CHAPTER II

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Lactic acid bacteria are Gram-positive, non-sporulating, catalase negative, aero-tolerant, acid tolerant and fermentative microorganisms that are without cytochromes and produce lactic acid as the main outcome of carbohydrate metabolism. It has a long history of safe use in fermented foods (El-Ghaish et al., 2011). Furthermore, to obtain useful and genetically-stable strains for industrially-important products, LABs need to be isolated from many natural sources. The group of LAB comprises of several bacterial genera: *Tetragenococcus, Vagococcus, Aerococcus, Weissella, Microbacterium, Bifidobacterium, Propionibacterium, Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus* and *Streptococcus*. Screening procedures and modifying the growth media and enzyme production media have always been the most powerful means for obtaining useful and genetically-stable strains for industrially-important products (Sato et al., 2004). Microbial enzymes produced are required in the production of fermented food, such as cheese, fermented sausages, fermented soybean products and soy sauce (Fernandez-Garcia et al., 1999).

Different species of LABs such as *Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Enterococcus, Vagococcus, Lactobacillus* and *Carnobacterium* have adapted to grow under very different environmental conditions (Farzanfar, 2006). LAB plays an essential role in the majority of food fermentations, in extending the shelf life of fermented products and improvement on the nutritional and sensory characteristics of end products (De vuyst & Leroy, 2007; Abd El Gawad et al., 2010).

These bacteria are broadly used by researchers and industries (Nikita & Hemangi, 2012) in the production of fermented food products, such as cheeses (*Lactococcus* spp.) and yogurt (*Streptococcus* spp. and *Lactobacillus* spp.). LABs play a critical role in the biochemical events that occur in the process of cheese ripening (Widyastuti et al., 2014). Visser (1993) asserted that the majority of LAB isolated from the fermentation of dairy products have a range of amino acids auxotrophy and be able to grow in protein rich medium and, they rely on the production of a complex proteolytic system to degrade casein, the main protein in milk. Furthermore, proteinase has an important function in the formation of cheese texture because of protein degradation (Olson, 1990).

Lactobacilli make up the bulk of the non-starter LAB (NSLAB) population (Fitzsimons et al., 1999; Swearingen et al., 2001). Other bacterial groups for instance, *pediococci, micrococci* and *leuconostocs* also have been proven to exist in the microflora of artisanal dairy products (Manolopoulou et al., 2003). NSLAB affect both the development of taste and texture, particularly of homemade fermented dairy products at specific ecological locations. These bacteria reflect the local, specific

microflora and it is the popular belief that variations in the quality of such products are due to the existence of NSLAB (Cogan et al., 1996; Beresford et al., 2001).

It is known that for centuries, LAB has been utilized to flavor, texturize and preserve food. LAB such as *Lactobacillus lactis* and *Streptococcus thermophilus* prevent food spoilage and the growth of pathogenic bacteria in dairy milk such as yogurt, thus ensuring the preservation of the nutritional value of milk and ensuring longer shelf life. In recent times there has also been discussion on the use of metabolites of LAB as a source of bio-preservatives for food. The antimicrobial effect of LAB is mostly due to the lactic and organic acid production, which reduces the pH of the growth environment. A low pH tends to transform organic acids to soluble lipids, which causes their diffusion through the cell membrane into the cytoplasm. Several halophiles LABs are often found on salted food, such as salted fish, meat and other food products (Tanasuoawat & Komagata 1995; Benito et al 2007; Hajar & Hamid, 2013). Sim el al. (2009) declared that microorganisms predominantly found during Bakasang fermentation were *Micrococcus*, *Streptococcus* and *Pediococcus*, and have been detected in a range of foods and fermented products including meat, milk products, vegetables, beverages and bakery products.

Lactic acid bacteria identification can be succeeded by phenotypic analyses and molecular methods such as 16S rDNA (Yeung et al., 2002). Phenotypic identification of LAB is based on physiological, metabolic biochemical and chemotaxonomic methods (Khedid et al., 2009). The molecular techniques have been rapidly developed, including PCR, which is based on amplification of ribosomal RNA and

electrophoresis of the PCR production. Analysis using PCR has been broadly applied in the study of microbial communities from environments (Zoetendal et al., 1998; Temmerman et al., 2004; Lee et al., 2005). The 16S rDNA gene sequence has been used to identify the LAB from different sources. The identification is based on the similarity with other sequences within the data base (Ali et al., 2012).

