 2W4R-211D) at 37 ℃ and 150 rpm for 18 hours to allow sufficient growth.

ii. The next day, plasmid isolation was performed. The cells from the 5 mL LB broth culture were harvested by centrifugation using an Eppendorf 5810 R centrifuge at 7000 rpm for 10 minutes. The cell pellet was then resuspended in 250 µL of the provided resuspension buffer and vortexed thoroughly using a Labnet vortexer.

iii. Subsequently, 250 µL of the lysis solution was added to the resuspended cells. The contents of the tube were mixed by gently inverting it 4-6 times until the solution became viscous and slightly clear. To neutralize the lysate and stabilize the DNA, 350 µL of the neutralization solution was added and mixed thoroughly by inverting the tubes.

iv. The mixture was then centrifuged at 12000 rpm for 5 minutes using an Eppendorf microcentrifuge 5430 R to pellet down the cell debris. The supernatant, containing the plasmid DNA, was carefully transferred to a GeneJET spin column provided in the kit. The column was then centrifuged for 1 minute at 12000 rpm to bind the DNA to the silica membrane.

v. To remove impurities, 500 µL of wash solution was added to the column, followed by centrifugation for 60 seconds at 12000 rpm. The flow-through

was discarded, and this washing step was repeated once. The GeneJET spin column, now containing the purified plasmid DNA bound to the silica membrane, was transferred to a new 1.5 mL microcentrifuge tube.

vi. To elute the plasmid DNA, 50 µL of elution buffer was added to the column and incubated at 37 ℃ for 20 minutes. After incubation, the column was centrifuged at 12000 rpm for 2 minutes using an Eppendorf microcentrifuge 5430 R, and the eluted plasmid DNA was collected in the microcentrifuge tube.

vii. Finally, the eluted plasmid DNA was stored at -20 ℃ for future use.

2.15.1 Agarose gel electrophoresis of purified *Chymosin/pTZ57R/T* plasmid

After the Plasmid isolation, the purified plasmid was subjected to 1% agarose gel electrophoresis as described in **Section 2.7** to confirm the plasmid and to quantify it.

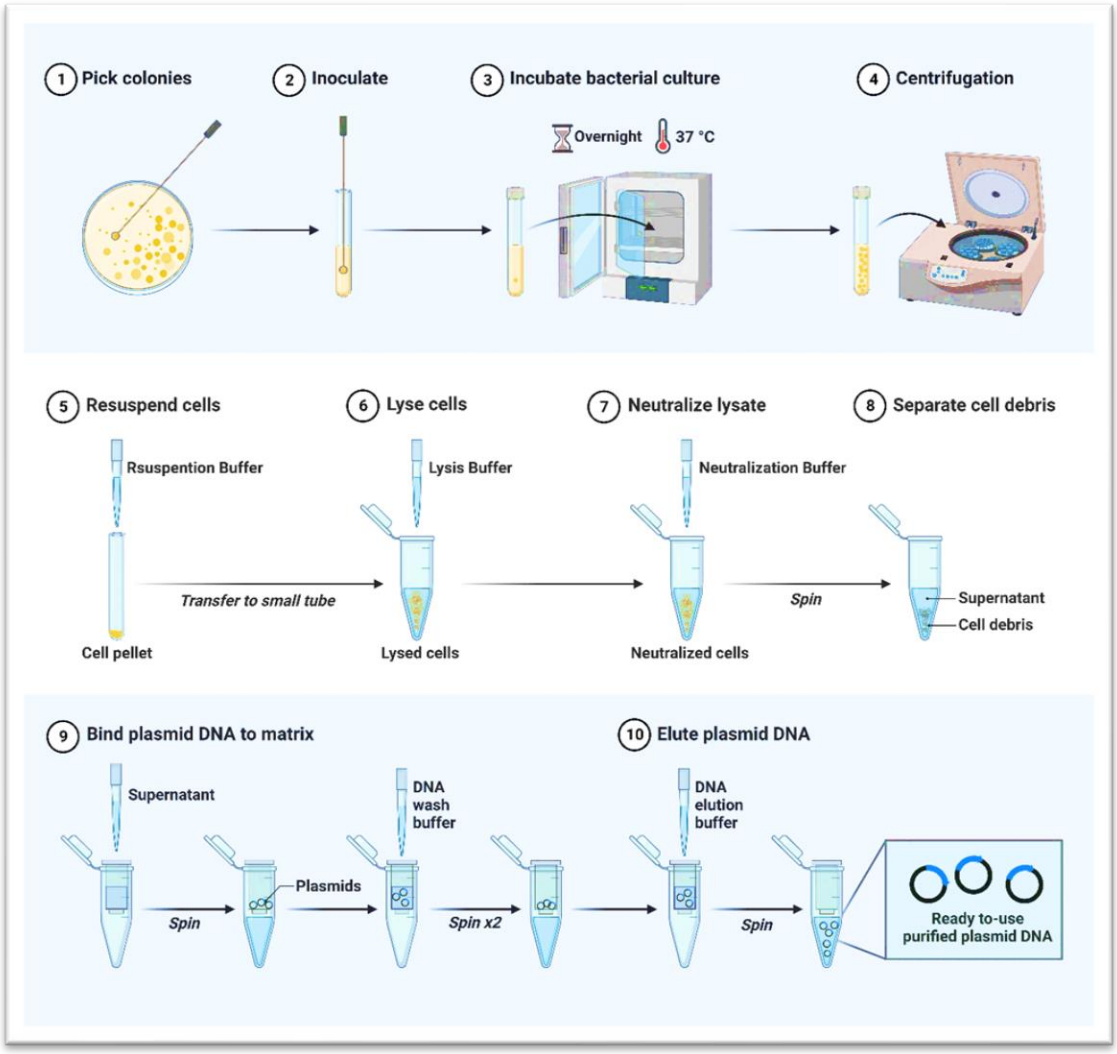

Figure 2.6 Plasmid Purification (Miniprep) Overview

2.16 DNA Quantification

The concentration of purified plasmid and the PCR product was determined by NanoDrop™ 2000/2000c Spectrophotometers (Catalogue number: ND-2000), first of all, the wavelength verification was done and then blank measurement was performed using 1 µL of elution buffer, following the sample measurement was carried in a similar way and values at 260/280 nm ratio were recorded.

2.17 Restriction Digestion of Purified Plasmids

Chymosin/pTZ57R/T was subjected to restriction analysis, for that purpose the two enzymes: HindIII (Thermo Fisher Scientific, Cat # ER0501) and EcoR I (Thermo Fisher Scientific, Cat # ER0271) were selected were the zero cutters of respective plasmids. By consulting the restriction enzymes buffer activity chart, red buffer 10x (Thermo Fisher Scientific, Cat # BR5) was selected for performing the restriction reaction. A restriction reaction of 20 µL for single and 40 µL for double digestion was set up as described in **Tables 14, 15, and 16.** Restriction of plasmid was carried at 37 ℃ for 4 hours. Agarose gel (1%) electrophoresis was done to visualize the restricted DNA fragments. After that the band was cut and the chymosin gene double digested from *pTZ57R/T* was purified using the Fermentas GeneJETTM Gel Extraction Kit (#K0692).

Table 2.14 Double digestion reaction mixture *Chymosin/pTZ57R/T*

Components.	Amount.
Plasmid	10 µl
R buffer(10X)	4 µl
EcoRI	3 µl
HindIII	3 µl
H_2O	20µl
Total volume	40 µl

Table 2.15 Single digestion reaction mixture with HindIII

Components.	Amount.
Plasmid	5µl
R buffer(10X)	2 µl
HindIII	2 µl
H_2O	13µl
Total volume	20 µl

Table 2.15 Single digestion reaction mixture with EcoRI

Components.	Amount
Plasmid	5µl
R buffer(10X)	2 µl
EcoRI	2 µl
H_2O	13µl
Total volume	20 µl

2.18 Ligation of Chymosin to *pET28a* and its Transformation to *DH5a* cells

Initially, my supervisor provided me with the *pET28a* vector. To amplify it, I performed a transformation of the vector into *HD5α* cells, following the protocol outlined in **Section 2.14**. Positive colonies were selected from the transformation plates and used to inoculate overnight cultures in 20 ml of LB media. The following day, plasmid isolation was conducted using the standard protocol described in **Section 2.15**. To prepare the *pET28a* vector for ligation experimentation, the purified plasmid was subjected to double digestion with HindIII and EcoRI restriction enzymes, employing the same procedure detailed in **Section 2.17,** and the composition of the digestion mixture is provided in **Table 2.17**. The resulting digested vector was visualized using agarose gel electrophoresis, as described in **Section 2.7**. The band corresponding to the double undigested vector was excised from the gel and purified using the Fermentas GeneJETTM Gel Extraction Kit (#K0692). To determine the quantity of the restricted plasmid and chymosin, nanodrop spectrophotometry was employed, facilitating the setup of an efficient ligation reaction. The ligation reaction was conducted following the standard protocol outlined in **Section 2.13**. The composition of the ligation mixture is provided in **Table 2.18**. Following the ligation

process, the resulting ligation mixture was transformed into *DH5α* competent cells, employing the protocol discussed in **Section 2.14.**

Table 2.16 Double digestion reaction mixture *Chymosin/ pET28a*

Components.	Amount.
Plasmid	10 µl
R buffer(10X)	4 µl
EcoRI	3 µl
HindIII	3 µl
H_2O	20µl
Total volume	40 µl

Table 2.17 Composition of the ligation reaction

Components	Ligation Reaction (µL)
Linear *pET28a* expression vector	10
Chymosin gene	5
T4 ligase buffer	**3**
T4 ligase	1
Water	11
Total volume	**30**

2.19 Isolation of *Chymosin/pET28a* from *DH5a* and transformation into *E. coli* Strain *BL21 (DE3)-RIL*

After the successful transformation of *chymosin/ pET28a* in *DH5a*, a single colony was selected and inoculated into 20 ml of LB media. The culture was then placed in an incubator at 37 °C for 16-18 hours to allow for growth. Subsequently, plasmid miniprep was performed using the Thermo Scientific GeneJET Plasmid

Miniprep Kit (Cat. #. K0502), following the instructions provided in the previous section. Next, 2µl of the *chymosin/ pET28a* construct obtained from the plasmid miniprep was taken and transformed into *BL21 (DE3)-RIL* competent cells. The competent cells were prepared according to the procedure described in **Section 2.11**. The transformation process followed a standard protocol as previously discussed in **Section 2.14**. The transformed cells were plated onto LB agar supplemented with ampicillin, and positive colonies were identified. To increase the number of single positive colonies, the positive colonies on the LB agar/ampicillin plates were restreaked. The restreaked plates were then stored at 4 °C for further studies related to expression analysis.

2.20 Colony PCR confirmation of *Chymosin/ pET28a / BL21 (DE3)-RIL*

Colony PCR was conducted to confirm the successful transformation of *Chymosin/ pET28a* plasmid in *BL21 (DE3)-RIL* cells following the protocol discussed in **Section 2.14.2.** The PCR reaction mixture mentioned in **Table 2.19** was prepared by adding the necessary components, including PCR buffer, dNTPs, forward and reverse primer, Taq polymerase, and sterile distilled water.

Table 2.18 Colony PCR Cycle Reaction Mixture

Components	Stock Concentration	Required Concentration	Required Volume (µl)
10X Taq buffer	10X	1X	2
Magnesium chloride ($MgCl_2$)	25Mm	2mM	1.5
dNTP's	2.5mM	0.2mM	1.5
Forward primer	10 µM	0.1 µM	0.5
Reverse primer	10 µM	0.1 µM	0.5
Taq Polymerase	5U/ µl	0.15U/ µl	1
Bacterial colony			
ddH_2O			13
Total Volume			20

2.21 Expression of *Chymosin/ pET28a* in *E. coli BL21 (DE3)-RIL*

To analyze the expression of *chymosin/ pET28a* in *E. coli BL21 (DE3)-RIL*, a single transformed colony was selected based on positive confirmation through colony PCR. This colony was then inoculated into 20 mL of LB broth supplemented with 100 µg/mL ampicillin and 60 µg/mL chloramphenicol. As a control, *BL21 (DE3)-RIL* cells were also inoculated into 20 mL of LB broth under the same conditions. The cultures were incubated at 37 ºC for 16 hours in a shaking. The following day, 1 mL of each bacterial culture was transferred into 5 separate flasks, each containing 20 mL of LB broth supplemented with 100 µg/mL ampicillin and 60 µg/mL chloramphenicol. All the flasks were incubated at 37 ºC in a shaking incubator until the optical density at 600 nm (OD600) reached a range of 0.7 to 0.8.

Subsequently, a stock solution of 100 mM IPTG (Isopropyl β-D-thiogalactoside) was prepared according to the specified recipe in **Table 2.20.** The bacterial cells were then induced with different concentrations of IPTG (0 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM). The induced flasks were incubated at 25 ºC for 16 hours in a shaking incubator. The following day, the post-induction OD600 of all cultures was measured using a spectrophotometer. Based on the OD600 readings obtained at different IPTG concentrations, graphs were generated to depict the IPTG induction, as shown in **Figure 2.7**. To visualize the proteins on SDS PAGE, an equal volume of cells with normalized cell density was lysed. To achieve this, an optical density of 10 at 600 nm was selected as the standard for cell density normalization.

Table 2.19 Preparation of 100 mM IPTG Solution (10 mL)

Components	Concentration	Amount required
IPTG (Isopropyl β-Dthiogalactoside)	100 mM	0.2383 g
MilliQ Water	-	Up to 10 mL

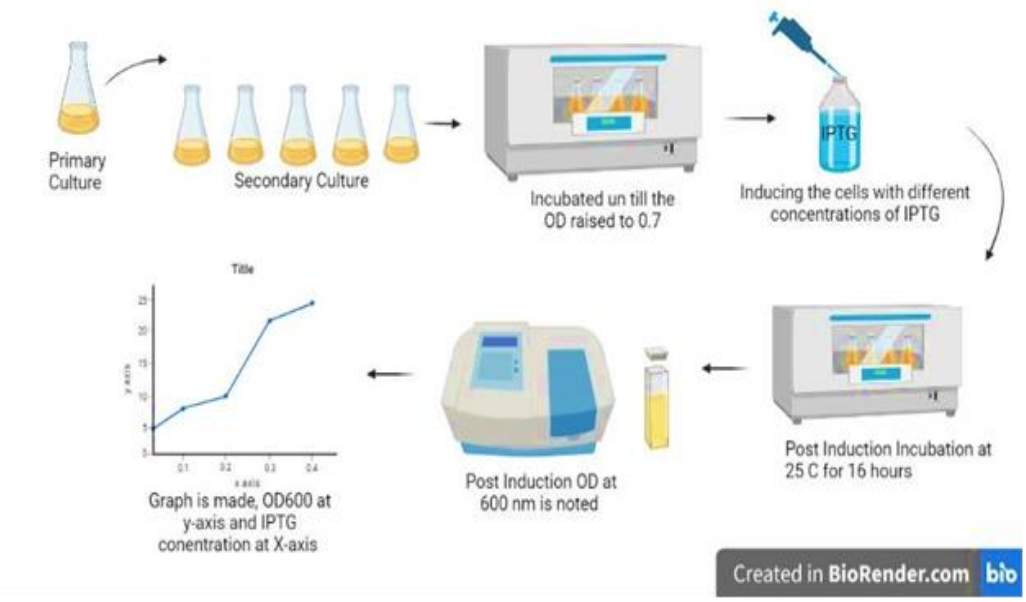

Figure 2.7 Overview of the protocol used for the IPTG induction to carry out the expression of *Chymosin/ pET28a* in *E. coli BL21 (DE3)-RIL*

2.22 Cell Lysis

Before the lysis of cells, the optical density of cell cultures was normalized considering 1 OD at 600 nm equals to 1×10^9 Cells (Elbing & Brent, 2019; Mira, 2022). For this, an amount of buffer was added into harvested bacterial cells to make the final OD_{600} to 10 using the formula mentioned in equation 1,

$$\frac{OD_{600} \times \text{Volume of Bacterial culture to make cell pellet (mL)}}{\text{Required Number of OD's}} \quad \text{..... (Equation 1)}$$

To initiate cell lysis, the cell culture was harvested through centrifugation using an Eppendorf microcentrifuge 5430 R at a speed of 12,000 rpm for 15 minutes. The resulting supernatant was discarded, and the cell pellet was then re-suspended in a solution of 50 mM Tris-Cl at pH 8.0. The re-suspended cells underwent lysis using a sonication method employing 4 cycles, with each cycle consisting of 10 seconds of sonication followed by 20 seconds of rest at 60% amplitude. Subsequently, the entire lysate was subjected to another round of centrifugation at 12,000 rpm for 30 minutes at 4 ºC using an Eppendorf centrifuge 5430R. The resulting cell pellet was once again re-suspended in a 50 mM Tris-Cl solution at pH 8.0. The samples were stored at 4 ºC for subsequent processing. The specific steps involved in the cell lysis protocol are visually represented in **Figure 2.8.**

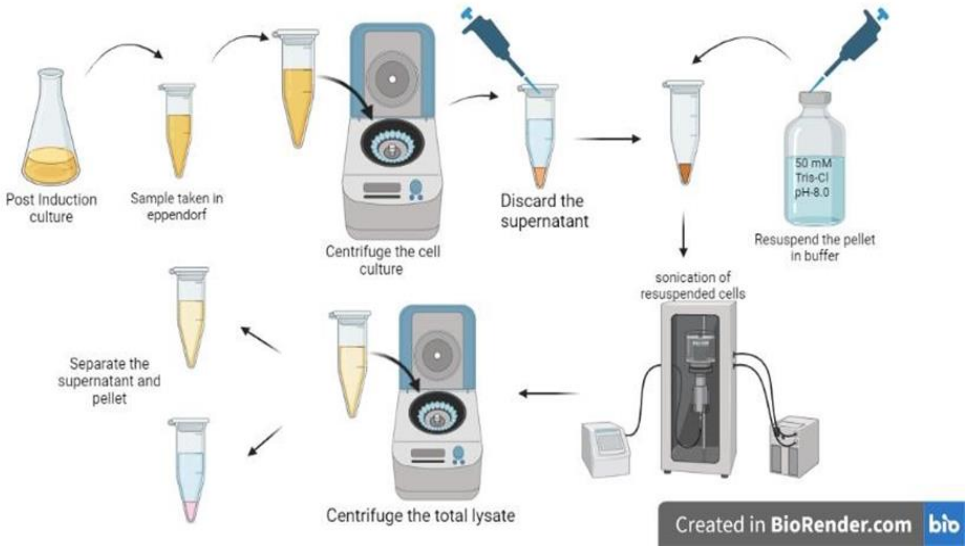

Figure 2.8 The general layout of carrying out the cell lysis.

