PROTEASE ACTIVITY OF *Streptococcus thermophilus* TISTR 894 IN FERMENTED MILK

ABSTRAK

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Susu fermentasi merupakan produk susu yang difermentasi oleh mikrobia, umumnya bakteri asam laktat. Selain menggunakan laktosa untuk pertumbuhan dan produksi asam, protease yang dihasilkan oleh bakteri asam laktat akan mendegradasi protein susu menjadi peptide rantai pendek dan asam amino. Bakteri asam laktat tidak dapat mensintesis asam amino yang diperlukan untuk pertumbuhan dan pemeliharaan fungsi sel bakteri.

Oleh karenanya tujuan penelitian ini adalah mempelajari aktivitas protease selama fermentasi susu oleh *Streptococcus thermophilus* TISTR 894. Kultur S. thermophilus TISTR 894 diinokulasikan ke dalam susu dan diinkubasi selama 48 jam pada suhu 30°C dan diamati pertumbuhan, dan aktivitas proteasenya. Produksi protease juga diamati selama fermentasi susu pada berbagai suhu fermentasi. Aktivitas protease duji pada berbagai suhu pH (4, 5, 6, dan 7) dan suhu (30, 40, 50°C). Aktivitas enzim protease tertinggi diperoleh pada jam ke-24 dengan nilai aktivitasnya sebesar 0.0003 U/ml. Produksi protease tertinggi diperoleh pada fermentasi susu pada suhu 30°C. Kondisi optimal aktivitas enzim protease diperoleh pada pH 5 dan suhu 40°C.

Kata Kunci : *susu fermentasi, aktivitas protease, Streptococcus thermophilus* TISTR 894, *pH,* dan *suhu*

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ABSTRACT

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Fermented milk is product of milk that is fermented by microorganism, it is commonly used lactic acid bacteria (LAB). In addition to use lactose for growing and lactic acid producing, protease is also produced by LAB to cleave protein into small peptides and free amino acids that cannot be synthesized by lactic acid bacteria. Amino acids are essential for the growth and maintenance the cell of bacteria. Therefore, this research aims to study the protease activity during milk fermentation by *Streptococcus thermophilus* TISTR 894. Milk was inoculated with *S. thermophilus* TISTR 894 and incubated to be grown at 30°C for 48 hours. Its growth and protease activity was observed during fermentation. Protease production determined at various milk fermentation temperatures. Enzyme activity was assayed at various pHs (4, 5, 6, and 7) and temperatures (30, 40, and 50°C). The highest protease activity was obtained at 24 hours, which was 0.0003 U/ml. The highest protease production was obtained at milk fermentation of 30°C. The optimum protease activity were obtained at pH 5 and 40°C.

Keywords : *Fermented Milk, the activity of protease, Streptococcus thermophilus* TISTR 894, pH, and temperature.

CHAPTER 1

INTRODUCTION

1.1 Background

Fermented milk products have been produced for a long time as the preserved food. They are produced by the microorganism involved in the fermentation. There are large number of researches about the fermented milk which applied lactic acid bacteria (LAB). Moreover, milk as a medium fermentation has abundantly favorable component for their growth such as, lactose, protein, vitamins, and minerals. Thus, milk is suitable medium for supporting the growth of LAB.

LAB that commonly used in production of fermented milk, utilize the lactose as source of energy in milk for the growth and acid production. Furthermore, some LAB possess proteolytic system to cleave milk protein into peptides and free amino acids. Those products contribute to build and maintain the cell of bacteria. In addition, they are fundamental for ruining the metabolic pathway of bacteria.

The ability of lactic acid bacteria to produce protease subsequently to cleave milk protein or the activity of protease is a highlight in this research because it is essential to generate peptides and free amino acids. Thus, the activity of protease has an important role for fulfilling their need of peptides and amino acids.

There are a lot of development in finding the other potential lactic acid bacteria to produce protease in fermented milk. However, there is a lack of related research from the second most widely applied strain in the dairy industry, which is *S. thermophilus*. In addition, *S. thermophilus* is well known strain for yoghurt

production with *Lactobacillus bulgaricus*. This is the only strain from *Streptococcus sp*. which is applied in food industry because it was proven by Food and Drug Association (FDA). This strain was validated safety because it have been incorporating in food for centuries and it is categorized as Generally Recognized as Safe (GRAS) bacteria. Furthermore, this strain possesses protease to produce amino acids and peptides. Therefore, this study was observed the protease activity of *S. thermophilus* TISTR 894 in during milk fermentation. In this study, the activity of protease from *S. thermophilus* TISTR 894 was also compared to others lactic acid bacteria.