2.2 Extracellular Proteinase from Lactic Acid Bacteria

Lactic acid bacteria as large group of beneficial bacteria that have similar properties and all produce lactic acid as the end product of the fermentation process. They are widespread in nature and found in our digestive systems. However, they are more popular in relation to their roles in the preparation of fermented food, LAB produce proteolytic enzymes and many of the investigations focused on the degradation of milk (Udomsil et al., 2010; Xing et al., 2012).

One of the important features of LAB is their ability to produce extracellular proteinases responsible for the biochemical events taking place during cheese ripening. The proteolytic system of LAB has received special interest especially in the food industry. These LABs produce an extracellular cell-bound proteinase (Law & Kolstad, 1983; Zaurari et al., 1992; Abraham & Antoni, 1993). Some of these bacteria possess an exopolysaccharide layer (EPS) which has been claimed to improve yogurt body and to enhance yogurt smoothness (Cerning et al., 1992; Zaurari et al., 1992; Malik et al., 1994).

2.3 Proteolytic Activity of Lactic Acid Bacteria

Lactic acid bacteria are added as starter culture in milk fermentation. Majority of the starter culture species are nutritionally demanding, requiring many amino acids and growth factors for adequate growth (Nadra, 2007). LABs are only mildly proteolytic compared to bacteria such as *Bacillus* and *Pseudomonas* (Fadda et al., 1998). Lactic acid bacteria utilize the polypeptides generated by MCE and by bacterial cell wall proteinases and, therefore are responsible for the casein degradation. The combined action of proteinases and peptidases provides the cells with peptides and free amino acids. Then peptides and amino acids are transported across the membrane via specific transport systems. The internalized peptides are hydrolyzed by cytoplasmic peptidases (Wouters, 2002). The proteolytic enzymes of LABs are important for protein degradation when producing fermented milk products to allow these bacteria to grow on milk products as well as to develop product texture and flavor (Fox, 1989; Savijoki et al., 2006). Hence, it is very important, especially when using LABs in food production to tap more information on the varieties of proteinases that emerge extracellularly during growth of LABs in terms of the substrate specificity and biochemical characteristics of the proteinases, and factors that enhance production of the enzymes which vary in different strains.

2.4 Milk-Clotting Enzymes

Milk-clotting enzymes are one of the most important enzymes in today's field of dairy industries, especially due to their necessity for cheese production. Milk coagulation is

a basic step in cheese manufacturing. One of the most widely and acceptable coagulant used in cheese making globally is calf rennet, this enzyme extracted from the calf's fourth stomach.

The use of microorganisms to produce enzymes has enormous economic and technical advantages, (Couto & Sanroman, 2006). Microbial sources of milk clotting enzymes (MCE) have been used commercially such as from *Rhizomucor miehei* (Merheb-Dini et al., 2010), while others are being considered as potential sources that include *Aspergillus oryza* (Shata, 2005), *Amylomyces rouxii* (Pei et al., 2005). *Thermomucor indicae-seudaticae* (Reps et al., 2006) and *A. niger* (Moosavi-Nasab et al., 2010). Shieh et al. (2009) studied the production of MCE from *Bacillus subtilis* natto, while El-Bendary et al. (2007), Kathiresan and Manivannan (2007) and Keila et al. (2001) concentrated on *B. sphaericus*, *Streptomycetes* sp. and *S. clavuligerus,* respectively.