The pellet was suspended by vertexing and sonicated with a pulse amplitude of 60% for 10-15 sec while samples were kept on ice during the sonication procedure. The expression was detected in the form of inclusion bodies observed by

lysis results. The gel samples were prepared as mentioned above and analyzed by SDS PAGE.

2.23 Sample preparation for SDS PAGE analysis

For preparing the samples for SDS PAGE, 5**x** reducing dye was prepared as per the recipe mentioned in **Table 2.21**. 1**x** working concentration of reducing dye was used. The sample and dye were mixed in 4:1 and then heated at 95 ^{o}C for 10 minutes on.

Table 2.20 Recipe of 5x Reducing Dye for SDS PAGE (10 mL)

Component	Amount required
1M Tris-Cl (pH-6.8)	2.5 mL
10% SDS	2 mL
Bromophenol blue dye	0.1 g
50% Glycerol	5 mL
2- MercaptoEthanol	0.5 mL

2.24 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE, a widely used biochemical method for protein separation based on molecular weights (Pavlova et al., 2018), was employed to analyze the expressed proteins in the cells. The CBS Scientific vertical apparatus was utilized for conducting the SDS PAGE. The procedure began by wrapping sealing material around the edges of a large glass plate and properly aligning the spacers on the plate. Subsequently, a second plate was placed on top of the first plate, and the entire

assembly was secured using three clamps. The apparatus was checked for any signs of water leakage.

To prepare the gel, a 12% resolving gel **(Table 2.22)** was created and poured between the plates. Isopropanol was then poured above the resolving gel, and the gel was left at room temperature for polymerization. After polymerization, the isopropanol was discarded, and a 5% stacking gel **(Table 2.23)** was poured on top of the resolving gel. A 10-well comb with a width of 1 mm was inserted into the stacking gel. The gel was allowed to polymerize at room temperature for approximately 40 minutes. Concurrently, a 10x Tris-glycine (TG) buffer **(Table 2.24)** was prepared, and a 1x TG buffer was subsequently made from it. Once the gel had polymerized, the plates were placed into the tank, and the buffer was added. The comb was carefully removed, and the wells were washed with the buffer. Following this, the prepared SDS PAGE samples were loaded into the wells. In the first well of the SDS PAGE gel, 8µL of Thermo Scientific PageRuler Unstained Protein Ladder (Cat.no # 26614) was loaded as a molecular weight marker. Subsequent wells were loaded with the respective protein samples. It was further ensured that there were no air bubbles left at the bottom of the gel because they can disrupt the flow of electric current. The lid of the apparatus was closed, and the anode and cathode plugs were inserted into the power supply. Initially, the voltage was set at 80 V to allow the protein samples to stack, and once stacking was complete, the voltage was increased to 120 V. The gel was run until the dye was released from the bottom of the gel and samples migrated sufficiently through the gel and into the buffer. Finally, the gels were carefully removed from the apparatus for further analysis. The overview of the SDS PAGE analysis is depicted in **Figure 2.9**.

Table 2.21 Composition of 12% resolving gel (5 mL)

Components	Required volume per gel
Water	1.6 mL
30% Acrylamide	2.0 mL
1.5 M Tris-Cl (pH – 8.8)	1.3 mL
10% SDS	0.05 mL
10% Ammonium persulfate	0.05 mL
TEMED	0.002 mL

Table 2.22 Composition of 5% stacking gel (2 mL)

Components	Required volume per gel
Water	1.4 mL
30% Acrylamide	0.33 mL
1M Tris-Cl (pH – 6.8)	0.26 mL
10% SDS	0.02 mL
10% Ammonium persulfate	0.02 mL
TEMED	0.002 mL

Table 2.23 10x TG Buffer Recipe for SDS PAGE Analysis (1 L)

Components	Required volume per gel
Trizma base	30.0 g
Glycine	144 g
10% SDS	100 mL
Distilled water	Up to 1000 mL

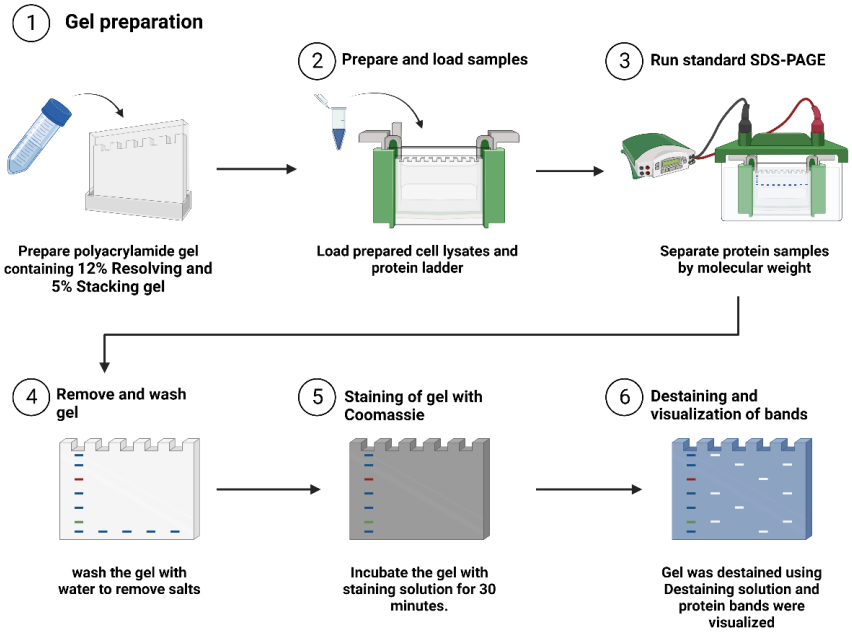

Figure 2.9 Overview of SDS PAGE

2.24.1 Staining and Destaining

Firstly, the staining solution is made as per the recipe mentioned in **Table 2.25.** Gel was soaked in the staining solution on the glass petri plate. The Petri plate was settled on the orbital shaker overnight. The next day the destaining solution was made (**Table 2.26**) and the gel was taken out of the staining solution. Now the gel was washed 2-3 times with the distilled water to remove the extra stain from it and then it was put into the destaining solution for 2 hours and then gel was put into the distilled water. The staining and destaining solution can be preheated up to 50-60 °C to speed up the process of staining and destaining within 15-30 minutes. After complete destaining, the gel was observed under visible light.

Table 2.24 Composition of Staining solution (1 L)

Components	Amount required
Methanol	500 mL
Glacial Acetic acid	100 mL
Coomassie Brilliant Blue (R-250)	0.5 g
Distilled water	Up to 1000 mL

Table 2.25 Composition of Destaining solution (1 L)

Components	Amount required
Methanol	300 mL
Glacial Acetic acid	100 mL
Distilled water	Up to 1000 mL

2.25 Large Scale Expression of *Chymosin/ pET28a in E. coli BL21 (DE3)-RIL*

The large-scale expression was obtained at a 2-liter scale in four flasks while each flask contained 500 ml of LB media. The large-scale expression was obtained following the strategy of small-scale expression as described in **Section 2.21** without any IPTG induction because the expression was leaky and was maximum without any IPTG induction. The media was centrifuged at 6000 rpm or 7,200 g for 10 minutes at 4 °C and wet pellet weight was measured which was 10 g.

2.25.1 Preparation of Inclusion Bodies

The inclusion bodies of chymosin were prepared by sonication. The total time of sonication was 25 minutes, and the sample was kept on ice during the

whole procedure. The 5g pellet was suspended in 50 ml of wash buffer (**Table 2.27)** and sonicated with a pulse amplitude of 60% with a pulse of 10 sec on and 20 sec off. After the first 10 minutes of sonication, the sonicated sample was centrifuged at 6500 rpm or 5243 g for 20 min. The supernatant was stored, and the pellet was suspended in 50 ml of 20 mM Tris-HCl pH 8.0 and sonicated for 10 minutes according to the above sonication parameters and the same procedure (sonication and centrifugation) was repeated every 5 minutes sonication cycle, until 25 minutes of complete sonication. 200 µl of the sample was removed before every centrifugation for PAGE analysis.

Table 2.26 Composition of Wash Buffer

Components	Required Concentration
Triton X100	3%
Tris-Cl pH;8	50mM
EDTA pH;8	10 mM
Sodium chloride	0.2 M
DTT	4mM

2.25.2 Protein Analysis

Analysis of protein samples i.e., whole cell lysate, supernatants, and inclusion bodies was done by using 12% of SDS PAGE gel (**Sections 2.23**).

2.25.3 Quantification of Inclusion Bodies by UV Spectrophotometer

Inclusion bodies (suspended in 50 ml of 20 mM Tris-HCl, pH 8.0) were prepared in **Section 2.24.1** and quantified by UV spectrophotometry. To determine the amount of inclusion bodies, the OD_{280} obtained from the spectrophotometer was multiplied by the dilution factor of 20 and then further multiplied by the total volume of the suspended inclusion bodies.

Amount of inclusion bodies = OD_{280} x Dilution Factor x Total volume of the suspension.

930 OD_{280} were obtained from a 10g pellet of 2-liter culture.

2.26 Solubilization and Refolding of Chymosin Inclusion Bodies

2.26.1 DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) Assay

The DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) assay, also known as Ellman's assay, is commonly used to measure the concentration of sulfhydryl groups (thiols) in a sample. The reagents required for a basic DTNB assay include:

2.26.1.1 100 ml 1 M Disodium Hydrogen Phosphate ($Na2HPO_4$)

To create a 1 M disodium hydrogen phosphate solution, 14.195 g of Na2HPO4 was dissolved in 60 ml of distilled water, and the final volume was adjusted to 100 ml using distilled water.

2.26.1.2 100 ml 1 M Sodium Dihydrogen Phosphate

1 M sodium dihydrogen phosphate solution was obtained by adding 13.799 g of NaH_2PO4 to 50 ml of distilled water, followed by adjusting the final volume to 100 ml with distilled water.

2.26.1.3 300ml 1 M NaH_2PO_4 Buffer pH 7.0

For the 0.1 M sodium dihydrogen phosphate buffer with a pH of 7.0, a mixture of 12.6 ml of disodium hydrogen phosphate and 17.31 ml of sodium hydrogen phosphate was prepared in a reagent bottle. The solution was then diluted to a final volume of 300 ml with distilled water, autoclaved for 20 minutes, and stored at room temperature.

2.26.1.4 20ml 7.4 mM DTNB reagent

The DTNB reagent, with a concentration of 7.4 mM, was prepared by combining 20 ml of 0.1 M sodium dihydrogen phosphate buffer (pH 7.0) with 58.8 mg of DTNB in a dark reagent bottle. This reagent was stored at 4 °C and allowed to reach room temperature before use.

2.26.1.5 200 ml Assay Buffer

The assay buffer, consisting of a 200 mM Tris-HCl solution with a pH of 8.7, was prepared by dissolving 4.84 g of Tris base in 100 ml of distilled water. The pH was adjusted to 8.7 using concentrated HCl, and the final volume was adjusted to 200 ml with distilled water. The assay buffer was stored at room temperature.

2.26.1.6 The procedure of DTNB Assay

The UV spectrophotometer was adjusted to a wavelength of 412 nm. For the DTNB assay, a glass cuvette was filled with 900 µl of assay buffer and 100 µl of DTNB reagent. The spectrophotometer was auto-zeroed using this solution as the reference. Subsequently, 10 µl of the sample was introduced into the cuvette, and the absorbance was recorded. A plot of absorbance versus time was generated to visualize the data obtained from the assay.

2.26.2 Solubilization of Chymosin Inclusion Bodies

The inclusion bodies were solubilized in 8M urea as a solubilizing agent. The urea solubilization buffer of pH 11.0 was used as described below in **Table 2.28**.

Table 2.27 Urea solubilization buffer (110ml)

Component	Required concentration	Amount Required
Urea	8 M	52.8 g
Tris-Cl	50Mm	5.5ml of 1M without pH adjustment
Glycine	50mM	412 mg
DTT	4mM	68.8 mg

The urea solubilization buffer was prepared by dissolving 52.8 g of urea, 5.5 ml of autoclaved 1 M Tris-HCl, and 412 mg of glycine in 70 ml of autoclaved distilled water, with the pH adjusted to 11.0 using NaOH pellets. The final volume was adjusted to 110 ml with autoclaved distilled water. Just before use, 67.8 mg of DTT was added. A total of 440 OD_{280} out of 930 OD_{280} of inclusion bodies were suspended in 110 ml of the solubilization buffer through sonication. The suspended inclusion bodies underwent sonication for 2 minutes at a pulse amplitude of 60%, with a cycle of 10 seconds on and 20 seconds off. Subsequently, they were incubated at 55°C for 30 minutes, followed by another round of sonication and an additional incubation at 37°C for 1.5 hours. Sonication was performed every 30 minutes during the solubilization process. The solubilized inclusion bodies were then centrifuged at 17000 rpm and 4 °C for 20 minutes. The resulting supernatant was quantified at 280 nm against the solubilization buffer, yielding a reading of 420 OD_{280} units in solubilized form. The solubilized inclusion bodies were subsequently subjected to in vitro refolding.

2.26.3 Refolding of Solubilized Chymosin

For the refolding process, a total of 420 OD_{280} units of solubilized inclusion bodies of chymosin were utilized. To prepare the refolding buffer (**Table 2.29)** with a volume of 750 ml, the following components were added: 75 ml of autoclaved 1 M Tris-HCl, 75 ml of 99.99% glycerol, 3 ml of autoclaved 0.5 M EDTA, 40 g of urea, and 75 ml of 50 mM cystine. Additionally, 60 mg of cysteine was dissolved in 530 ml of autoclaved distilled water. The pH of the solution was adjusted to 11 by the addition of NaOH pellets. Before reaching the final volume, 750 µl of 0.1 mM PMSF was incorporated. Autoclaved distilled water was added to achieve a total volume of 750 ml.

Table 2.28 Refolding Buffer (pH:11)

Components	Required Concentration
Tris-HCl (Without pH adjustment pH;10.95)	0.1 M
Glycerol	10%
EDTA	2 mM
Cystine (From 50 mM in 0.3 N HCl solution)	5 mM
Cysteine	0.5 Mm
PMSF (Phenylmethylsulphonyl fluoride, prepared in 100 % isopropanol)	0.1 M
Urea	2 M

2.26.3.1 Reverse Dilution Refolding Method

For refolding, both solubilized inclusion bodies and refolding sink (buffer) were placed in an ice bath. Using a peristaltic pump at a flow rate of

approximately 5 – 6 ml per minute, the refolding buffer was transferred to solubilized inclusion bodies and placed in an ice bath, with a gentle stirring. The flow rate was adjusted so that the refolding buffer was transferred into the inclusion bodies sink within 2 hours. The final concentration of refolded reconstitute (solubilized inclusion bodies and refolding buffer) was 0.5 OD280/ml. Thiol contents were monitored by DTNB assay after every half an hour. 200 µl of protein sample was alkylated after every half an hour.

2.26.4 Alkylation of Protein Sample and their PAGE Analysis

A freshly prepared solution of 0.5 M iodoacetamide was prepared by dissolving 9.2 mg of iodoacetamide in 100 µl of autoclaved distilled water. The iodoacetamide was placed in a microfuge tube wrapped in aluminum foil and kept on ice till for use. Protein samples were collected, both before and during the refolding process, at different time intervals. These protein samples were then treated with 10 mM iodoacetamide by adding 4 µl of the 0.5 M iodoacetamide solution to a microfuge tube covered in aluminum foil. The samples were subsequently incubated at 37 °C for 45 minutes and then stored at -20 °C. The next day, a 12% PAGE (both SDS and Native on the same gel because they only differ in the composition of loading dye, and the analysis was performed as mentioned in **Section 2.23** to examine the refolding profile of chymosin. The composition of Native PAGE dye is mentioned in following **Table2.30.**

Table 2.29 Recipe of 5x Native PAGE loading dye (10 mL)

Component	Amount
1 M Tris-Cl (pH-6.8)	2.5 mL
Water	2 mL
Bromophenol blue dye	0.1 g
50% Glycerol	5 mL
2-Mercaptoethanol	0.5 mL

2.26.5 Dialysis after Refolding

Dialysis is a biochemical method that allows the separation of small molecules from a mix of different size molecules. The sample is added to a dialysis membrane that has a specific pore size. Small molecules diffuse through the pores into the solvent and bigger molecules remain in the membrane. After the refolding process, dialysis was performed to eliminate salts from the protein solution. Dialysis was conducted using Spectra/Pro® dialysis membrane, specifically a dialysis tubing with a pore size of 3.5 kDa, a flat width of 45 mm, and a diameter of 29 mm. The refolding reconstitute was dialyzed against a 20 mM Tris-HCl buffer at pH 8.0 (**Figure 2.10**). The buffer solution was changed every 2-3 hours, with a total of 5-6 buffer exchanges. Following the dialysis procedure, the protein concentration was determined by measuring its absorbance at 280 nm using a UV spectrophotometer.