1.2 Objectives

Based on the research rationale above, these are the purpose of the research.

1. To study and compare protease activity of 5 strains of Lactic Acid Bacteria (*Lactobacillus plantarum* SKKL 1, *Lactobacillus casei* TISTR 1463, *Lactobacillus plantarum* MW3, *Streptococcus thermophilus* TISTR 894, and *Lactobacillus plantarum* TISTR 2075) which were grown in MRS Broth.
2. To study protease activity of *S. thermophilus* TISTR 894 during milk fermentation.
3. To study the protease activity of *S. thermophilus* TISTR 894 at various pH and temperatures.

1.3 Benefits

The information of protease activity of *S. thermophilus* TISTR 894 during milk fermentation and the optimum pH and temperature for protease activity can be used to design milk fermentation condition in order to get the more produced peptides and free amino acids for supporting its growth.

1.4 Expected Outcome

Based on purpose of Research above, these are the expected outcome.

1. Information of activities of protease from 5 strains of Lactic Acid Bacteria (*Lactobacillus plantarum* SKKL 1, *Lactobacillus casei* TISTR 1463, *Lactobacillus plantarum* MW3, *Streptococcus thermophilus* TISTR 894, and *Lactobacillus plantarum* TISTR 2075) which were grown in MRS Broth.
2. There is an information about the activity of the protease from the *S. thermophilus* TISTR 894) during milk fermentation.
3. The information of optimum pH and temperature for protease activity from *S. thermophilus* TISTR 894.

CHAPTER 2
LITERATURE REVIEW

2.1 Milk and Milk Components

Milk is considered as a completely nutritional food because containing energy, protein, lipid, vitamins, and minerals. Those composition fulfill daily fundamental need of human because milk has approximately 62 kcal of energy, 3.3 g of total protein, and 3.3 g of total lipid in 100 g of milk. In addition, it provides some vitamins (vitamin A, vitamin E, vitamin C, vitamin B_{12}, vitamin B_6, thiamin, retinol, and carotene) and minerals (zinc, calcium, magnesium, iron, potassium, copper, selenium, manganese, phosphorus) for supporting human physiological system (FAO, 2013). Each component in milk has an essentially specific role contributing many benefits .Thus, milk is beneficial dairy food.

Milk as a source of energy is obtained from lactose, essential carbohydrate in milk. As a disaccharide, it is able to be split by lactase in the form of monosaccharide, glucose and galactose. Furthermore, lactose maintains the growth and increases the absorption of water and mineral. However, in case of lactose intolerance, the lactose results discomfort in digestive tract and diarrhea (FAO, 2013)

Milk protein are commonly found in the form of whey protein and casein. Whey protein is soluble material of milk that is obtained from cheese production. It provides amino acids and peptides (FAO, 2013). Casein as the major protein component, which is 80% in milk (Tian *et al.*, 2018), is considered as the valuable

source to earn amino acids, which are fundamental for the growth. As mentioned before, the peptides can be produced from milk proteins by protease activity during fermentation. The protease can splits the proteins become protein fragment, which is arranged from two until 20 amino acids (Chen *et al.*, 2014).

Milk fat is consisted by large number of fatty acids and other lipid molecules. For example, 1.9 g of saturated fatty acids (SFAs)/100 g is contained by cow milk. In addition, it has abundant amount of oleic acid (C18:1), monounsaturated fatty acids (MUFA), which is 0.8 g/100 g in milk. Also, the PUFA is detected as well in milk (about 0.2 g/100 g) (FAO, 2013). Moreover, it contains cholesterol concentration, which is about 0.03 g /100 g in milk (Barłowska, Szwajkowska and Litwi, 2011) . Moreover, milk fat also provides energy value.

Milk as food ingredient has some characteristics. For example, the presence of abundantly nutritional milk components can be a favorable environment for growth of spoilage and pathogenic microorganism. In addition, the number of lactic acid bacteria was commonly found in raw milk, which was $5 \times 10^2 - 8 \times 10^2$ CFU/ml. The unsaturated fatty acids in milk stimulate the undesirable effect, for example, rancidity (Stulova, 2013).