The ability to be mass-cultured and with a variety of properties that can be used for the production of different types of cheese made microbial enzymes more preferable to other sources of enzymes. Varieties of MCEs from plants such as *Ananas comosus, Carica papya* and *Lactuca sativa* (Egito et al., 2007); *Bromelia hieronymi* (Bruno et al., 2010); *Solanum dubium* (Isma et al., 2009); artichoke flowers *Cynara scolymus* L., (Chazarra et al., 2007); fruits of plants *Balanites aegyptiaca, Albizia lebbeck* and *Helianthus annus* (Egito et al., 2007) have received attention especially, when the use of animal rennet might be limited for religious reasons, vegetarianism diet or consumer concern regarding genetically engineered foods such as in Germany, Netherlands and France (Egito et al., 2007). In cheese making, MCEs are the primary

active agents, which involve the enzyme-mediated cleavage of κ-casein which covers the protein micelles at the peptide bond Phe 105-Met 106 which renders the casein micelles unstable and eventually causes aggregation that yields a clot or a gel.

In soft cheese, 50 to 80% moisture of the main proteolytic agent is the residual MCE, because the moisture content and the absence of cooking process enhance its retention in the curd and its activity on proteins (Noomen, 1978) in addition, to the native enzyme action in the milk (plasmin) (Fox & Stepaniak, 1993). Cheese contains high quality protein and calcium, and also several minor nutrients including phosphorus, zinc, vitamin A, riboflavin, and vitamin B12. Moreover, due to its nutritional food value, cheese is a popular item in human diet and there is widespread belief that consuming cheese minimizes the risk of dental caries through various mechanisms (Kashket & DePaola, 2002). Proteolysis is one of the most complex biochemical events in cheese ripening. The activities of these enzymes hydrolyze caseins (αs1-, αs2-, β- and κ-casein) to smaller peptides and amino acids, which contribute to flavor and texture of the cheese (McSweeney & Sousa, 2000). Proteolysis has been widely used as a basis for classification of cheese (Sousa & McSweeney, 2001).

Microbial rennet seems to have more commercial potential as its production is more cost effective, it is biochemically more diverse, and genetic modification is easier. It was shown that, many species of microorganism can also produce an MCE with the potential to replace calf rennet. Although cheeses made with vegetable coagulants are usually produced on an artisanal scale, in farmhouse or small dairy, most of them proved unsuitable for commercial cheese making owing to their high proteolytic

activity which lowers cheese yield and produce bitter flavors in the final cheese (Roseiro et al., 2003).

Lactic acid bacteria play an important role in the production of fermented food products, such as yogurt and cheeses. They play a vital role in the biochemical events that taking place during milk clotting in cheese processing and during ripening. LABs are also known to produce extracellular proteinases and LABs such as *L. helveticus* (Hebert et al., 2001), *L. paracasie*) (Haq-ul & Mukhtar, 2006) and *Enterococcus faecalis* 2495L, *E. faecalis* IAM10065 and *E. faecalis* 156 (Sato et al., 2004) were reported to have enzymes with milk clotting activity (MCA).

The proteolytic activity varies with types of microorganisms used. Most microbial sources of MCE are from fungi such as *A. niger* (Moosavi-Nasab et al., 2010) and *T. indicae-seudaticae* N31: (Merheb-Dini et al., 2010), but the MCE from fungi showed high proteolysis which leads to a weak body, bitter flavor defects and reduced cheese yield during storage (Moosavi-Nasab et al, 2010).

2.5 Milk Composition

Milk is containing more than 100 substances that are either in emulsion or suspension in solution for instance, casein, the major protein of milk, is scattered as a large number of solid particles ultrafine that they do not resolve, but remain in suspension. These particles are called micelles. The fat and fat soluble vitamins are in the form of an emulsion in the milk (Atamian et al., 2014). Some of the whey proteins, lactose,

mineral salts and other substances are soluble. The casein micelles and the fat globules give milk most of its physical characteristics. Many factors influence the composition of the milk such as breed of animals, feed, stage of lactation and season of the year. Milk is a normal product of mammary gland secretion. Ng-Kwai-Hang (2002) asserted that milk protein is a complex group of peptides in which over 200 different molecules have been characterized. Milk generally contains about 3.5% to 4.5% protein, of which approximately 80% are caseins and 20% of whey proteins. The isoelectric point of casein is at pH 4.6 while whey proteins consisting of β-lactoglobulin A (β-LG A), β-lactoglobulin B (β-LG B), α-lactalbumin (α-LA) has its at isoelectric point at pH 4.5 to 5.35. Milk caseins are fundamental in cheese making process because they form the gel network that entraps the other constituents of cheese. Casein consists of αS1-CN, αS2-CN, β-CN, and κ-CN in approximate proportions 4:1:4:1 (Ng-Kwai-Hang, 2002).