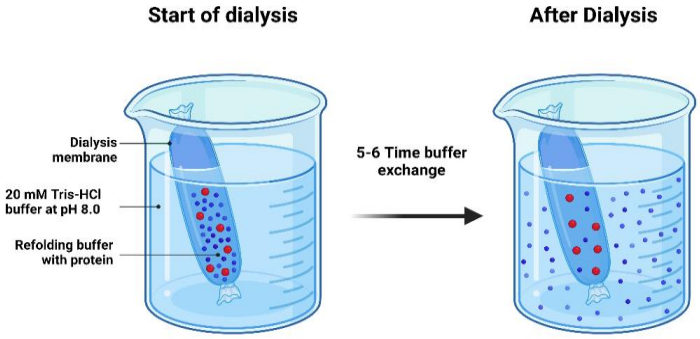

Figure 2.10 Dialysis After Refolding

2.26.6 The concentration of the Dialyzed Protein Solution by Lyophilization

To concentrate the dialyzed chymosin protein, a Christ Alpha 1-2LD Plus Lyophilizer was employed in the experimental procedure. Initially, the samples were pre-frozen with liquid nitrogen in freeze-drying round bottom flasks. Prior to initiating the main drying process, it was essential to ensure that the operation temperature was reached and that the vacuum pump was loaded with condensable gases. The vacuum pump was either warmed up for at least 15 minutes or switched on at least 15 minutes before commencing the primary drying process. The pre-frozen samples in freeze-drying round bottom flasks were fixed on a lyophilizer and airtight with the knob. The temperature of the ice condenser was set at -40 ºC, and the pressure was adjusted to 0.120 mbar. The volume of dialyzed protein solution was reduced to 400 ml from 1200 ml by lyophilizer.

2.27 Purification of Chymosin by Anion Exchange Chromatography

Ion-exchange chromatography is a powerful technique used in protein purification that exploits the differences in the charge properties of biomolecules. This method relies on the reversible adsorption of charged molecules to a solid support, such as ion exchange resins, based on their electrostatic interactions with charged

functional groups present in the resin matrix (Giacometti, 2013). The principle behind ion-exchange chromatography is that proteins or other charged molecules of interest can be selectively bound to the resin by exploiting their differences in net charge or surface charge distribution (Shekhawat, 2000). By controlling the pH and ionic strength of the buffer solutions used, the adsorption and subsequent elution of the target protein can be precisely manipulated. This enables the separation and purification of the desired protein from complex mixtures (Kumari, 2022).

A Bio-Rad glass column with dimensions of 20 cm in length and 3 cm in diameter was utilized for the purification of chymosin through ion-exchange chromatography. To prepare the column, approximately 40-45 ml of pre-soaked DEAE Sephadex slurry was carefully poured into the column. The column was washed with 5 column volumes (200 ml) of distilled water to remove any preservatives. Subsequently, the column was equilibrated with 2 column volumes of 20 mM Tris-HCl at pH 8.0, ensuring a suitable environment for the subsequent purification steps.

For protein sample loading, a peristaltic pump was used to load the protein sample onto the column at a rate of 2-2.5 ml/min. The protein sample, with an optical density at 280 nm of 350 OD_{280}/400ml, was carefully loaded onto the column. The flow through during the loading phase (400 ml) was collected and quantified at 280 nm to monitor the presence of chymosin. Following the confirmation of chymosin binding to the DEAE Sephadex column, unbound proteins were removed by washing the column with 2 column volumes (100 ml) of 20 mM Tris-HCl at pH 8.0. This step ensured the removal of any non-specifically bound or weakly interacting molecules. To elute the chymosin, a step-gradient elution was employed using 30 ml of NaCl at various concentrations (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M,

0.6 M, and 1 M) prepared in 20 mM Tris-HCl at pH 8.0. Elution fractions of 10 ml were collected and quantified at 280 nm to assess the presence of chymosin and monitor its elution pattern (**Figure 2.11**).

To ensure the proper maintenance of the DEAE Sephadex resin, it was thoroughly cleaned and regenerated. The DEAE Sephadex resin was washed sequentially with 200 ml of the following solutions: 3 M NaCl, distilled water, 1 N HCl, distilled water, 1 N NaOH, and distilled water. The DEAE Sephadex slurry was removed and stored in 20% ethanol at 4 °C. Finally, the column was washed with distilled water, air-dried, and stored at room temperature for future use (Cummins, 2017).

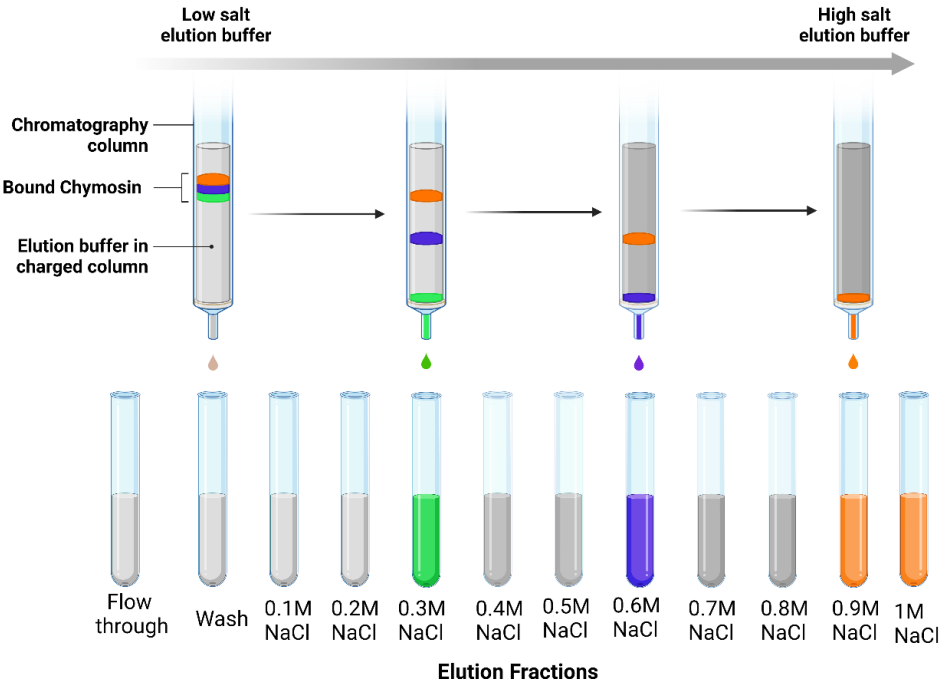

Figure 2.11 Overview of Ion Exchange Chromatography

2.27.1 Concentrating Purified Protein by Ultra Centrifugal Filtration (Concentrator)

To concentrate the purified protein through ion exchange chromatography the eluted fractions with maximum OD_{280} in the concentrator column were used. The column underwent a series of washing steps. Initially, the column was washed with distilled water through centrifugation at 4 ℃ and 3500 rpm using an Eppendorf 5810 R centrifuge, lasting for 4 minutes. Subsequently, the column was equilibrated with the dialysis buffer and re-centrifuged at 4 ℃ and 3500 rpm for 4 minutes. The purified protein fractions were mixed and added to the equilibrated column and centrifuged at 4℃ and 3500 rpm for 5 to 6 minutes. To prevent protein aggregates from settling at the bottom of the column, gentle mixing was performed using a pipette after removing the column. The column was then centrifuged again at 4 ℃ and 3500 rpm for 5 to 6 minutes. This process was repeated until the protein was concentrated to the desired volume. Finally, the concentrated protein was carefully retrieved from the column and stored at -20 ℃ in 40% glycerol for future use.

2.28 Activation of pro-Chymosin to Active Chymosin

Like other zymogens found in the stomach, prochymosin undergoes limited proteolysis to transform into an active enzyme, specifically at a pH below 5. The rate of this conversion process is significantly enhanced by lowering the pH from 5 to 2. The initial studies on calf prochymosin activation provided insights into the mechanism of converting gastric zymogens into active enzymes. These studies suggested that the first step involves a pH-dependent conformational change that exposes the active site crevice. At pH 2, cleavage of calf prochymosin occurs at the Phe25p-Leu26p site, resulting in the formation of an intermediate known as pseudo chymosin, which remains stable at pH 2. The maturation of chymosin takes place

through activation at pH 4-5, and pseudo-chymosin is subsequently converted into chymosin at pH 5.5 (Aboulnaga, 2019).

So, to convert prochymosin to chymosin the pH of the enzyme solution was decreased to 2 with HCL, and the mixture was placed in the incubator for 30 minutes at 37 °C. After the reaction mixture was taken out and the pH was increased to 6 with 5 N NaOH. The reaction mixture was paced at -20 °C for further activity assays and Tricine PAGE analysis to confirm the proteolytic cleavage and activation of prochymosin to active chymosin. Tricine PAGE is known for its better resolution as compared to SDS PAGE. The active chymosin and the released peptide of about 4.9 kDa were analyzed by 15% Tricine-PAGE as resolving gel while 5% staking gel was the same as for SDS PAGE.

CHAPTER 3

RESULTS

3.1 Derivation of the Chymosin Gene Sequence of *Bos taurus* (Bovine)

The chymosin gene sequence of *Bos taurus* (Bovine) was taken from NCBI accession number *(NM_180994.2*) is mentioned in following **Figure 3.1**.

AGCAGCGGCTGGACCCAGATCCAAGATGAGGTGTCTCGTGGTGCTACTTGCTGTCTT
CGCTCTCTCCCAAGGCGCTGAGATCACCAGGATCCCTCTGTACAAAGGCAAGTCTCT
GAGGAAGGCGCTGAAGGAGCATGGGCTTCTGGAGGACTTCCTGCAGAAACAGCAGT
ATGGCATCAGCAGCAAGTACTCCGGCTTCGGGGAGGTGGCCAGCGTGCCCCTGACCA
ACTACCTGGATAGTCAGTACTTTGGGAAGATCTACCTCGGGACCCCGCCCCAGGAGTT
CACCGTGCTGTTTGACACTGGCTCCTCTGACTTCTGGGTACCCTCTATCTACTGCAAG
AGCAATGCCTGCAAAAACCACCAGCGCTTCGACCCGAGAAAGTCGTCCACCTTCCA
GAACCTGGGCAAGCCCCTGTCTATCCACTACGGGACAGGCAGCATGCAGGGCATCCT
AGGCTATGACACCGTCACTGTCTCCAACATTGTGGACATCCAGCAGACAGTAGGCCT
GAGCACCCAGGAGCCCGGGGACGTCTTCACCTATGCCGAATTCGACGGGATCCTGGG
GATGGCCTACCCCTCGCTCGCCTCAGAGTACTCGATACCCGTGTTTGACAACATGATG
AACAGGCACCTGGTGGCCCAAGACCTGTTCTCGGTTTACATGGACAGGAATGGCCAG
GAGAGCATGCTCACGCTGGGGGCCATCAACCCGTCCTACTACACAGGGTCCCTGCAC
TGGGTGCCCGTGACAGTGCAGCAGTACTGGCAGTTCACTGTGGACAGTGTCACCATC
AGCGGTGTGGTTGTGGCCTGTGAGGGTGGCTGTCAGGCCATCTTGGACACGGGCACC
TCCAAGCTGGTCGGGCCCAGCAGCGACATCCTCAACATCCAGCAGGCCATTGGAGCC
ACACAGAACCAGTACGGTGAGTTTGACATCGACTGCGACAACCTGAGCTACATGCCC
ACTGTGGTCTTTGAGATCAATGGCAAAATGTACCCACTGACCCCCTCCGCCTATACCA
GCCAAGACCAGGGCTTCTGTACCAGTGGCTTCCAGAGTGAAAATCATTCCCAGAAAT
GGATCCTGGGGGATGTTTTCATCCGAGAGTATTACAGCGTCTTTGACAGGGCCAACAA
CCTCGTGGGGCTGGCCAAAGCCATCTGATCACATCGCTGACCAAGAACCTCACTGTC
CCCACACACCTGCACACACACATGCACACATGTACATGAGCACATGTGCACACACAC
AGATGAGGTTTCCAGACAGATGATTCTCAATAAACGTTGTCTTTC

Figure 3.1 Gene sequence of Chymosin: ATG: Start codon; ATC: Stop codon.

3.2 Selection of only the Chymosin protein sequence and its Codon Optimization

The heterologous expression is carried out to produce recombinant bovine chymosin. For this purpose, only the chymosin gene sequence was selected from the

whole coding sequence. Codon is considered as the information carrier for protein synthesis. According to the already established data and knowledge, more than one codon can encode for the same amino acid and this feature has been named as the synonymous codon (Menzella, 2011). During the translation process, the usage of synonymous codons varies from organism to organism. Organisms may use one or several synonymous codons for an amino acid, these codons are known as optimal codons or codon usage biasness. Heterologous expression of the proteins is highly related to the codon usage biasness. Rare codons have the potential to reduce the translation rate. To increase the expression, during gene synthesis, codon optimization is carried out and the codon-optimized sequence of chymosin is mentioned in **Figure 3.2**.

ATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAATCACTGAGAAAAGCGCTG
AAAGAACACGGACTGCTGGAAGACTTTCTGCAAAAACAACAATATGGAATTTCATCA
AAATATTCAGGATTTGGAGAAGTGGCGTCAGTGCCGCTGACAAACTATCTGGACTCA
CAATATTTTGGAAAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTG
ACACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGCGTGTAA
AAACCACCAAAGATTTGACCCGAGAAAATCATCAACATTTCAAAACCTGGGAAAACC
GCTGTCAATTCACTATGGAACAGGATCAATGCAAGGAATCCTGGGATATGACACAGTG
ACAGTGTCAAACATTGTGGACATTCAACAAACAGTGGGACTGTCAACACAAGAACC
GGGAGACGTGTTTACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCA
CTGGCGTCAGAATATTCAATTCCGGTGTTTGACAACATGATGAACAGACACCTGGTGG
CGCAAGACCTGTTTTCAGTGTATATGGACAGAAACGGACAAGAATCAATGCTGACAC
TGGGAGCGATTAACCCGTCATATTATACAGGATCACTGCACTGGGTGCCGGTGACAGT
GCAACAATATTGGCAATTTACAGTGGACTCAGTGACAATTTCAGGAGTGGTGGTGGC
GTGTGAAGGAGGATGTCAAGCGATTCTGGACACAGGAACATCAAAACTGGTGGGAC
CGTCATCAGACATTCTGAACATTCAACAAGCGATTGGAGCGACACAAAACCAATATG
GAGAATTTGACATTGACTGTGACAACCTGTCATATATGCCGACAGTGGTGTTTGAAAT
TAACGGAAAAATGTATCCGCTGACACCGTCAGCGTATACATCACAAGACCAAGGATTT
TGTACATCAGGATTTCAATCAGAAAACCACTCACAAAAATGGATTCTGGGAGACGTG
TTTATTAGAGAATATTATTCAGTGTTTGACAGAGCGAACAACCTGGTGGGACTGGCGA
AAGCGATT

Figure 3.2 Codon Optimized Gene sequence of Chymosin: ATG: Start codon; ATT: Stop codon.