2.2 Fermented Milk

- **Definition and Benefits of Fermented Milk**

Fermented milk is one of the dairy products and is preserved food. It has a lot sort of form, such as fermented milk, butter milk, yoghurt, kefir, koumis, tarag, kurut, dabi, and others fermented milk. Fermented milk has been contributed a lot

of beneficial effect to human digestive system and also reduced serum of cholesterol. The advantage from this product is obtained by inoculating or growing the amount of micro-organism in milk. They stimulate the health of host by their existence or their metabolism. For instance, in fermented foods, which are incorporated with the bacteria from lactobacilli and bifidobacteria, stimulate the immune system, decrease the risk of gastrointestinal problems, mitigate the intolerance of lactose, keep the allergy at bay, inhibit the growth of pathogenic bacteria in gastrointestinal, etc. Additionally, milk protein and lactose in fermented milk is more digestible than in original milk. In addition to provide the digestible component, it also increases the shelf-life (FAO, 2013).

- **Single and Mixed Cultures in Milk Fermentation**

As mentioned before about the microorganism involved during fermentation, lactic acid bacteria is the commonly applied strain in each of fermentation products. There are a lot of fermented milk product involved either single or mixed cultures. Both of cultures catabolize three major milk components during fermentation: (i) degradation of lactose into lactic acid (fermentation), (ii) cleavage of casein into free amino acids and peptides (proteolysis), (iii) change of milk fat into free fatty acids (lipolysis). Those products contribute to the decrease of pH, the semi-solid texture, and the distinctive flavor. However, the acceleration of those products to be produced are different in single and mixed cultures. For example, in yoghurt production, the mixed culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus)* reached the desirable pH for yoghurt at 4 hours of fermentation

meanwhile the single culture required more than 4 hours (Settachaimongkon, 2014).

Both of cultures mostly can grow in milk because the milk component is nutritionally suitable to support their activities during fermentation. For instance, the lactose as source to fulfill their energy and the protein to maintain their cells.

- **Changes of Milk Components during Fermentation**

Milk components has been altered during fermentation. For instance, the protein is partial split by the presence of bacterial proteolytic system and is changed to be amino acids for supporting the growth of bacteria during milk fermentation. In case of lactose, it is decreased by enzyme of bacteria converting lactose into glucose and galactose. This condition provide a better tolerated food for lactose-intolerance issue because the less lactose. In addition, the glucose is used by bacteria in milk to support their growth and is altered to lactic acid. It creates sour taste in each of fermented milk products. Also, it inhibits the growth of pathogenic bacteria by lowering the pH with an amount of lactic acid in fermented milk. Furthermore, this condition increases the shelf-life of fermented milk products and microbiological safety of food (FAO, 2013). Moreover, the milk fat is transformed to free fatty acids by lipolitic enzyme to contribute the distinctive flavor (Settachaimongkon, 2014).

2.3 Lactic Acid Bacteria

o General Characteristic of Lactic Acid Bacteria

Lactic acid bacteria (LAB) is commonly applied as starters in dairy products. There are four genera of LAB, *Lactococcus, Streptococcus, Leuconostoc,* and *Lactobacillus.,* are widely used in dairy products to produce some products, such as, cheese, butter, yoghurt, fermented milk, etc. The characteristics of LAB for dairy products are acid tolerant, Gram-positive, non-motile, catalase-negative, facultative anaerobic micro-organisms, and nonsporulating. LAB divide into homo-fermentative and hetero-fermentative, based on main product from their metabolism. The homo-fermentative LAB mostly produces lactic acid meanwhile hetero-fermentative LAB, in addition to provide lactic acid, results amount of other fermentation products, such as ethanol, acetic acid, carbon dioxide, and formic acid. Furthermore, LAB initiates the production of other favorable products, such as enzymes, flavor precursor, and some products contributing the body and texture of cheese (Stulova, 2013).

LAB vary in their optimum temperatures for growth. The mesophilic bacteria thrive at 20-30°C meanwhile the thermophilic bacteria lives at 35-45°C. Mesophilic LAB are applied in the production of fermented milk products, cheese, and ripened cream butter. In case of thermophilic bacteria, they are used for yoghurt and cheese making with the high processing temperatures (Stulova, 2013).

o Metabolism of LAB during Milk Fermentation

LAB can metabolize the lactose, primarily major substrate during fermentation for providing energy. Lactose is transferred from milk medium into the cell of

bacteria by enzyme, phosphotransferase (PTS) and permease. In the cell of bacteria, lactose is split into glucose and galactose by intracellular enzyme, β-galactosidase. The glucose is used as a source of energy (Stulova, 2013).