The concentration of calcium generally found in milk would cause precipitation of αS1-CN, αS2-CN, and β-CN; the calcium-sensitive proteins bind to their phosphoserine residues. The k-CN, however, is soluble in calcium and stabilizes the other caseins in a colloidal state (Farrell et al., 2006). It was also shown that about 95% of the caseins are aggregated in colloidal structures in milk (Farrell et al., 2006). The three main whey proteins are β-LG, α-LA and blood serum albumin (BSA), representing approximately 50, 20 and 10% of total whey proteins, respectively. Most whey proteins are globular with organized secondary and tertiary structure, which, in contrast to the caseins, make them sensitive to heat denaturation at temperatures above 60°C (Creamer et al., 2004).

2.6 Casein

Casein is one of the most important components of milk proteins. It is a phosphoprotein that represents 80% of the total milk proteins and precipitate at pH 4.6. There are three subgroups of casein αs1-, αs2-, ß- and κ-casein. The α-casein is the major casein (Walstra et al., 1999). α- Casein contains two proteins, including αs1- and αs2-casein. The αs1-casein has the highest charge and phosphate content, while α_{s2}-casein has the highest hydrophilic content due to the presence of phosphoserine. ß-casein is also a phosphoprotein, while κ-casein is a glycoprotein containing carbohydrate groups, κ- casein remains soluble in the presence of calcium salts and it plays an important role in stabilizing casein micelles (Fox & McSweeney, 1998; Walstra et al., 1999).

2.6.1 Casein Micelles

Casein micelles are generally spherical in shape as observed by the scanning electron microscopy with diameters ranging from 50 to 500 nm. About 95% of casein in normal milk is not present in solution but in large colloidal particles known as micelles. Micelles contain about 94% protein (on dry matter basis) and 6% low molecular weight species as colloidal calcium phosphate (CCP), and minerals calcium, magnesium, phosphate and citrate resulting the white color in milk (Fox & McSweeney, 1998). The α, β and κ-casein monomers form small and roughly spherical aggregates known as sub micelles which are stabilized by hydrophobic interactions and calcium bridges. The casein micelles are about 12 to 15 nm in

diameter each of them contains 20 to 25 casein molecules (Walstra et al., 1999). These sub micelles contain a hydrophilic layer. Sub micelles are aggregated by colloidal calcium phosphate (CCP) linkages to form casein micelles. The κ-casein is set nearby outside of the micelles with the hydrophilic part swollen from the micelles surface to form a hairy layer that will prevent aggregation of casein micelles by steric and electrostatic repulsion, resulting in stable casein micelles.

Figure 1: Sub-micelle Model (cross section) of a Casein Micelle (Walstra, 1999)

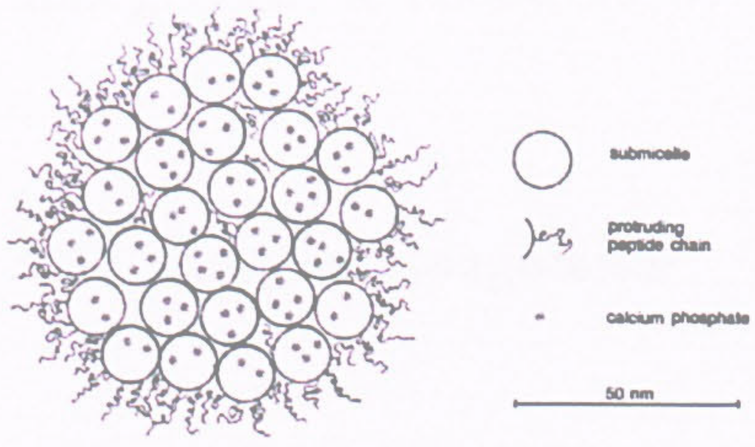
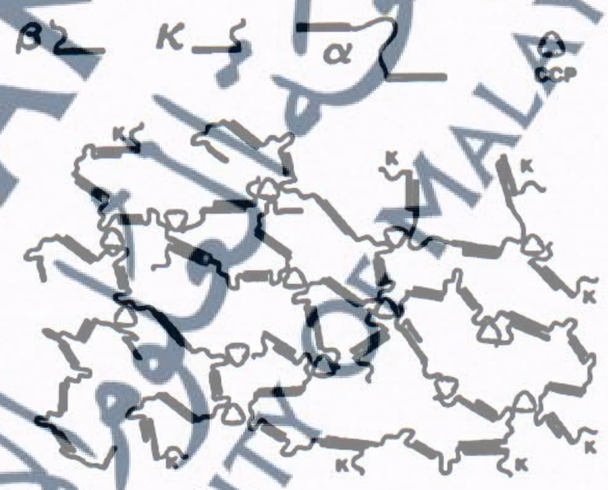