Initially, the codon optimization of the chymosin was carried out according to the *Bacillus subtilis* expression system. But in the current study, I am using the same codon-optimized chymosin gene to study its expression in *E. coli* because due to the universal nature of codon and codon redundancy mostly it works well across the species. The sequence was synthesized, mainly focused on reducing the GC content, reducing the number of inverted and direct repeats to prevent secondary structures and enhance the life of mRNA. The ideal GC content percentage is 30 to 70% and in the optimized sequence, GC count was acquired as 43.3% (**Figure 3.3).**

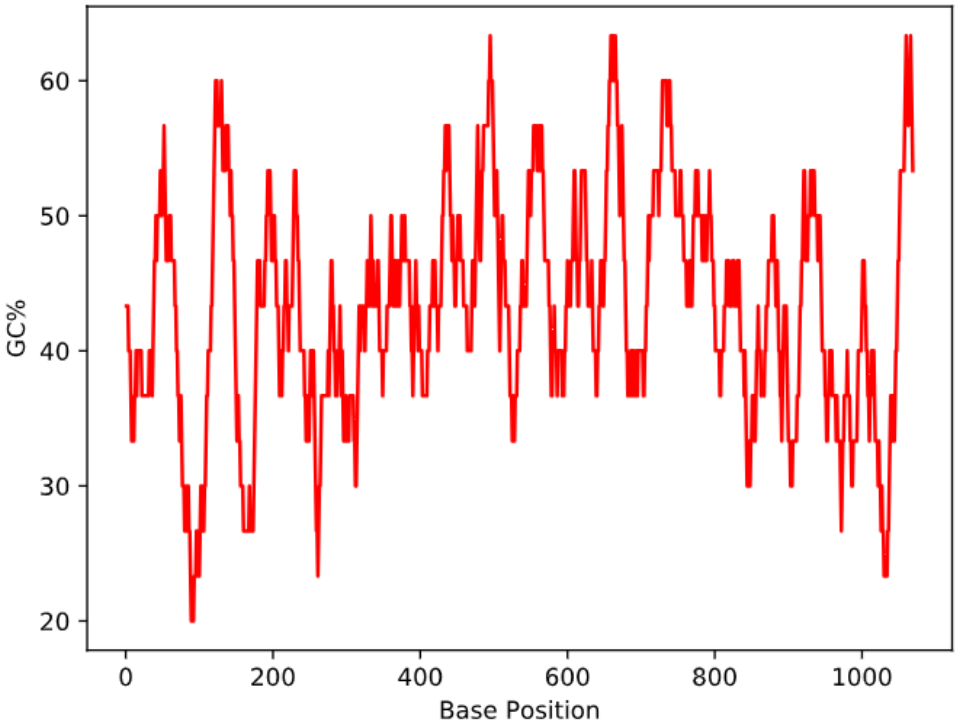

Figure 3.3 %GC Content in Codon Optimized Chymosin Gene

3.3 Sequence of Nucleotide Encoding the Amino Acids

The nucleotide sequence of the codon-optimized Chymosin Gene was analyzed for protein translation by using Expasy Translate Tool (https://web.expasy.org/translate/). The results obtained for encoded protein are given below.

atg gcg gaa att aca aga atc ccg ctg tat aaa gga aaa tca ctg aga aaa gcg ctg aaa

M A E I T R I P L Y K G K S L R K A L K

gaa cac gga ctg ctg gaa gac ttt ctg caa aaa caa caa tat gga att tca tca aaa tat

E H G L L E D F L Q K Q Q Y G I S S K Y

tca gga ttt gga gaa gtg gcg tca gtg ccg ctg aca aac tat ctg gac tca caa tat ttt

S G F G E V A S V P L T N Y L D S Q Y F

gga aaa att tat ctg gga aca ccg ccg caa gaa ttt aca gtg ctg ttt gac aca gga tca

G K I Y L G T P P Q E F T V L F D T G S

tca gac ttt tgg gtg ccg tca att tat tgt aaa tca aac gcg tgt aaa aac cac caa aga

S D F W V P S I Y C K S N A C K N H Q R

ttt gac ccg aga aaa tca tca aca ttt caa aac ctg gga aaa ccg ctg tca att cac tat

F D P R K S S T F Q N L G K P L S I H Y

gga aca gga tca atg caa gga atc ctg gga tat gac aca gtg aca gtg tca aac att gtg

G T G S M Q G I L G Y D T V T V S N I V

gac att caa caa aca gtg gga ctg tca aca caa gaa ccg gga gac gtg ttt aca tat gcg

D I Q Q T V G L S T Q E P G D V F T Y A

gaa ttt gac gga atc ctg gga atg gcg tat ccg tca ctg gcg tca gaa tat tca att ccg

E F D G I L G M A Y P S L A S E Y S I P

gtg ttt gac aac atg atg aac aga cac ctg gtg gcg caa gac ctg ttt tca gtg tat atg

V F D N M M N R H L V A Q D L F S V Y M

gac aga aac gga caa gaa tca atg ctg aca ctg gga gcg att aac ccg tca tat tat aca

D R N G Q E S M L T L G A I N P S Y Y T

gga tca ctg cac tgg gtg ccg gtg aca gtg caa caa tat tgg caa ttt aca gtg gac tca

G S L H W V P V T V Q Q Y W Q F T V D S

gtg aca att tca gga gtg gtg gtg gcg tgt gaa gga gga tgt caa gcg att ctg gac aca

V T I S G V V V A C E G G C Q A I L D T

gga aca tca aaa ctg gtg gga ccg tca tca gac att ctg aac att caa caa gcg att gga

G T S K L V G P S S D I L N I Q Q A I G

gcg aca caa aac caa tat gga gaa ttt gac att gac tgt gac aac ctg tca tat atg ccg

A T Q N Q Y G E F D I D C D N L S Y M P

aca gtg gtg ttt gaa att aac gga aaa atg tat ccg ctg aca ccg tca gcg tat aca tca

T V V F E I N G K M Y P L T P S A Y T S

caa gac caa gga ttt tgt aca tca gga ttt caa tca gaa aac cac tca caa aaa tgg att

Q D Q G F C T S G F Q S E N H S Q K W I

ctg gga gac gtg ttt att aga gaa tat tat tca gtg ttt gac aga gcg aac aac ctg gtg

L G D V F I R E Y Y S V F D R A N N L V

gga ctg gcg aaa gcg att

G L A K A I

3.4 Translated Protein Sequence

MAEITRIPLYKGKSLRKALKEHGLLEDFLQKQQYGISSKYSGFGEVASVPLTN

YLDSQYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDPRKS

STFQNLGKPLSIHYGTGSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTY

AEFDGILGMAYPSLASEYSIPVFDNMMNRHLVAQDLFSVYMDRNGQESMLTL

GAINPSYYTGSLHWVPVTVQQYWQFTVDSVTISGVVVACEGGCQAILDTGTS

KLVGPSSDILNIQQAIGATQNQYGEFDIDCDNLSYMPTVVFEINGKMYPLTPSA

YTSQDQGFCTSGFQSENHSQKWILGDVFIREYYSVFDRANNLVGLAKAI

3.5 PCR Amplification of Chymosin from pUC57

The template for the preparation of the bovine chymosin gene was taken from the synthetic gene of bovine chymosin with a signal peptide, which was present in the *pUC57* vector. The PCR reaction and conditions used for amplification are detailed in the "Materials and Methods" section, specifically in **Section 2.6**. The forward primer, Chymosin-F, and the reverse primer, Chymosin-R, were utilized for the amplification process. The specific characteristics of these primers are provided in **Section 2.5** of the "Materials and Methods" chapter. Verification of the resulting amplification product was performed by running it on a 1% agarose gel, as described in **Section 2.7**. The amplification result of the PCR product, as shown in **Figure 3.4**, was analyzed using 1% agarose gel electrophoresis. Lanes 3-5 displayed a distinct band at 1095 bp (1.095 Kb), indicating the successful amplification of the chymosin gene. In contrast, lane 6, which served as the negative control where the template was not added, confirmed the absence of any band in the gel.

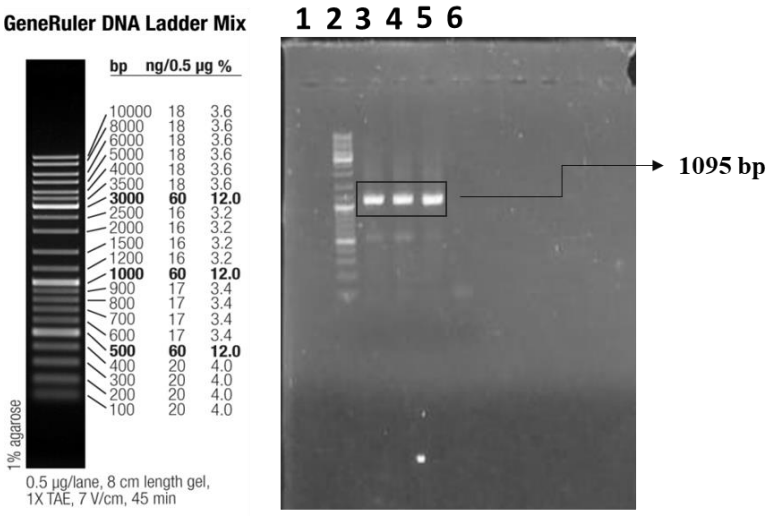

Figure 3.4; 1% agarose gel of PCR Amplification of Chymosin from pUC57

Note. **Lane 1:** Blank; **Lane 2:** Ladder (Thermo ScientificTM GeneRuler # SM0331)

Lane 3-5: Chymosin PCR product; **Lane 6:** Negative Control

3.6 Gene Purification of PCR product of Chymosin

The gel purification of the amplified PCR product measuring 1095 base pairs (bp) was performed by excising it from the gel (**Figure 3.4**) and subsequently purifying it using the GeneJET Gel Extraction Kit (Catalog number: K0692). The specific procedure for DNA purification from agarose gel was outlined in **Section 2.8** of the materials and methods. To verify the successful purification, 5 µl of the purified product was loaded and analyzed on a 1% agarose gel (**Fig. 3.5**).

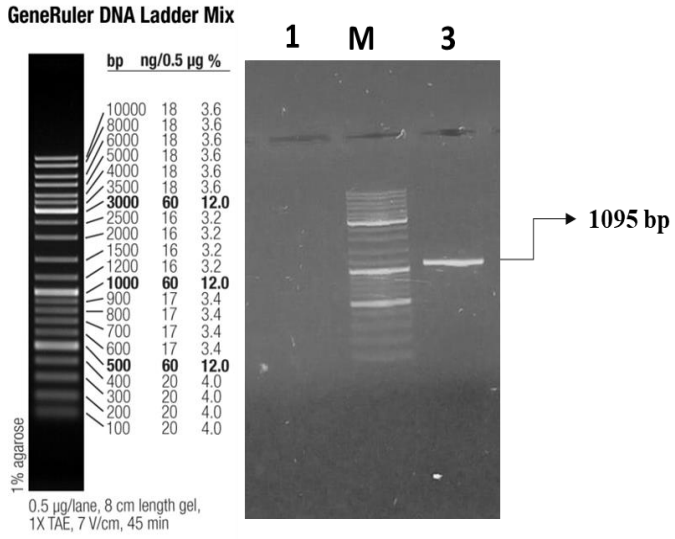

Figure 3.5 1% Agarose Gel of Purified PCR Product of Chymosin

Note. **Lane 1:** Blank**; Lane 2:** Marker (Thermo ScientificTM GeneRuler # SM0331)
Lane 3: Purified Chymosin PCR product

3.7 Blue-White Screening of colonies of *Chymosin in pTZ57 R/T* for Confirmation of Transformation into *DH5α*

Chymosin was ligated to the *pTZ57 R_T* cloning vector and the ligated vector was transformed into *DH5α* cells (**Figure 3.6)**.

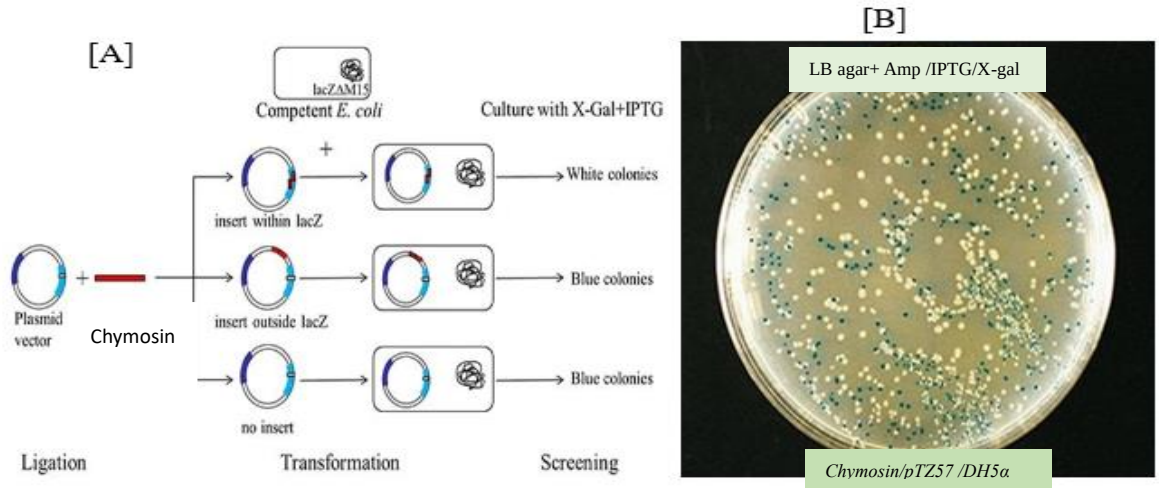

Figure 3.6 Blue-White screening of Chymosin /pTZ57R_T transformed into DH5α cells

Note. Panel **[A]** is depicting the graphical flowchart of the blue-white screening procedure and the possibilities of gene insertion points in the plasmid vector and as a result the color of colonies. Panel **[B]** is showing the white colonies of *DH5α* cells with successful transformation of *Chymosin /pTZ57R_T.*

3.8 Colony PCR of *Chymosin / pTZ57* R/T in *DH5α*

After transformation, the colony PCR was carried out by following the methodology as described in **Section 2.14.2**, all the colonies showed positive results on agarose gel (1%) electrophoresis as presented in **Figure 3.7**.

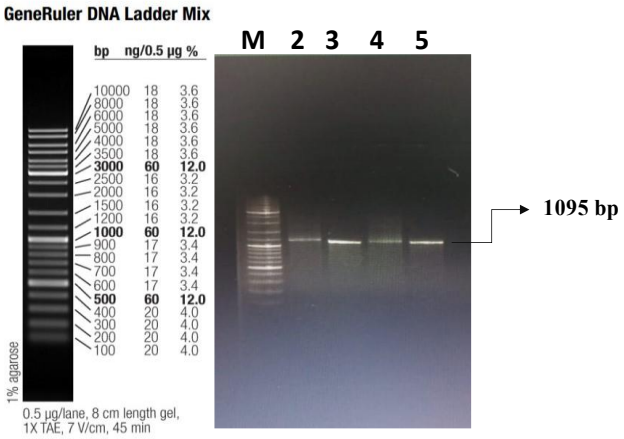

Figure 3.7 Confirmation of Chymosin in pTZ57 R/T in DH5α by Colony PCR

Note. **Lane 1:** Marker (Thermo ScientificTM GeneRuler # SM0331)**; Lane 2:** Colony 1**; Lane 3:** Colony 2**; Lane 4:** Colony 3**; Lane 5:** Colony 4

3.9 *Chymosin/pTZ57 R/T* Plasmid Isolation from *DH5α*

Chymosin/pTZ57 R/T plasmid was extracted from *Escherichia coli DH5a* cells using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Catalog number: K0502) following the procedure outlined in the materials and methodology section (**Section 2.15**). The presence of the *Chymosin/pTZ57 R/T* plasmid DNA band was confirmed through agarose gel electrophoresis (1%), in accordance with the protocol described in **Section 2.7**. The gel analysis revealed the presence of a DNA band corresponding to the *Chymosin/pTZ57 R/T* plasmid in lane 1 (**Figure 3.8**). The *Chymosin/pTZ57 R/T* plasmid was observed to be in a nicked circular conformation.

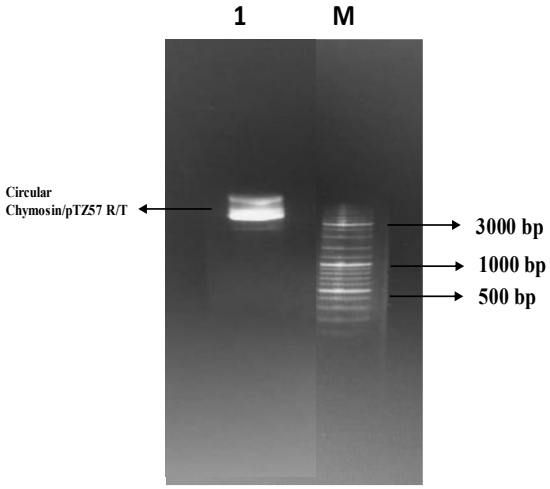

Figure 3.8 Confirmation of Chymosin/pTZ57 R/T Plasmid isolation from DH5α

Note. **Lane 1:** Marker (Thermo ScientificTM GeneRuler # SM0331)**; Lane 2:** *Chymosin/pTZ57 R/T* Plasmid

3.10 DNA Quantification

The concentration of purified plasmid and the PCR product was determined by NanoDrop™ 2000/2000c Spectrophotometers (Catalogue number: ND-2000), first of all, the wavelength verification was done, and then blank measurement was performed using 1 μL of elution buffer, following the sample measurement was

carried in similar way and values at 260/280 nm ratio were recorded and the concentration is mentioned in **Table 3.1.**

Table 3.1 DNA concentration using Nanodrop.

DNA name	Concentration	260/280	260/230
Chymosin purified PCR product	51.71 ng/µl	1.88	0.18
Chymosin/pTZ57 R/T Miniprep	287.39 ng/µl	1.88	2.24

3.11 Restriction Digestion Analysis *Chymosin/pTZ57 R/T*

After the plasmid isolation, the single and double digestion of *Chymosin/pTZ57 R/T* was carried out to confirm the successful ligation and to determine the actual size of the plasmid and gene. To do this single and double restriction digestion was carried out using Hind III and EcoRI according to the method mentioned in **Section 2.17**. Agarose gel (1%) electrophoresis has shown that single restriction digestion has produced a single DNA band of approximately 4 Kbp (corresponding to *Chymosin/pTZ57 R/T*) whereas double restriction digestion has produced two DNA bands, one at approximately 1.1 Kbp (corresponding to Chymosin gene) and the other one at about 2.8 Kbp (corresponding to double digested *pTZ57 R/T*) (**Figure 3.9**).

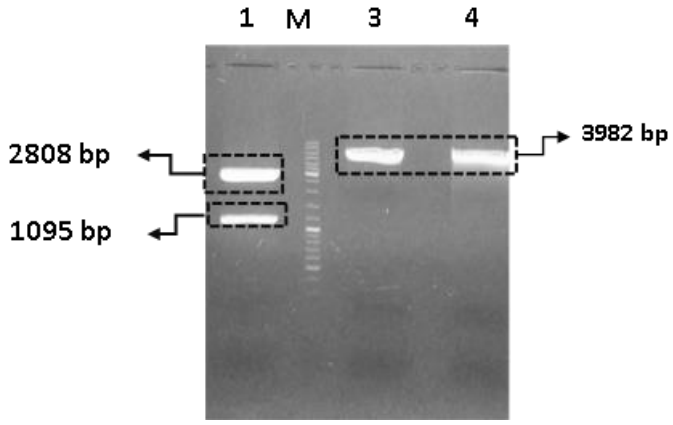

Figure 3.9 Restriction Digestion Analysis Chymosin/pTZ57 R/T

Note. **Lane 1:** Double Digested *Chymosin/pTZ57 R/T* by Hind III and EcoRI; **Lane 2:** Marker (Thermo ScientificTM GeneRuler # SM0331); **Lane 3:** Single Digested *Chymosin/pTZ57 R/T* by Hind III; **Lane 4:** Single Digested *Chymosin/pTZ57 R/T* by EcoRI

3.12 Gene Sequencing of *Chymosin/pTZ57 R/T* Plasmid and its Alignment

The plasmid *Chymosin/pTZ57 R/T* was sequenced using the forward and reverse primer through BT-Sequencing by Celemics. The result of the sequence is shown below. The nucleotides of *Chymosin/pTZ57 R/T* and their corresponding codons are shown in bold letters.

>***Chymosin/pTZ5 R/T***

CCGGAATTC**ATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAAT**

CACTGAGAAAAGCGCTGAAAGAACACGGACTGCTGGAAGACTTTCTG

CAAAAACAACAATATGGAATTTCATCAAAATATTCAGGATTTGGAGAAG

TGGCGTCAGTGCCGCTGACAAACTATCTGGACTCACAATATTTTGGAA

AAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGACA

CAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGC

GTGTAAAAACCACCAAAGATTTGACCCGAGAAAATCATCAACATTTCA

AAACCTGGGAAAACCGCTGTCAATTCACTATGGAACAGGATCAATGCA

AGGAATCCTGGGATATGACACAGTGACAGTGTCAAACATTGTGGACAT

TCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTTTA

CATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTGG

CGTCAGAATATTCAATTCCGGTGTTTGACAACATGATGAACAGACACC

TGGTGGCGCAAGACCTGTTTTCAGTGTATATGGACAGAAACGGACAAG

AATCAATGCTGACACTGGGAGCGATTAACCCGTCATATTATACAGGATC

ACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTACAGT

GGACTCAGTGACAATTTCAGGAGTGGTGGTGGCGTGTGAAGGAGGAT

GTCAAGCGATTCTGGACACAGGAACATCAAAACTGGTGGGACCGTCA

TCAGACATTCTGAATATTCAACAAGCGATTGGAGCGACACAAAACCAA

TATGGAGAATTTGACATTGACTGTGACAACCTGTCATATATGCCGACAG

TGGTGTTTGAAATTAACGGAAAAATGTATCCGCTGACACCGTCAGCGT

ATACATCACAAGACCAAGGATTTTGTACATCAGGATTTCAATCAGAAAA

CCACTCACAAAAATGGATTCTGGGAGACGTGTTTATTAGAGAATATTAT

TCAGTGTTTGACAGAGCGAACAACCTGGTGGGACTGGCGAAAGCGAT

TTAAAAGCTTGGG

Chymosin's original codon-optimized gene sequence was aligned with the gene sequence obtained after sequence analysis from the *pTZ57R_T* vector by using the CLUSTALW tool (https://www.genome.jp /tools-bin/clustalw). Results are depicted in **Figure 3.10**.

```
Original          ---------ATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAATCACTGAGAAAA
Chym/pTZ57R/T     CCGGAATTCATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAATCACTGAGAAAA
                            **************************************************

Original          GCGCTGAAAGAACACGGACTGCTGGAAGACTTTCTGCAAAAACAACAATATGGAATTTCA
Chym/pTZ57R/T     GCGCTGAAAGAACACGGACTGCTGGAAGACTTTCTGCAAAAACAACAATATGGAATTTCA
                  ************************************************************

Original          TCAAAATATTCAGGATTTGGAGAAGTGGCGTCAGTGCCGCTGACAAACTATCTGGACTCA
Chym/pTZ57R/T     TCAAAATATTCAGGATTTGGAGAAGTGGCGTCAGTGCCGCTGACAAACTATCTGGACTCA
                  ************************************************************

Original          CAATATTTTGGAAAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGAC
Chym/pTZ57R/T     CAATATTTTGGAAAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGAC
                  ************************************************************

Original          ACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGCGTGTAAAAAC
Chym/pTZ57R/T     ACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGCGTGTAAAAAC
                  ************************************************************

Original          CACCAAAGATTTGACCCGAGAAAATCATCAACATTTCAAAACCTGGGAAAACCGCTGTCA
Chym/pTZ57R/T     CACCAAAGATTTGACCCGAGAAAATCATCAACATTTCAAAACCTGGGAAAACCGCTGTCA
                  ************************************************************

Original          ATTCACTATGGAACAGGATCAATGCAAGGAATCCTGGGATATGACACAGTGACAGTGTCA
Chym/pTZ57R/T     ATTCACTATGGAACAGGATCAATGCAAGGAATCCTGGGATATGACACAGTGACAGTGTCA
                  ************************************************************

Original          AACATTGTGGACATTCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTTT
Chym/pTZ57R/T     AACATTGTGGACATTCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTTT
                  ************************************************************

Original          ACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTGGCGTCAGAATAT
Chym/pTZ57R/T     ACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTGGCGTCAGAATAT
                  ************************************************************

Original          TCAATTCCGGTGTTTGACAACATGATGAACAGACACCTGGTGGCGCAAGACCTGTTTTCA
Chym/pTZ57R/T     TCAATTCCGGTGTTTGACAACATGATGAACAGACACCTGGTGGCGCAAGACCTGTTTTCA
                  ************************************************************

Original          GTGTATATGGACAGAAACGGACAAGAATCAATGCTGACACTGGGAGCGATTAACCCGTCA
Chym/pTZ57R/T     GTGTATATGGACAGAAACGGACAAGAATCAATGCTGACACTGGGAGCGATTAACCCGTCA
                  ************************************************************

Original          TATTATACAGGATCACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTACA
Chym/pTZ57R/T     TATTATACAGGATCACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTACA
                  ************************************************************
```

Figure 3.10 DNA Alignment of Chymosin/pTZ57 R/T with original construct

3.13 Plasmid isolation and Restriction digestion of *pET28a*

After the gene purification of chymosin (restricted from *pTZ57 R/T*), the *pET28a* plasmid was isolated and digested with the same enzymes (Hind III and EcoRI). Panel A in **Figure 3.11** shows the isolated *pET28a* plasmid, while B depicts the linear plasmid digested by restriction enzymes.

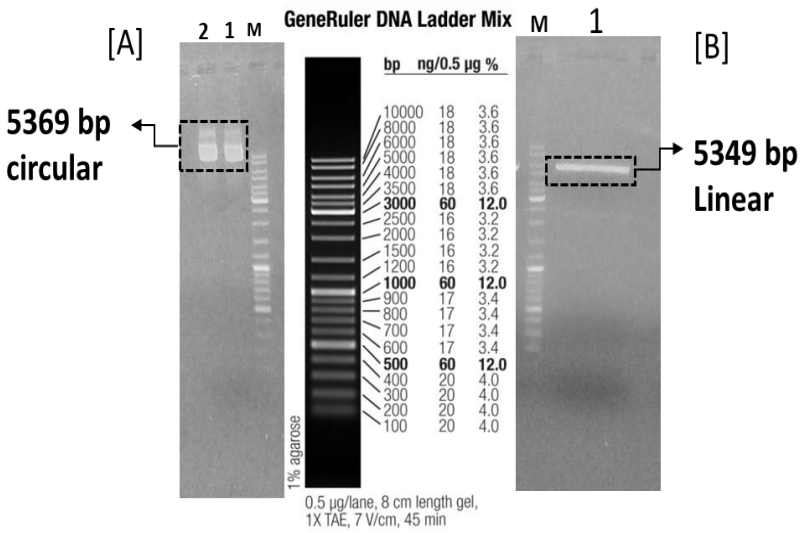

Figure 3.11 1% agarose gel of isolated and double-digested *pET28a* vector

Note. **Panel [A]** is depicting the gel of the isolated *pET28a* vector in which; **Lane M:** Marker (Thermo ScientificTM GeneRuler # SM0331); **Lane 1&2:** Isolated *pET28a* vector; **Panel [B]** is depicting the gel of double digested *pET28a* vector in which; **Lane M:** Marker (Thermo ScientificTM GeneRuler # SM0331); **Lane 1:** Double digested *pET28a* vector.

3.14 Colony PCR confirmation of *Chymosin/ pET28a / BL21 (DE3)-RIL*

The ligation of chymosin into the *pET28a* was done and it was transformed into *DH5a* for amplification of positive construct. Following the successful construct synthesis, the *Chymosin/ pET28a* plasmid was isolated and transformed into the *BL21 (DE3)-RIL* cell for expression studies. After transformation, the colony PCR was carried out by following the methodology as described in **Section 2.14.2**, all the colonies showed positive results on agarose gel (1%) electrophoresis as presented in **Figure 3.12**

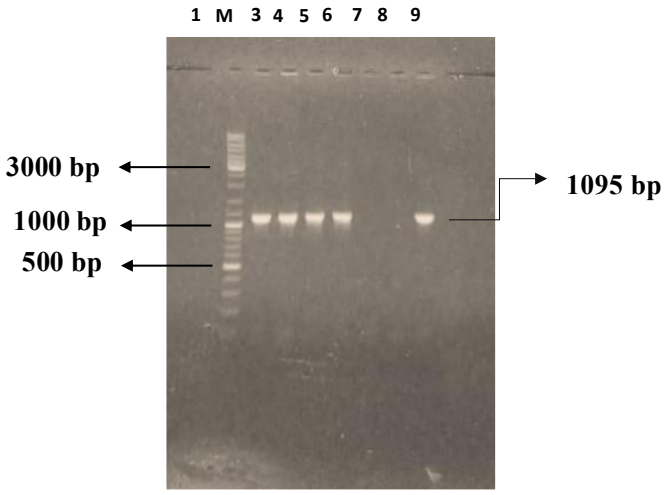

Figure 3.12 Confirmation of *Chymosin/ pET28a / BL21 (DE3)-RIL* by Colony PCR

Note. **Lane 1:** Negative Control; **Lane 2:** Marker (Thermo ScientificTM GeneRuler # SM0331)**; Lane 3-6:** Positive colonies of *Chymosin/ pET28a / BL21 (DE3)-RIL*; **Lane 7&8:** Negative Colonies; **Lane 9:** Positive Control

3.15 Gene Sequencing of *Chymosin/pET28a* Plasmid and its Alignment

The plasmid *Chymosin/pET28a* was sequenced using the forward and reverse primer through BT-Sequencing by Celemics. The result of the sequence is shown below. The nucleotides of *Chymosin/pET28a* and their corresponding codons are shown in bold letters.

> ***Chymosin/pET28a***

TCCGGAATTC**ATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAA**

TCACTGAGAAAAGCGCTGAAAGAACACGGACTGCTGGAAGACTTTCT

GCAAAAACAACAATATGGAATTTCATCAAAATATTCAGGATTTGGAGAA

GTGGCGTCAGTGCCGCTGACAAACTATCTGGACTCACAATATTTTGGA

AAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGAC

ACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACG

CGTGTAAAAACCACCAAAGATTTGACCCGAGAAAATCATCAACATTTC

AAAACCTGGGAAAACCGCTGTCAATTCACTATGGAACAGGATCAATGC

AAGGAATCCTGGGATATGACACAGTGACAGTGTCAAACATTGTGGACA

TTCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTTT

ACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTG

GCGTCAGAATATTCAATTCCGGTGTTTGACAACATGATGAACAGACAC

CTGGTGGCGCAAGACCTGTTTTCAGTGTATATGGACAGAAACGGACAA

GAATCAATGCTGACACTGGGAGCGATTAACCCGTCATATTATACAGGAT

CACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTACAG

TGGACTCAGTGACAATTTCAGGAGTGGTGGTGGCGTGTGAAGGAGGA

TGTCAAGCGATTCTGGACACAGGAACATCAAAACTGGTGGGACCGTC

ATCAGACATTCTGAATATTCAACAAGCGATTGGAGCGACACAAAACCA

ATATGGAGAATTTGACATTGACTGTGACAACCTGTCATATATGCCGACA

GTGGTGTTTGAAATTAACGGAAAAATGTATCCGCTGACACCGTCAGCG

TATACATCACAAGACCAAGGATTTTGTACATCAGGATTTCAATCAGAAA

ACCACTCACAAAAATGGATTCTGGGAGACGTGTTTATTAGAGAATATTA

TTCAGTGTTTGACAGAGCGAACAACCTGGTGGGACTGGCGAAAGCGA

TTTAAAAGCTTGCG

Chymosin original codon-optimized gene sequence was aligned with the gene sequence obtained after sequence analysis from *Chymosin/pET28a* vector by using the CLUSTALW tool (https://www.genome.jp /tools-bin/clustalw). Results are shown in **Figure 3.13.**

```
Original        ----------ATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAATCACTGAGAAA
Chymo/pET28a    TCCGGAATTCATGGCGGAAATTACAAGAATCCCGCTGTATAAAGGAAAATCACTGAGAAA
                          **************************************************

Original        AGCGCTGAAAGAACACGGACTGCTGGAAGACTTTCTGCAAAAACAACAATATGGAATTTC
Chymo/pET28a    AGCGCTGAAAGAACACGGACTGCTGGAAGACTTTCTGCAAAAACAACAATATGGAATTTC
                ************************************************************

Original        ATCAAAATATTCAGGATTTGGAGAAGTGGCGTCAGTGCCGCTGACAAACTATCTGGACTC
Chymo/pET28a    ATCAAAATATTCAGGATTTGGAGAAGTGGCGTCAGTGCCGCTGACAAACTATCTGGACTC
                ************************************************************

Original        ACAATATTTTGGAAAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGA
Chymo/pET28a    ACAATATTTTGGAAAAATTTATCTGGGAACACCGCCGCAAGAATTTACAGTGCTGTTTGA
                ************************************************************

Original        CACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGCGTGTAAAAA
Chymo/pET28a    CACAGGATCATCAGACTTTTGGGTGCCGTCAATTTATTGTAAATCAAACGCGTGTAAAAA
                ************************************************************

Original        CCACCAAAGATTTGACCCGAGAAAATCATCAACATTTCAAAACCTGGGAAAACCGCTGTC
Chymo/pET28a    CCACCAAAGATTTGACCCGAGAAAATCATCAACATTTCAAAACCTGGGAAAACCGCTGTC
                ************************************************************

Original        AATTCACTATGGAACAGGATCAATGCAAGGAATCCTGGGATATGACACAGTGACAGTGTC
Chymo/pET28a    AATTCACTATGGAACAGGATCAATGCAAGGAATCCTGGGATATGACACAGTGACAGTGTC
                ************************************************************

Original        AAACATTGTGGACATTCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTT
Chymo/pET28a    AAACATTGTGGACATTCAACAAACAGTGGGACTGTCAACACAAGAACCGGGAGACGTGTT
                ************************************************************

Original        TACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTGGCGTCAGAATA
Chymo/pET28a    TACATATGCGGAATTTGACGGAATCCTGGGAATGGCGTATCCGTCACTGGCGTCAGAATA
                ************************************************************

Original        TTCAATTCCGGTGTTTGACAACATGATGAACAGACACCTGGTGGCGCAAGACCTGTTTTC
Chymo/pET28a    TTCAATTCCGGTGTTTGACAACATGATGAACAGACACCTGGTGGCGCAAGACCTGTTTTC
                ************************************************************

Original        AGTGTATATGGACAGAAACGGACAAGAATCAATGCTGACACTGGGAGCGATTAACCCGTC
Chymo/pET28a    AGTGTATATGGACAGAAACGGACAAGAATCAATGCTGACACTGGGAGCGATTAACCCGTC
                ************************************************************

Original        ATATTATACAGGATCACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTAC
Chymo/pET28a    ATATTATACAGGATCACTGCACTGGGTGCCGGTGACAGTGCAACAATATTGGCAATTTAC
                ************************************************************
```

```
Original        AGTGGACTCAGTGACAATTTCAGGAGTGGTGGTGGCGTGTGAAGGAGGATGTCAAGCGAT
Chymo/pET28a    AGTGGACTCAGTGACAATTTCAGGAGTGGTGGTGGCGTGTGAAGGAGGATGTCAAGCGAT
                ************************************************************

Original        TCTGGACACAGGAACATCAAAACTGGTGGGACCGTCATCAGACATTCTGAACATTCAACA
Chymo/pET28a    TCTGGACACAGGAACATCAAAACTGGTGGGACCGTCATCAGACATTCTGAATATTCAACA
                ************************************************** ********

Original        AGCGATTGGAGCGACACAAAACCAATATGGAGAATTTGACATTGACTGTGACAACCTGTC
Chymo/pET28a    AGCGATTGGAGCGACACAAAACCAATATGGAGAATTTGACATTGACTGTGACAACCTGTC
                ************************************************************

Original        ATATATGCCGACAGTGGTGTTTGAAATTAACGGAAAAATGTATCCGCTGACACCGTCAGC
Chymo/pET28a    ATATATGCCGACAGTGGTGTTTGAAATTAACGGAAAAATGTATCCGCTGACACCGTCAGC
                ************************************************************

Original        GTATACATCACAAGACCAAGGATTTTGTACATCAGGATTTCAATCAGAAAACCACTCACA
Chymo/pET28a    GTATACATCACAAGACCAAGGATTTTGTACATCAGGATTTCAATCAGAAAACCACTCACA
                ************************************************************

Original        AAAATGGATTCTGGGAGACGTGTTTATTAGAGAATATTATTCAGTGTTTGACAGAGCGAA
Chymo/pET28a    AAAATGGATTCTGGGAGACGTGTTTATTAGAGAATATTATTCAGTGTTTGACAGAGCGAA
                ************************************************************

Original        CAACCTGGTGGGACTGGCGAAAGCGATT------------
Chymo/pET28a    CAACCTGGTGGGACTGGCGAAAGCGATTTAAAAGCTTGCG
                ****************************
```