Figure 2: Dual-binding Model (Horne, 1998)



2.7 Mechanism of Milk Coagulation

The unique characteristic texture of many dairy products is the result of coagulation of casein to form gels. Milk protein is able to form different types of network structures depending on many factors such as temperature, pH, and salts. Milk coagulation is the first step in cheese manufacture. Coagulation is essentially the formation of a gel by destabilizing the casein micelles causing them to aggregate and form a network which entraps fat. Coagulation can be initiated by the following:

2.7.1 Acidification

Under normal condition, at pH of 6.5 to 6.8 of natural milk, caseins have net negative charges which result in electrostatic repulsion that stabilizes casein micelles. Acidification causes the casein micelles to destabilize or aggregate by decreasing their electric charge to that of the isoelectric point at pH 4.6. As the charges of casein molecules are neutralized and electrostatic repulsion is reduced, hydrophobic interactions between caseins occurs resulting in aggregation of casein micelles to acid milk gel (Lee & Lucey, 2010).

2.7.2 Enzyme Coagulants

The enzymes used for milk coagulation originated from a number of sources: animal, plants and microorganisms. They are initiated by proteolysis of κ-casein, followed by calcium-induced micelles aggregation. Rennet from calf stomach is commonly used. This enzyme hydrolyzes peptide bond specifically the Phe105-Met106 of κ-casein, producing para- κ-casein and macropeptide. The hydrophilic macropeptides of κ-casein diffuse into the surrounding aqueous, which removes the steric stabilizing layer while the para-κ-casein remains to the micelles core. As a result, both negatively charged groups and steric stabilization reduces, resulting to aggregation into gel of para- κ-casein and other caseins under influence of Ca^{2+} (Choi et al., 2007). Various proteolytic enzymes that are obtained from plants, animals and microbial sources could serve as measures in achieving the coagulation of casein micelles in milk. Enzymes traditionally used in the manufacture of cheese are rennet containing a

mixture of chymosin and pepsin, the former being extracted from calf stomach and the later from cow stomach. In the gel formation process, three phases can be distinguished (Dalgleish, 1992). In the basic phase, the κ-casein of the casein micelles is hydrolysed by the enzyme to yield two peptides of different properties, a hydrophilic macropeptide which is split off from the micelle and the hydrophobic para- κ-casein which remains in the micelle. The progressive hydrolysis of κ-casein leads to the change in the properties of the casein micelles resulting in aggregation in the presence of Ca^{2+}.

2.8 Effect of Nitrogen Source in Enzyme Production Media on Milk Clotting Enzyme Production

Different substrates significantly affect milk clotting enzyme production (Merheb-Dini et. al., 2010). According to Edesfer et al. (2007), LABs have numerous nutritional requirements for growth, especially nitrogen sources. Generally biomass synthesis of LAB is predominantly from building blocks present in the culture medium. These variances in proteinase production which use a variety of nitrogen sources may show dissimilarities in the amino acids which encourage proteolytic activity.