Figure 3.13 DNA Alignment of *Chymosin/pET28a* with original construct

3.16 Protein Sequence Alignment

The protein sequence of *Chymosin/ pET28a* was obtained by applying ExPasy translate tool to the nucleotide sequence obtained after the sequencing of the gene. Protein sequence alignment was done with the original chymosin protein translated from codon-optimized sequence by using CLUSTALW and the results are given in **Figure 3.14**.

```
Orignal          ---MAEITRIPLYKGKSLRKALKEHGLLEDFLQKQQYGISSKYSGFGEVASVPLTNYLDS
Chymo/pET28a     PEFMAEITRIPLYKGKSLRKALKEHGLLEDFLQKQQYGISSKYSGFGEVASVPLTNYLDS
                    ************************************************************

Orignal          QYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDPRKSSTFQNLGKPLS
Chymo/pET28a     QYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDPRKSSTFQNLGKPLS
                 ************************************************************

Orignal          IHYGTGSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTYAEFDGILGMAYPSLASEY
Chymo/pET28a     IHYGTGSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTYAEFDGILGMAYPSLASEY
                 ************************************************************

Orignal          SIPVFDNMMNRHLVAQDLFSVYMDRNGQESMLTLGAINPSYYTGSLHWVPVTVQQYWQFT
Chymo/pET28a     SIPVFDNMMNRHLVAQDLFSVYMDRNGQESMLTLGAINPSYYTGSLHWVPVTVQQYWQFT
                 ************************************************************

Orignal          VDSVTISGVVVACEGGCQAILDTGTSKLVGPSSDILNIQQAIGATQNQYGEFDIDCDNLS
Chymo/pET28a     VDSVTISGVVVACEGGCQAILDTGTSKLVGPSSDILNIQQAIGATQNQYGEFDIDCDNLS
                 ************************************************************

Orignal          YMPTVVFEINGKMYPLTPSAYTSQDQGFCTSGFQSENHSQKWILGDVFIREYYSVFDRAN
Chymo/pET28a     YMPTVVFEINGKMYPLTPSAYTSQDQGFCTSGFQSENHSQKWILGDVFIREYYSVFDRAN
                 ************************************************************

Orignal          NLVGLAKAI----
Chymo/pET28a     NLVGLAKAI-KLA
                 *********
```

Figure 3.14 Protein Alignment of *Chymosin/pET28a* with original construct

3.17 Expression of *Chymosin/ pET28a in E. coli BL21 (DE3)-RIL*

After confirming through colony PCR, colony 2 was selected for the expression of *Chymosin/pET28a in the E. coli BL21 (DE3)* strain. As control *only pET28a* in *E. coli BL21 (DE3)-RIL* was used and given similar conditions as off to the construct. The expression of the chymosin protein was carried out at 37℃ with varying concentrations of IPTG (0 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM) as described in **Section 2.21**. The absorbance of the cell culture at 600 nm was measured after induction for each IPTG concentration, as outlined in **Table 3.2**. The impact of IPTG concentration on *E. coli* cell growth was graphically presented in **Figure 3.15**. Following lysis of the bacterial cells, fractions were subjected to SDS PAGE (12%) analysis using the method described in **Section 2.23**. Upon staining and destaining the gel, distinct bands corresponding to the desired 40.61 kDa prochymosin were

observed (**Figure 3.16**). The results demonstrated that the highest expression was observed in the absence of induction, while the lowest expression was seen with 1 mM IPTG induction. This indicates that the expression of chymosin in *E. coli BL21 (DE3)-RIL* cells was not tightly regulated and exhibited a leaky nature.

Table 3.2 Post Induction absorbance of *Chymosin/ pET28a in E. coli BL21 (DE3)-RIL* and *pET28a* in *E. coli BL21 (DE3)-RIL* cell culture at 600 nm under different IPTG concentrations.

IPTG Concentration(mM)	*Chymosin/ pET28a in E. coli BL21* OD_{600}/mL	*pET28a* in *E. coli BL21* OD_{600}/mL
0 mM	6.80	5.45
0.25 mM	2.85	2.52
0.5 mM	1.03	1.50
0.75 mM	0.55	0.73
1.0 mM	0.25	0.35

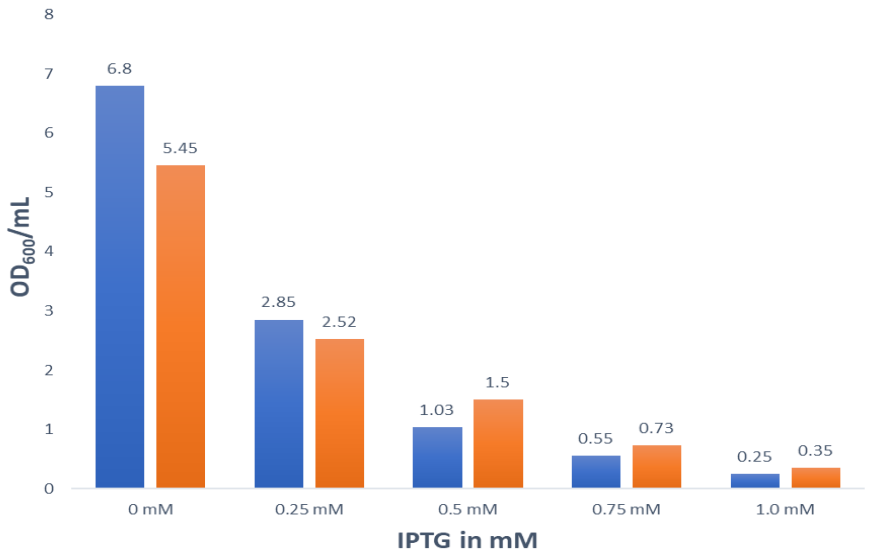

Figure 3.15 Graphical depiction of post-induction absorbance of *Chymosin/ pET28a* in *E. coli BL21 (DE3)-RIL* and *pET28a* in *E. coli BL21 (DE3)-RIL* cell culture at 600 nm under different IPTG concentrations.

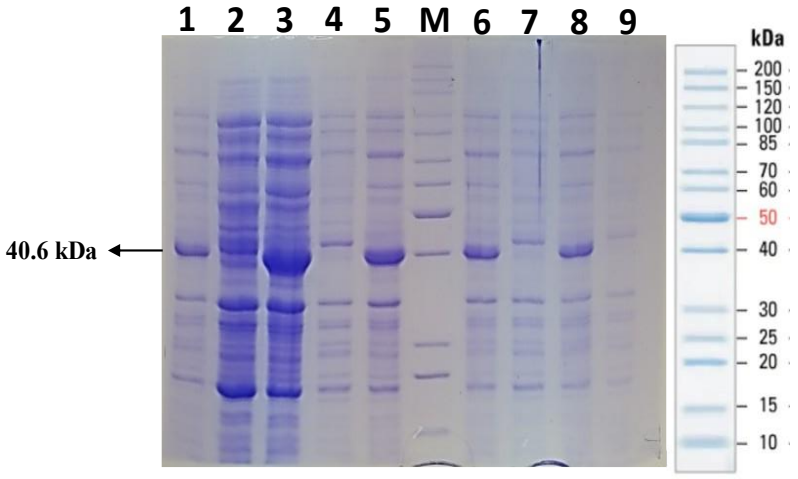

Figure 3.16 12% SDS PAGE for *Chymosin/ pET28a* in *E. coli BL21 (DE3)-RIL* expression analysis.

Note. **Lane M:** PageRular^{TM} Unstained Protein Ladder (10-200kDa); **Lane 1:** *Chymosin/ pET28a* induced with 1mM IPTG; **Lane 2:** *pET28a* uninduced; **Lane 3:** *Chymosin/ pET28a* uninduced; **Lane 4:** *pET28a* induced with 0.25 mM IPTG; **Lane 5:** *Chymosin/ pET28a* induced with 0.25 mM IPTG; **Lane 6:** *Chymosin/ pET28a* induced with 0.5 mM IPTG; **Lane 7:** *pET28a* induced with 0.5 mM IPTG; **Lane 8:** *Chymosin/ pET28a* induced with 0.75 mM IPTG; **Lane 9:** *pET28a* induced with 0.75 mM IPTG.

3.18 Inclusion Bodies Preparation and Analysis

After the confirmation of expression, the cell pellet of 10 g was subjected to cell lysis yielding soluble and insoluble fractions as inclusion bodies described in **Section 2.25**. During the whole procedure, representative samples (whole cell lysate, supernatants, and pellets) were collected after repeated washings with 20 mM Tris-HCl pH 8.0 to analyze chymosin. **Figure 3.17** shows that the chymosin is expressed in the form of inclusion bodies and due to repeated sonication and successive washings, the amount of soluble protein in the supernatant is reduced. The band of ~40.6kDa in lanes 6 and 7 shows that chymosin was expressed as insoluble inclusion bodies.

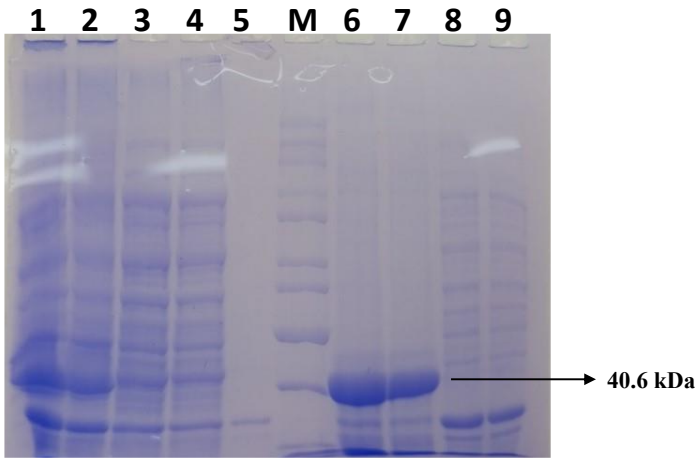

Figure 3.17 12% SDS-PAGE for Chymosin inclusion bodies analysis

Note. **Lane M:** PageRular^{TM} Unstained Protein Ladder (10-200kDa); **Lane 1:** Whole cell lysate reductive (20µL); **Lane 2:** Whole cell lysate reductive (10µL); **Lane 3:** Supernatant after 1st washing reductive (20µL); **Lane 4:** Supernatant after 2nd washing reductive (20µL); **Lane 5:** Supernatant after 2nd washing reductive (10µL); **Lane 6:** Pellet after 3rd washing reductive (20µL); **Lane 7:** Pellet after 2nd washing reductive (20µL); **Lane 8:** Supernatant after 3rd washing reductive (20µL); **Lane 9:** Supernatant after 3rd washing reductive (20µL)

The above figure shows distinct bands of required protein. It depicts that many bacterial proteins were present in whole cell lysate which were removed after continuous sonication and washings our required protein become more purified as inclusion bodies.

3.19 UV Quantification of Inclusion Bodies

A portion of inclusion bodies were UV quantified against 5% SDS by taking absorbance at 280 nm. The following formula was applied to calculate the protein concentration.

$1\ OD_{280} = 1$ mg of protein

$OD_{280} = OD_{280} - OD_{320}$

For applying zero baseline correction, OD_{320} was subtracted from OD_{280}. The amount of protein was calculated by multiplying OD_{280} with the dilution factor.

Table 3.3 Estimation of inclusion bodies of chymosin by UV quantification

Inclusion bodies	The volume of culture cultivated (L)	Cell pellet weight in g	Total protein (OD_{280})
Chymosin	2	10	940

3.20 Solubilization and Refolding of Inclusion Bodies of Chymosin

Inclusion bodies of chymosin were solubilized. About 420 OD_{280} were refolded in 750 ml of refolding buffer by reverse dilution method as mentioned in **Section 2.25**. During the refolding loss of total thiols in the medium was measured by DTNB assay as shown in **Table 3.4** and the graph plotted against these values is depicted in **Figure 3.18**. The redox content for thiols in refolding buffer remained constant till the end of refolding procedure while refolding reconstitute sink had similar thiols content as of refolding buffer. Refolded inclusion bodies were analyzed by 12 % SDSPAGE under reducing and non-reducing conditions as shown below.

Table 3.4 DTNB assay to monitor the loss of thiols during refolding of chymosin.

Sr.No.	Time (min)	ΔOD_{412}	ΔOD_{412}
		Refolding buffer	**Refolding mixture**
1	0	0.115	0.475
2	30	0.113	0.252
3	60	0.112	0.176
4	90	0.101	0.135
5	120	0.110	0.107

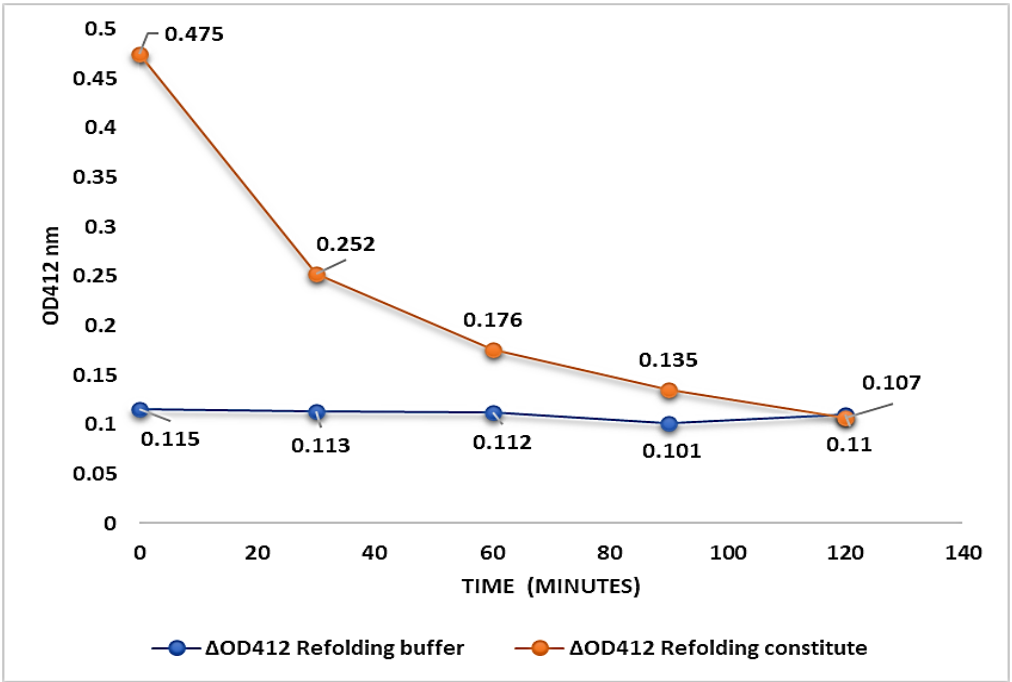

Figure 3.18 Graphical representation of loss of thiols during refolding of chymosin

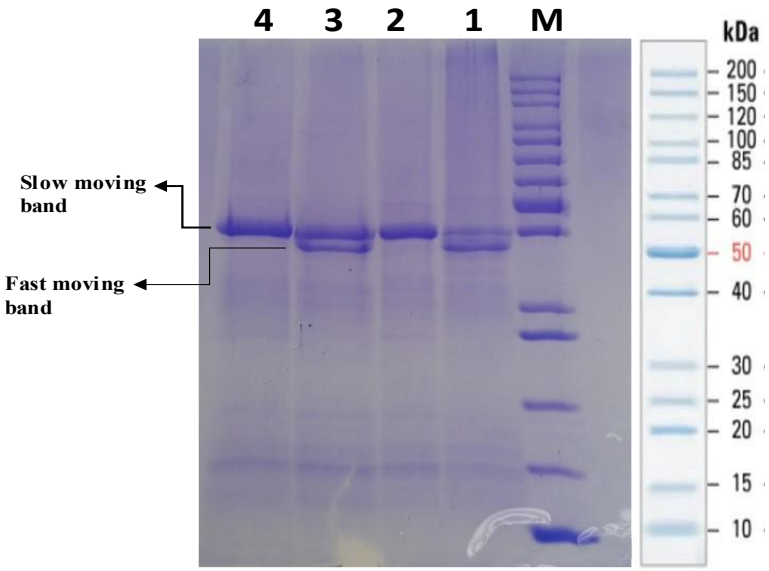

Figure 3.19 12% SDS-PAGE analysis of refolded chymosin

Note. **Lane M:** PageRular^{TM} Unstained Protein Ladder (10-200kDa)**; Lane 1:** 1 h non-reductive**; Lane 2:** 1 h reductive**; Lane 3:** 2h non-reductive**; Lane 4:** 2 h reductive

Figure 3.19 shows the distinct bands under both reducing and non-reducing conditions. Fast-moving bands under non-reducing conditions showed successful

refolding and revealed that the protein has attained its native globular structure, therefore, moving faster.

3.21 Purification of chymosin by anion exchange chromatography using DEAE Sephadex column

The refolded chymosin was purified by anion exchange chromatography as described in **Section 2.26**. Protein was loaded on the DEAE Sephadex column by adjusting the flow rate of 2-2.5 ml/min. Bound protein was eluted using various NaCl gradients 0.1-1.0 M as mentioned in **Section 2.26**. All the collected fractions were quantified by measuring UV, absorbance at 280 nm recorded in **Table 3.5**. The graphical profile of eluted fractions from DEAE Sephadex is shown in **Figure 3.20**.