Complex organic nitrogen sources such as casein, soya peptone, and tryptophan vary in free amino acid concentration and the amount of small peptides present. Casein plays an important role in microbial clotting enzyme and induces production of enzyme under both solid state fermentation and submerged fermentation conditions in

the case of *R. miehei* (Silveira et al., 2005). *Mucor. mucedo* DSM 809 is grown in enzyme production media containing 0.5% (w/v) casein (Yeing et al., 2010). In contrast, *R. nainitalensis* showed maximum MCA obtained when 1.5% (w/v) casein was used as nitrogen source in the production media (Khademi et al., 2013). De-Lima et al. (2008) reported that rennet obtained from *M. miehei* NRRL 3420 had the highest MCA obtained when using 0.4% casein and decreased MCA when casein concentration was increased to 0.8% in enzyme production media. The MCA and PA of *M miehei* NRRL 3420 can be affected by nitrogen sources (Moon & Parulekar, 1991; Chu et al., 1992; Sato et al., 2004; Patel et al., 2005). Therefore, each nitrogen source may possess the necessary amino acid in inadequate amounts to encourage activity in optimal concentrations for induction or in an excess repressing enzyme synthesis.

2.9 Factors Influencing Milk Clotting Activity

2.9.1 Effect of pH and Temperature on Milk Clotting Activity

Temperature, pH and concentration of $CaCl_2$ of the milk are very important and have significant effects on the MCA yield and rheological characteristics of the product (Najera et al., 2003). MCA is strongly influenced by the temperature which affects protein aggregation and gel formation (Najera et al., 2003). It was observed that the rate of gel firming increased with an increase in temperature which causes the protein matrix to shrink due to increased hydrophobic interaction (McMahon et al., 1984). Additionally, temperatures higher than standard milk pasteurization temperature causes extensive degradation of casein leading to bitter taste, reducing the yield of the

gel and totally denaturing the enzyme (Lucey, 2002). At higher incubation temperature up to 60ºC significantly decreases the MCA (Foda et al., 2012). A thermophillic neutral protease from *Bacillus* strain HS 08t showed optimum MCA at 65ºC. MCA is similarly greatly influenced by pH. A milk pH below 5.8 leads to a change in the distribution of casein between micelles and serum (Awad, 2007).

Several microbial extracellular proteinases show activity similar to rennin and are suitable for cheese-making, and the activity varies with bacteria, pH and temperature. It was reported that *B. subtilis natto* (Shieh et al., 2009) showed maximum enzyme activity at pH 6 and temperature at 37°C; *B. sphaericus* exhibited optimum activity at pH 5.7 to 7.5 and temperature at 55ºC (El-Bendary et al., 2007). A partially purified neutral protease from *B. subtilis* showed optimum MCA at pH 7.0 and at temperature of 40ºC (El-Safey et al., 2004). A halotolerant *B. aquimaris* VITP4 produced extracellular protease with optimum pH 7.5 and temperature 37°C (Shivanand et al., 2009).

2.9.2 Concentration of Calcium chloride

Calcium plays an important role in milk coagulation as well as in the gel formation. It has a significant role in casein aggregation in the second step (non-enzymatic) of milk clotting, as a result of the neutralization of casein micelles' negative residues (phosphoserine and carboxylic groups) by Ca^{2+} and calcium– phosphate complexes (Pires et al., 1999). Merheb-Dini et al. (2010) reported that calcium has a significant role in casein aggregation during milk coagulation. Increasing the concentration of

$CaCl_2$ over 10mM may have a negative effect on curd formation, as the additional calcium will increase the positive charge on the surface of the micelle, causing charge dissonance which produces weaker gel or no gelation at all (Arima et al., 1970; El-Bendary et al., 2007; Sandra et al., 2012; Verma et al., 2012) reported that the increasing calcium concentration to 20mM in goat or cow milk decreased milk clotting time. Vairo-Cavalli et al. (2005) suggested that the increased Ca^{2+} concentration in the substrate increased the ionic force or the saturation of negative residues of the casein micelles.

2.10 Purification of Enzyme

Researchers focused their investigation on discovering and characterizing novel and natural proteases from formerly ignored sources (Goud et al., 2009). Microorganisms are being investigated for their potential to be suitable biotechnological sources of relevant enzymes with promise of industrial viability (Peter et al., 2014). Thus, there is a need to identify novel and more active proteolytic enzymes from diverse habitats.