Table 3.5 The absorbance of the fractions collected during anion exchange chromatography, at 280 nm.

Sample	Fractions	OD_{280}	Volume (ml)	Total OD_{280}
Flow through	F1	0.052	100	5.2
	F2	0.065	50	3.25
	F3	0.043	50	2.15
Washing	F1	0.045	50	2.25
	F2	0.034	50	1.7
Elution 1 0.1 M NaCl	F1	0.110	10	1.1
	F2	0.105	10	1.05
	F3	0.156	10	1.56
Elution 2 0.2 M NaCl	F1	0.122	10	1.22
	F2	0.149	10	1.49
	F3	0.166	10	1.66
Elution 3 0.3 M NaCl	F1	0.172	10	1.72
	F2	0.186	10	1.86
	F3	0.211	10	2.11

Elution 4 0.4 M NaCl	F1	0.455	10	4.55
	F2	0.654	10	6.54
	F3	0.855	10	8.55
Elution 5 0.5 M NaCl	F1	1.155	10	11.55
	F2	1.881	10	18.81
	F3	2.950	10	29.50
Elution 6 0.6 M NaCl	F1	1.653	10	16.53
	F2	1.550	10	15.50
	F3	1.350	10	13.50
Elution 7 0.7 M NaCl	F1	1.225	10	12.25
	F2	0.454	10	4.54
	F3	0.356	10	3.56
Elution 8 0.8 M NaCl	F1	0.146	10	1.46
	F2	0.106	10	1.06
	F3	0.095	10	0.95
Elution 9 0.9 M NaCl	F1	0.084	10	0.84
	F2	0.065	10	0.65
	F3	0.079	10	0.79
Elution 10 1 M NaCl	F1	0.046	10	0.46
	F2	0.041	10	0.41
	F3	0.006	10	0.06

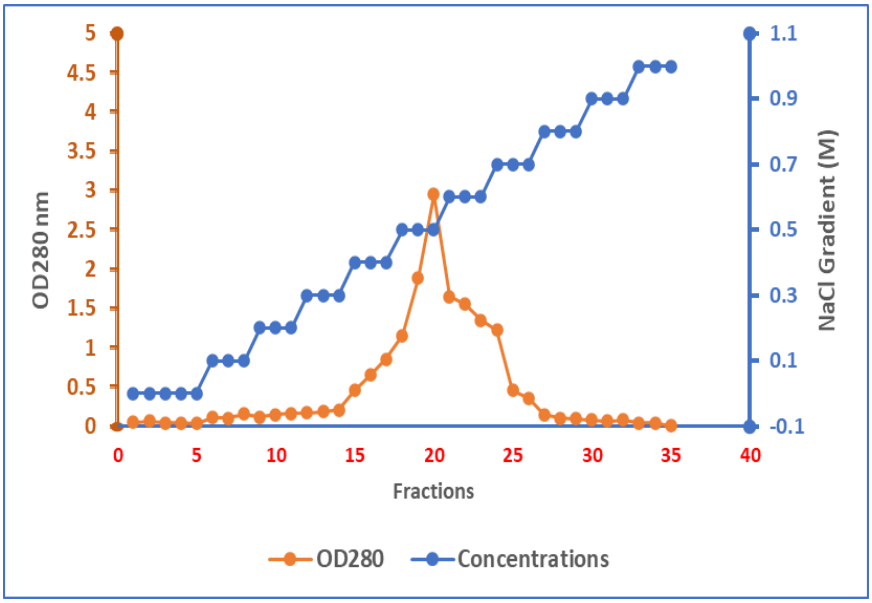

Figure 3.20 Graphical representation of fractions collected by DEAE Sephadex column.

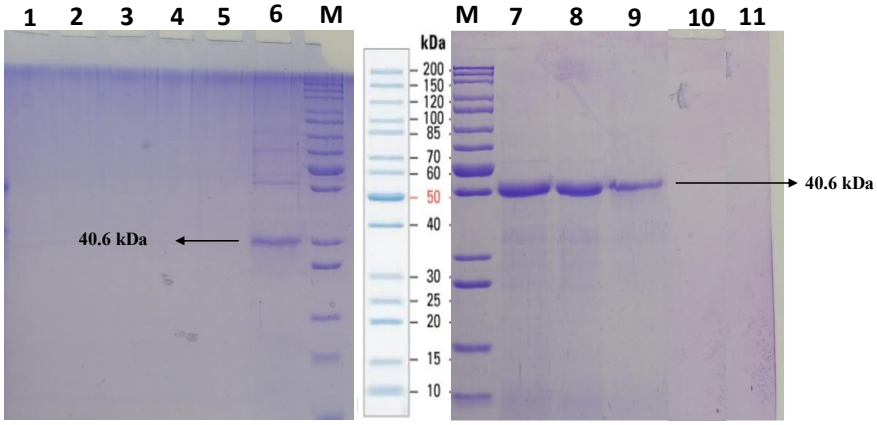

Figure 3.21 12% SDS PAGE analysis for fraction collected from DEAE Sephadex column.

Note. **Lane M:** PageRular^{TM} Unstained Protein Ladder (10-200kDa); **Lane 1:** Flow-through; **Lane 2:** Washing; **Lane 3:** 0.1 M NaCl fraction; **Lane 4:** 0.2 M NaCl fraction; **Lane 5:** 0.3 M NaCl fraction; **Lane 6:** 0.4 M NaCl fraction; **Lane 7:** 0.5 M NaCl fraction**; Lane 8:** 0.6 M NaCl fraction; **Lane 9:** 0.7 M NaCl fraction; **Lane 10:** 0.8 M NaCl fraction; **Lane 11:** 0.9 M NaCl fraction.

Form graphical representation of chymosin the purification by DEAE Sephadex column, showed that most of the bound protein (properly refolded) i.e., approximately 130 OD_{280} eluted in 0.4 M to 0.7 M NaCl fractions while the

remaining approximately 26 OD_{280} eluted in flow-through, washing and in NaCl gradients 0.1- 0.3 M. These fractions were analyzed under reductive conditions. For this purpose, the representative sample of the selected fractions was analyzed on 12% SDS-PAGE shown in **Figure 3.21**. Fractions eluted in 0.1 to 0.3 M NaCl gradient did not contain significant protein as analyzed by UV spectrophotometrically. At various steps of refolding and protein purification, the yield of chymosin is summarized in **Table 3.6 An overview of the yield of chymosin at different steps.**

Steps	**Specifications**
OD_{280} of inclusion bodies	440 OD_{280}
Vol. of Solubilization buffer added	110 ml
Total OD_{280} of solubilized inclusion bodies 1mg = 1 OD_{280} (for impure protein)	410 OD_{280}
The volume of refolding buffer added up to OD_{280} (0.5 mg/ml)	750 ml
The volume of refolding reconstitute.	860 ml
Volume after dialysis against 20 mM Tris-HCl pH 8.0	950 ml
Total protein after dialysis and loaded on the column	310 OD_{280}
Protein in flow-through (OD_{280})	13.2 OD_{280}
Protein eluted in NaCl gradients (OD_{280}) 0.4M – 0.7 M:	
0.4 M	19.64 OD_{280}
0.5 M	59.86 OD_{280}
0.6 M	45.83 OD_{280}
0.7 M	20.35 OD_{280}
Total recovered protein from DEAE Sephadex Colum	145.68 OD_{280}
Total purified protein after dialysis against 20 mM Tris-HCl pH 8.0	140.5 OD_{280}
% Yield with respect to solubilized inclusion bodies (Since it was purified, therefore for yield OD_{280})	34.26 %
% Yield with respect to protein loaded on a DEAE Sephadex Colum	45.32 %

3.22 The concentration of the purified protein by ultra-centrifugal filtration (concentrator)

To concentrate the purified protein through ion exchange chromatography the eluted fractions (from 0.4 M to 0.7M) with maximum OD_{280} were taken. The purified protein fractions were mixed and added to the equilibrated column and centrifuged at 4℃ and 3500 rpm for 5 to 6 minutes. Finally, the concentrated protein was carefully retrieved from the column and 12% SDS PAGE was performed to confirm the concentrated purified chymosin protein shown in **Figure 3.22**.

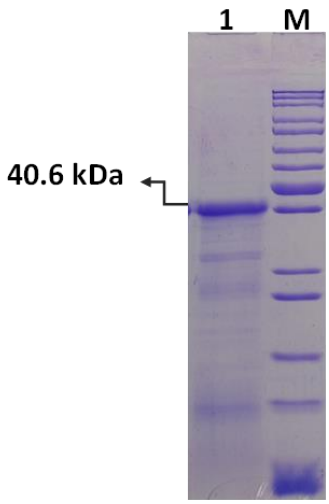

Figure 3.22 12% SDS PAGE of concentrated purified chymosin protein

Note. **Lane M:** PageRular^{TM} Unstained Protein Ladder (10-200kDa); **Lane 1:** Concentrated purified chymosin protein

3.23 Activation of pro-Chymosin to Active Chymosin

To convert prochymosin to chymosin the pH of the enzyme solution was decreased to 2 with HCL and the mixture was placed in the incubator for 30 minutes at 37 °C. After the reaction mixture was taken out and the pH was increased to 6 with 5 N NaOH. The reaction mixture was paced at -20 °C for further activity assays and Tricine PAGE analysis was done to confirm the proteolytic cleavage and activation of prochymosin to active chymosin (35.65 kDa). Tricine PAGE is known for its better resolution as compared to SDS PAGE. The active chymosin and the released peptide of about 4.97 kDa were analyzed by 15% Tricine-PAGE (**Figure 3.23**) as resolving gel while 5% staking gel was the same as for SDS- PAGE.

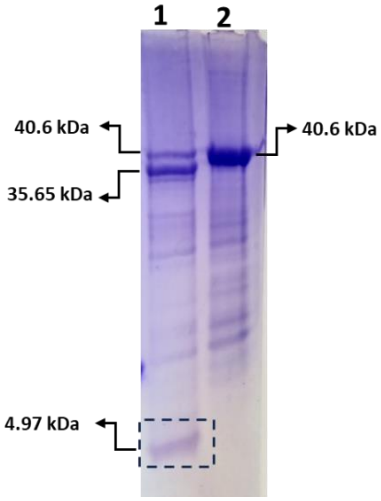

Figure 3.23 15% Tricine-PAGE for analysis of activation of prochymosin to active chymosin

Note. **Lane 1:** Activated chymosin along pro peptide; **Lane 2:** Non-active prochymosin

There are three bands visible in lane 1 which correspond to prochymosin, active chymosin, and the peptide released during activation. While on the other side in lane 2 (control) there is only a single band of 40.6 kDa corresponding to the

prochymosin. These results show that a portion of the prochymosin (may be completely in the native folded stage) undergoes cleavage on providing the acidic condition and some portion of it remain in inactive prochymosin form. This shows that our protein is now ready for further activity assay and characterization.

CHAPTER 4

DISCUSSION

The production of cheese is an ancient tradition dating back thousands of years and relies heavily on the use of chymosin, a vital enzyme responsible for the coagulation of milk proteins. Traditionally, chymosin was sourced from the abomasum of young ruminant animals, making it costly, labor-intensive, and subject to supply fluctuations. In recent decades, significant advancements in biotechnology and genetic engineering have paved the way for an innovative approach to cheese production by harnessing the power of recombinant DNA technology (Al-Zoreky et al., 2021). The demand for cheese continues to rise globally, driven by its diverse culinary applications, nutritional benefits, and appealing taste profiles. As cheese manufacturers strive to meet this escalating demand, the need for an abundant, reliable, and cost-effective source of chymosin becomes ever more crucial. The introduction of recombinant bovine chymosin emerges as a viable solution to address these challenges while simultaneously addressing concerns over animal welfare and supply chain stability. The utilization of genetic engineering techniques offers the opportunity to produce chymosin in a controlled, scalable manner, independent of animal sources, thus enhancing the sustainability of cheese production (Belenkaya et al., 2020).

Chymosin is a primary enzyme from the class of aspartyl proteinases and is found in the abomasum of newborn mammals. It has a very close resemblance to pepsin A which is the main protease found in grown-up mammals. These two proteases are produced in the stomach initially as an inactive precursor state known as prochymosin and become activated to chymosin through autocatalytic cleavage of the

pro sequence when exposed to acid (A. Kumar et al., 2010). Chymosin is highly effective at coagulating milk and prolonging the retention of milk protein precipitates in the stomach allowing them to be adequately exposed to proteolytic enzymes. The main function of chymosin is to cause milk clotting by breaking the peptide bond between Phe105-Met106 in the κ-casein protein chain. This process is necessary to produce cheese which is a highly nutritious milk product also and it brings changes to the texture and enhances flavor during the cheese ripening process (Justesen et al., 2009).

The current research also suggests that chymosin might be employed in the manufacture of therapeutic proteins since it has the most promising features for the precise removal of fusion tags. However, as the bovine population declines and the need for cheese manufacturing rises, other animal species are being examined as potential sources of chymosin (Roller et al., 1994). Although microbial alternatives are available, they do not give the required flavors during the cheese ripening process (Roseiro et al., 2003a). Plant-based proteolytic activity is also an option, but it can lead to extensive digestion of the curd which results in off flavors and impaired taste. So, these problems impede the use of animal chymosin in the dairy industry and promote the search for alternatives. While there are various methods available for producing such substitutes such as recombinant DNA technology is usually preferred to generate a technique to produce active chymosin that closely resembles its natural form (Akishev et al., 2023).

This research work investigates the heterologous production of recombinant bovine chymosin, a crucial enzyme used in cheese processing. The chymosin gene was expressed in *Escherichia coli* expression system due to their advantages such as fast growth, well defined genetics, and cost effectiveness. Initially, the chymosin gene

was amplified and cloned into an expression vector *pET28a* to facilitate its expression in *E. coli.* In *E. coli BL21- (DE3)-RIL*, *chymosin/ pET28a* was expressed as inclusion bodies. Due to its lack of omp T protease and Lon protease, which allows for a high degree of expression, this strain was chosen for expression (Rosano and Ceccarelli, 2014). Additionally, it has T7 polymerase, which is produced by induction, and T7 RNA polymerase promotor, which are both present in the *pET* vector (Tabor, 2001). By using a 12 % SDS-PAGE, the expression of chymosin was analyzed. Upon staining and destaining the gel, distinct bands corresponding to the desired 40.61 kDa prochymosin were clearly observed. The results demonstrated that the highest expression was observed in the absence of induction, while the lowest expression was seen with 1 mM IPTG induction. This indicates that the expression of chymosin in *E. coli BL21 (DE3)-RIL* cells was not tightly regulated and exhibited a leaky nature.

After the confirmation of expression, the cell pellet was subjected to cell lysis yielding soluble and insoluble fractions as inclusion bodies. **Figure 3.17** shows that the chymosin is expressed in the form of inclusion bodies and due to repeated sonication and successive washings, the amount of soluble protein in the supernatant is reduced. It depicts that many bacterial proteins were present in whole cell lysate which were removed after continuous sonication and washings and our required protein become more purified as inclusion bodies. OD280 440/liter of chymosin inclusion body yield was obtained. Inclusion bodies of chymosin were solubilized in 8M urea solubilization buffer pH 11.0 which gave 95-97 % of solubilization of chymosin as compared to the initial OD280 of inclusion bodies. Refolding of chymosin was proceeded by reverse dilution method in which the refolding buffer pH 11 was added to the solubilized chymosin sink. The refolding buffer contained a redox couple using cysteine/cystine in a ratio of 1:10, the refolding buffer was transferred

within the 2 hours giving the final amount of protein 0.5 OD/ml. The free thiol content was analyzed by DTNB assay. The thiol content of refolding buffer and refolding reconstitute was similar at 2 hours of refolding and the next day there was no free thiol remained in refolding reconstitute indicating that all free thiols had been oxidized. The refolding profile of chymosin was analyzed by 12% SDS PAGE, which shows the distinct bands under both reducing and non-reducing conditions. Fast-moving bands under non-reducing conditions showed successful refolding and revealed that protein has attained its native globular structure therefore moving faster as compared to reductive one which had linear molecule of protein. This analysis indicated proper refolding of chymosin, and it was completely refolded in 2 hours.

The refolded chymosin was purified by anion exchange chromatography followed by dialysis with 20 mM Tris-Cl pH 8.0. Anion exchange chromatography is the first choice for the purification of properly refolded protein from unfolded or misfolded protein bearing similar charges (Scheich et al., 2004). The DEAE Sephadex resin was used for anion exchange chromatography and 0.1-1 M NaCl step gradient was utilized for elution of chymosin, and fractions were collected. The fractions having more than 0.2 OD280 were analyzed by 12% SDS-PAGE and pure chymosin was eluted in 0.4-0.7 M of NaCl gradient.