Protein separation techniques have traditionally been used to isolate and to purify specific proteins in order to facilitate studies of their enzymatic, physical, chemical and structural properties. These kinds of studies are necessary in order to elucidate the biological role of individual proteins in the cell and to understand the mechanism by which the activity of specific enzymes is controlled. A number of alkaline proteases from different sources have been purified and characterized. It has become common to use the popular precipitation method to isolate and recover proteins from crude

biological mixtures. It also carries out steps for purification and concentration (William & Swiatek, 2009).

The biochemical characteristics of extracellular protease increase in specific activity after the ammonium sulfate precipitation and acetone precipitation (Ganesh et al., 2008). The property of enzyme severely restricts the choice of purification methods, making the majority of the conventional procedures unsuitable. Several methods are employed for concentration of the culture supernatant by ammonium sulphate or solvent precipitation (Tunga et al., 2003; Fernandez-Lahore et al., 1999; Hajji et al., 2007) and ultra-filtration through membrane (Bohdziewicz, 1994). There have been several works that indicate acetone being used at various volume concentrations, as a main agent of precipitation to recover alkaline proteases (Horikoshi, 1971; Tsujibo et al., 1990; Kim et al., 1996).

Protease can also be purified as well as protein by a combination of chromatographic procedures. However, too many steps make the method more cumbersome and hence, minimum steps for purification of concentrated enzyme can be a method of choice. Purification procedures for different proteins tend to be unique for that protein. Procedures are developed in a stepwise manner. For further purification, gel filtration technique was applied. The purity was judged by enzyme specific activity, SDS-PAGE and casein zymogram, indicating that there was homologous protease within the extracellular supernatant and activity of the active fraction is assessed.

Several research works have been carried out on lactic acid bacteria proteases, likewise several types have been purified and characterized. Several studies using *Streptococci* and *Lactococci* extracellular or cell wall-associated proteases have also been conducted by Thomast and Mills (1981). Kunji et al. (1996) and Juillard et al. (1995) found that cell wall is associated with extracellular protease produced by *L. lactis* subsp. *cremoris* that hydrolyses casein. An extracellular cysteine proteinase produced by *Micrococcus* sp. INIA 528 was also purified by chromatography using sephadex G-100 and G-50 pre-equilibrated with 50mmol- sodium phosphate buffer to achieve a 29-fold increase and 28% recovery of the proteinase activity (Fernandez et al., 1996). Microbial proteases play an important role in biotechnological processes accounting for approximately 59% of total enzymes used (Shumi et al., 2004).

2.10.1 Ammonium Sulphate Precipitation

To isolate and recover proteins from crude biological mixtures, researchers have commonly used ammonium sulphate precipitation (Bell et al., 1983), which is also capable of performing both purification and concentration steps. Adding reagents like salt or an organic solvent can result in effects like the lowering of the solubility of the desired proteins in an aqueous solution. Even though ammonium sulphate precipitation has been used for many years, it is however not the precipitating agent of choice in the case of detergent enzymes. On the other hand, ammonium sulphate precipitation has been widely used particularly in acidic and neutral pH values and developed ammonia under alkaline conditions (Aunstrup, 1980).

The precipitation of the enzymes by ammonium sulphate remains a common use for the concentration of the enzymes from microbes (Lee et al., 2002; Cheng et al., 2010) because of its high solubility, lower cost and stabilizing effect on most enzymes as well as can be used in acidic and neutral pH solutions (Rifaat et al., 2006). Therefore, using sodium sulphate or an organic solvent can be a preferred choice.

2.10.2 Gel Filtration Chromatography

Gel filtration chromatography otherwise called molecular filter chromatography is a technique that sees the separation of molecules based on size and shape. Separating the components in the sample mixture, with some exceptions, has a close correlation with their molecular weights. In these cases, gel filtration us a feasible analytical technique to for the determination of the molecular weight of an uncharacterized molecule. The most common applications of gel filtration chromatography are in purification of enzymes and other proteins and in estimation of molecular weight mainly for globular proteins. Gel filtration is also an important preparative technique since it is often a chromatographic step in the purification of proteins, polysaccharides and nucleic acids (Nouani et al., 2009).