Like other zymogens found in the stomach, prochymosin undergoes limited proteolysis to transform into an active enzyme, specifically at a pH below 5. The rate of this conversion process is significantly enhanced by lowering the pH from 5 to 2. The initial studies on calf prochymosin activation provided insights into the mechanism of converting gastric zymogens into active enzymes. These studies suggested that the first step involves a pH-dependent conformational change that exposes the active site crevice. At pH 2, cleavage of calf prochymosin occurs at the

Phe25p-Leu26p site, resulting in the formation of an intermediate known as pseudo chymosin, which remains stable at pH 2. The maturation of chymosin takes place through activation at pH 4-5, and pseudo-chymosin is subsequently converted into chymosin at pH 5.5 (Aboulnaga, 2019). So, to convert prochymosin to chymosin the pH of the enzyme solution was decreased to 2 with HCL, and the mixture was placed in an incubator for 30 minutes at 37 °C. The reaction mixture was analyzed through 15% Tricine PAGE because of its better resolution as compared to SDS PAGE. It confirmed the proteolytic cleavage and activation of prochymosin to active chymosin (35.65 kDa) with the released peptide of about 4.97 kDa. There are three bands clearly visible in lane 1 which correspond to prochymosin, active chymosin, and the peptide released during activation. While on the other side in lane 2 (control) there is only a single band of 40.6 kDa corresponding to the prochymosin. These results show that a portion of the prochymosin (may be completely in the native folded stage) undergo cleavage on providing the acidic condition and some portion of it remain in inactive prochymosin form. This shows that our protein is now ready for further activity assay and characterization.

In future investigations, comprehensive characterization, and activity studies of the obtained active chymosin will be conducted. Moreover, its potential application in the downstream cheese manufacturing process will be explored, aiming to offer a cost-effective alternative to commercially available expensive milk coagulants for the local cheese industry in Pakistan.

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Appendix A

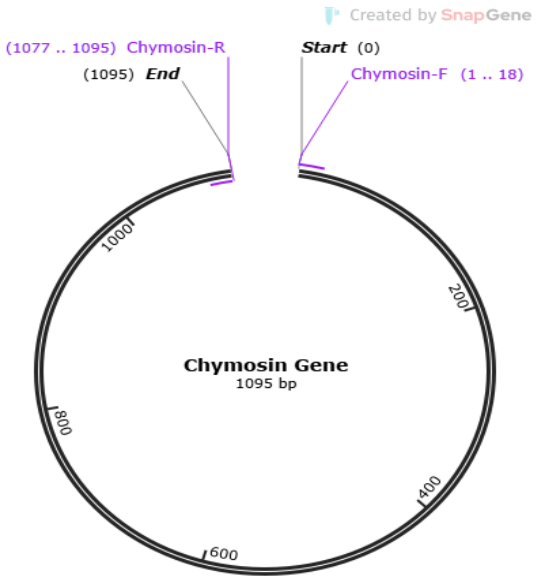

Figure A1 Primer designing for Chymosin gene.

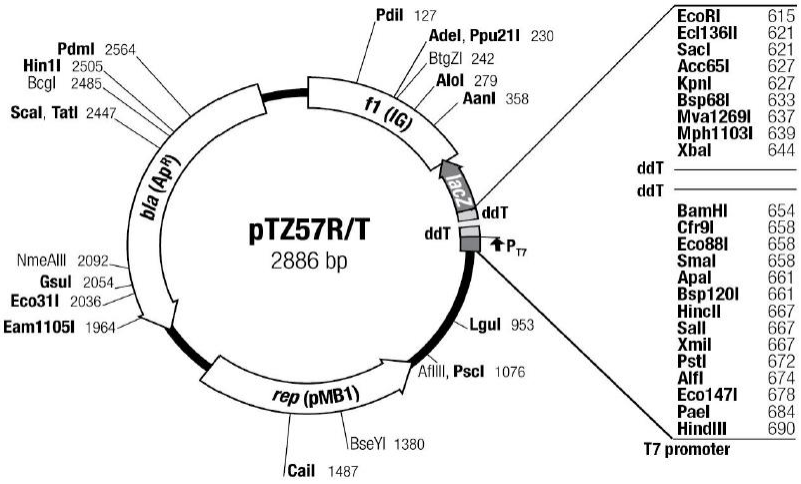

Figure A2 Map of the pTZ57R/T cloning vector

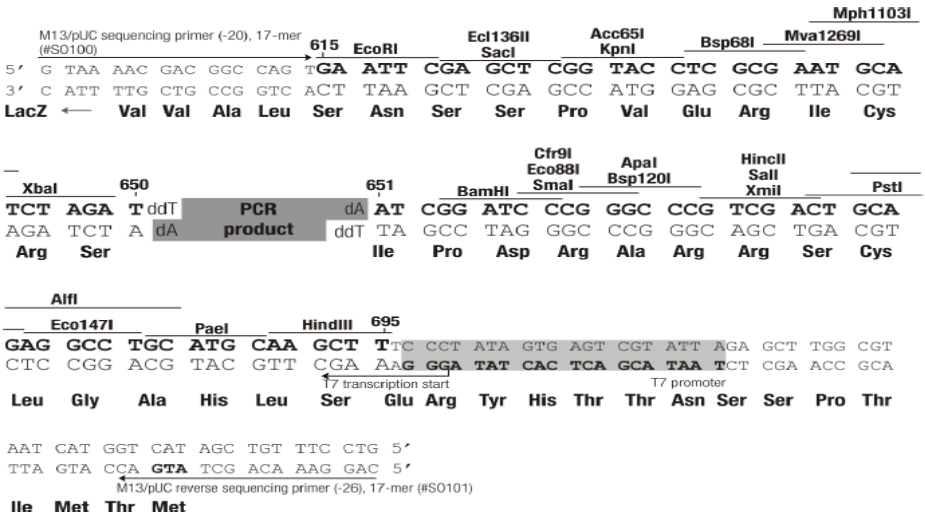

Figure A3 MCS of *pTZ57R/T* cloning vector

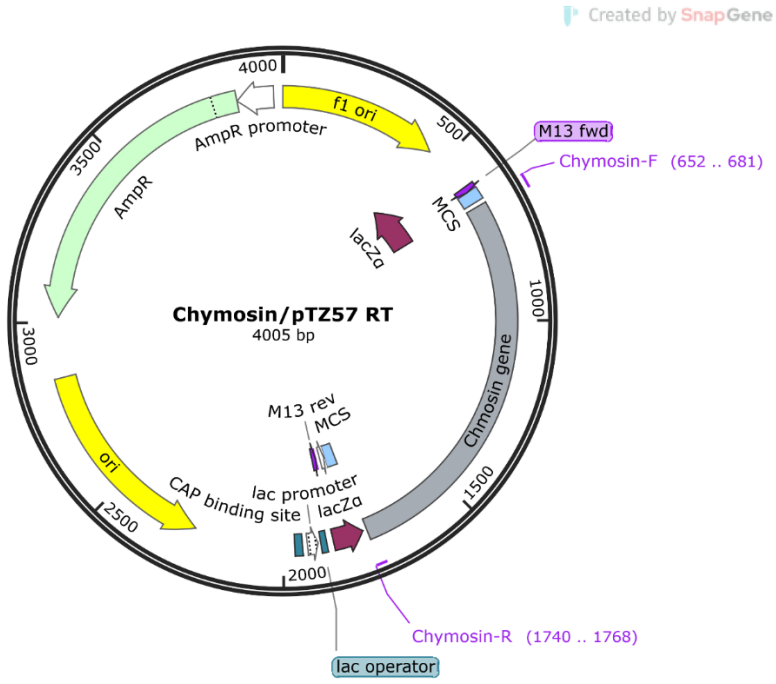

Figure A4 Map of the *chymosin/pTZ57R/T* construct

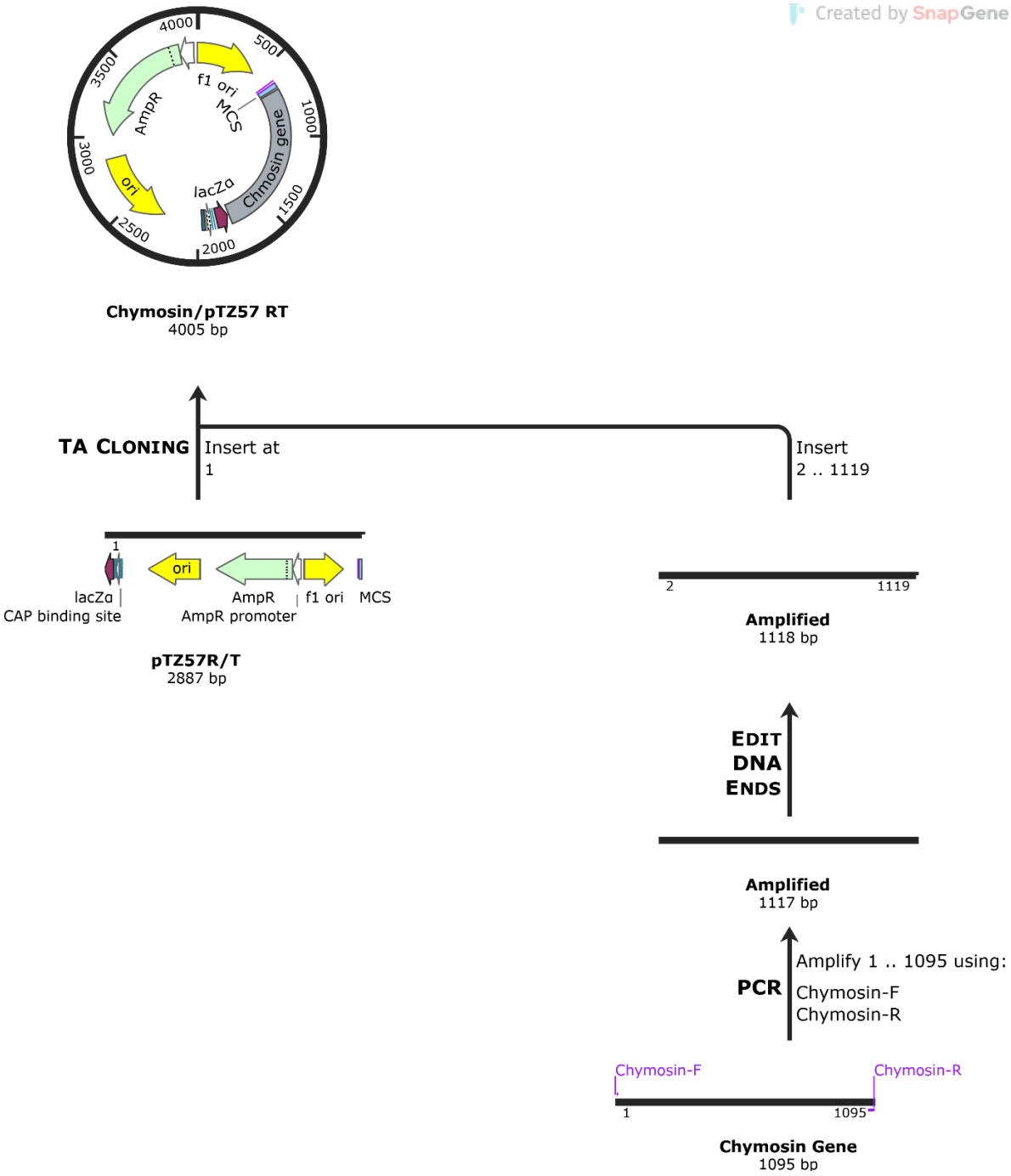

Figure A5 Cloning strategy for chymosin/pTZ57R/T construct.

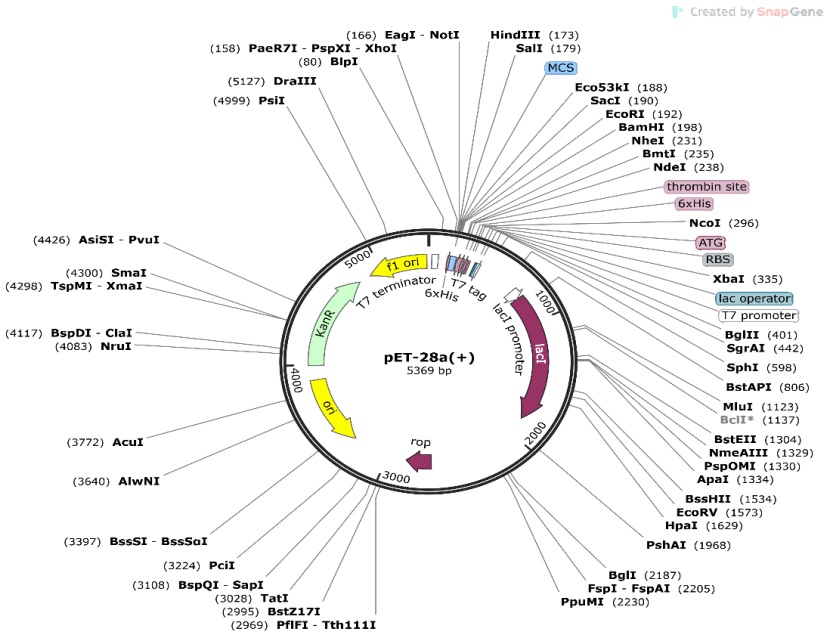

Figure A6 Map of the *pET28a* expression vector

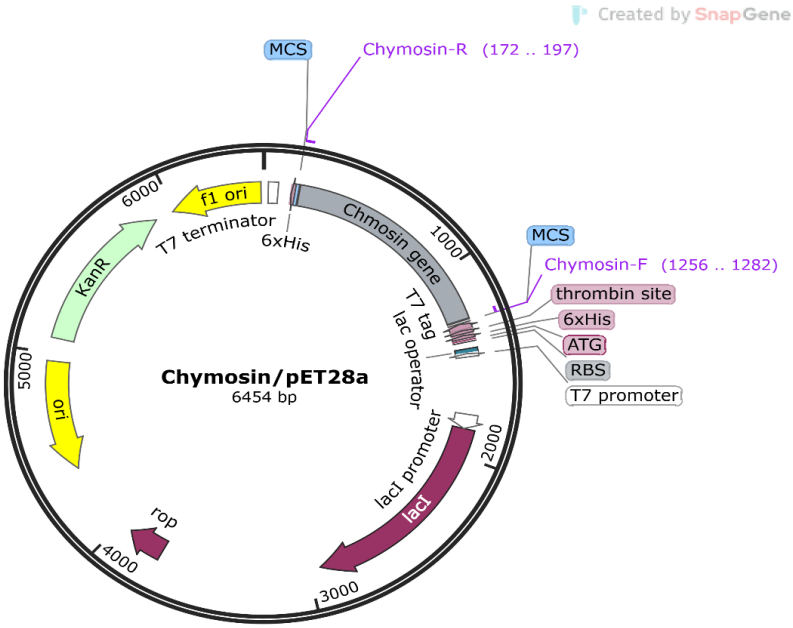

Figure A7 Map of the *chymosin/pET28a* construct

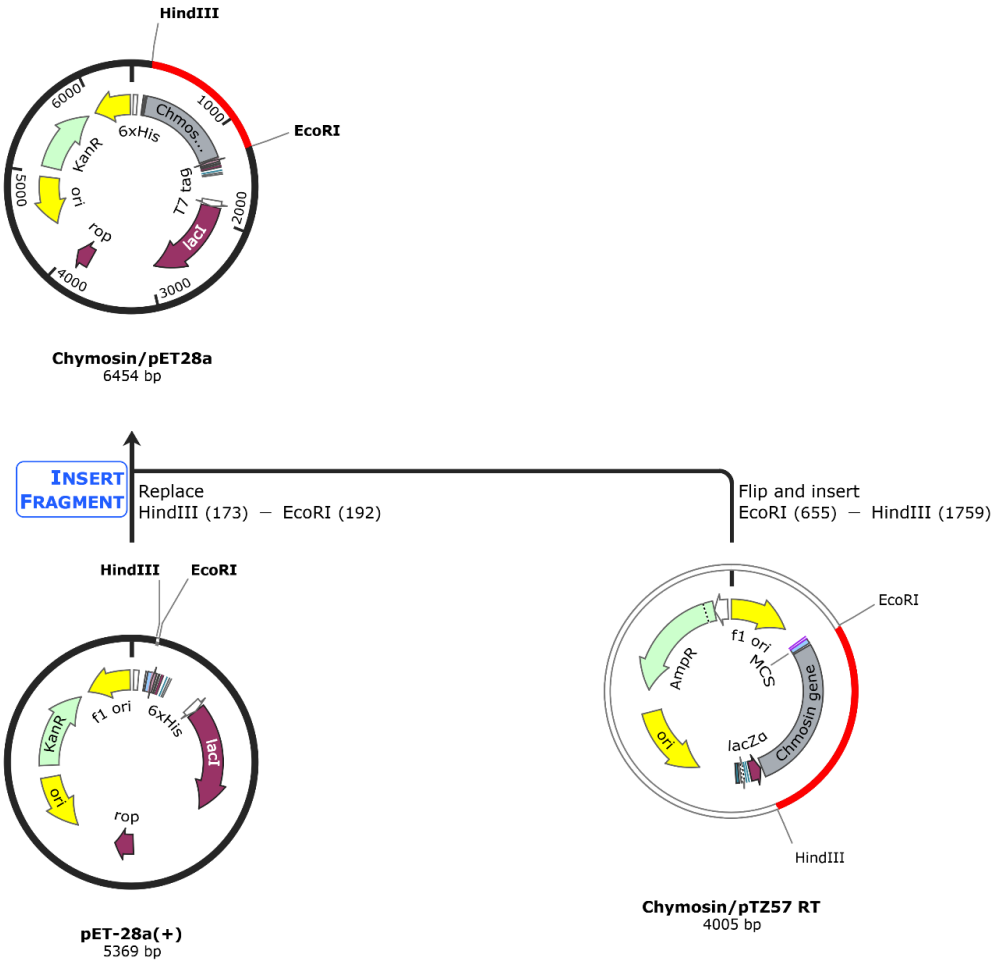

Figure A8 Cloning strategy for *chymosin/pET28a* construct.