An extracellular proteinase from *E. faecalis* subsp.*liquefaciens* was purified 780-fold using a process that included gel filtration on Sephadex G-50 and affinity chromatography. About 15% successful recovery was recorded of the original enzyme activity.

2.11 Milk Gel Texture

Various factors can affect the rheological and textural properties of coagulated casein as in cheese. Most of these effects are well recorded and others remain as topics of discussion, debate and ongoing research. Many factors influenced the rheological and textural properties of cheese such as flavor, appearance and other properties that matter to consumers. Texture is a very crucial aspect that affects consumer choice. Wendin et al. (2000) showed that texture characteristics are more critical in indicating differences between cheese samples than taste and flavor. Antoniou et al. (2000) categorized and differentiated French cheeses on the basis of instrumental and sensory measurements of texture. In general, the appropriate texture in cheese is taken by the consumer as the indication of the overall quality of the cheese. As such, the appearance and quality of the cheese texture is a critical determinant of consumer choice (McEwan et al., 1989). Texture can be described fundamentally as: the genuine, physical structure of material and that which is visually observed (Surmacka-Szcześniak, 2002).

Various characteristics of cheese, both visual and physical originate from the importance of foodstuff makeup and evaluation based on a sensory scheme. Therefore, this indicates that the human being's sensory processes and physical prototype of the material employed in mastication is vital in attempting to show conclusions related to a material's texture (Christensen, 1984). Ways to enhance techniques in evaluation have also been investigated. Awad et al. (2007) indicated that

evaluating by both hand and mouth can similarly differentiate the texture of a variety of cheeses.

Several scholars have employed the texture profile analysis (TPA) and the same uniaxial compression tests at temperatures ranging from 10 to 20°C to determine hardness and firmness of cheese. Mozzarella cheese was shown to have a distinct softening trend with increasing age and level of proteolysis (Tunik et al., 1993; Yun et al., 1995). However, Rudan et al. (1999) and Guinee et al. (2002) noticed that with increasing fat and/or moisture content, there was a reduction in Ca content and pH.

Foods being material have mechanical properties such as texture. Texture is determined by a combination of mechanical and fracture properties and their change and expression in the mouth during chewing. A number of factors can cause difficulties namely,

1. Foods are mechanically very complex
2. Mechanical processing and chewing are a combination of several processes that need to be separated, quantified and understood.
3. Food in mouth is all the time changing its properties with changes in temperature, water content, pH and so on.
4. The majority of food scientists have no training in material science
5. The majority of material scientists do not consider food as a material.

It is then not possible to measure it in a machine; it might possibly be able to identify the primary factors that determine the texture of a food material and to measure that.

For this there are to possible methods. The common and easier to do is to apply any type of mechanical deformation to evaluate the response and try to correlate the result with the findings from a sensory panel (Kilcase, 1999).

2.12 Syneresis

Syneresis is viewed as one of the most significant steps in the preparation of dairy products (Walstra, 1993). Re-arranging the casein micelles result in the shrinking of the casein matrix and subsequent expulsion of whey from curd pieces. The rate and extent of syneresis determine the moisture and lactose contents of curd (Castillo, 2006) which affects cheese moisture and pH, and ultimately the cheese texture, color, flavor and quality. Syneresis also affects protein and fat loss in whey and therefore, cheese yield. Enhanced control of the syneresis process improves the final cheese product's uniformity and quality (Mona et al., 2011).

Syneresis process and factors that impact its extent and kinetics have been well investigated in rennet-induced milk gels (Walstra, 1993). In spite of the significance of the syneresis process, it is still one of the least understood aspects of cheese making, particularly in mixed gels, like cottage cheese. Rate and extent of syneresis relies on the equilibrium between the pressure gradient within the gel network and the resistance to whey expulsion e.g., permeability (Walstra, 1993). Significant relationships between the syneresis reaction and coagulation factors and/or rheological properties of gel have been documented (Lucey, 2001; Dejmek et al., 2004).