In addition to cleave the lactose, LAB possess the extracellular proteolytic system to catabolize the casein, the major milk protein. This ability fulfill the amino acids need of LAB by degrading the protein into amino acids and peptides. The process involves three steps. In the first place, the protein is cleaved by proteinase into oligopeptides. Proteinase as cell envelope protease (CEP) initiates to degrade the casein. The presence of CEP is founded in some lactic acid bacteria. For example, *Lactobacillus plantarum and Lactobacillus casei*. They were proven secreting extracellular proteinase (Utami et al., 2015) (Genevois, 2019). However, most of *Streptococcus sp.* cultures have limited protease activity because it possesses a bit of cell-envelope protease (CEP) (Settachaimongkon, 2014). Then, the breakdown of protein product is transported into the cell by peptide and amino acid uptake system. Finally, the peptidase cleaves the peptides into free amino acids. The amino acids are used to support the growth of LAB in milk.

LAB catabolizes fat in milk as well. The bacteria transform the fat in milk into free fatty acids. Lipolysis facilitates the breakdown of milk fat by lipolitic enzyme system. This activity contributes to the distinctive flavor of fermented milk products (Settachaimongkon, 2014).

2.4 Protease from Fermented Milk

Proteases, according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, are enzymes which belong to hydrolases enzyme and peptide hydrolases or peptidases. They are divided become two group based on their site of action, endopeptidases and exopeptidases. Based on pH, proteases are classified into three groups, such as acid, neutral, and alkaline (Mamo, 2018). Also, they are divided into four group of protease based on the catalytic action, for instance aspartic, cysteine, metallo, and serine proteases.

Protease in metabolism of milk protein is classified into two group based on the substrate, proteinase and peptidase. Proteinase initiates the breakdown of casein into oligopeptides meanwhile peptidase cleaves the peptides into free amino acids (Stulova, 2013)

Protease, as an enzyme, can catalyzes a reaction of breakdown the protein into peptides and amino acids. It binds the substrates in the active site, followed by forming the bond between the protease and protein. This interaction is the key of a catalyst to decrease the activation energy, thus it can accelerates the breakdown of protein. Protease then cleaves the protein into smaller fragment and produces peptides and amino acids, the product of splitting the protein. Therefore, one unit (U) of protease is defined by the amount of casein about 1µmol that can be

transformed become peptide fragment per minute under the assay condition (Nelson and Cox, 1982)

Protease activity can be affected by some conditions, such as pH and temperature. The temperature increases the protease activity to a specific point. This can be explained by the more intense interaction between the substrate and protease as the increased temperature which creates the higher kinetic energy than before increasing temperature. However, the protease is eventually inactive and lost the function to transform the substrate when the temperature is gradually changed from the specific point or the optimum temperature. It can be caused by the denatured protease. This condition affect the structure of protease, primarily in the active site. Then, the protease is lost the function to bind the substrates (Daniel and Danson, 2013).

The condition of pH influences the protease structure and activity. In the optimum pH, the active site of protease is in the most effective state to bind substrates, thus the protease activity is increased. Nevertheless, it is decreased when the concentration of H^+ is changed from the optimum concentration of H^+. This change interferes the function of amino acids to stabilize the tertiary and quaternary structure of enzyme. Consequently, the active site is changed and lost the function to bind substrates (Yusriah and Kuswytasari, 2013) .

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

- **Bacteria Culture**

Lactobacillus casei TISTR 1463, *Streptococcus thermophilus* TISTR 894, and *Lactobacillus plantarum* TISTR 2075 were the strains of the Lactic Acid Bacteria that applied in this research and well provided by Thailand Institute of Scientific and Technological Research (TISTR) meanwhile *Lactobacillus plantarum* MW3 and *Lactobacillus plantarum* SKKL 1 were the strains that obtained from The Department of Biotechnology, Faculty of Technology, Khon Kaen University. The isolates were kept in MRS-Glycerol at -20 °C.

The stock culture of those strains were obtained from 1 mL of each strain in de Man, Rogosa, and Sharpe (MRS) Broth, which has been incubated for 24 hours and transferred afterward to micro tube to be centrifuged at 10,000 x *g* for 5 minutes at room temperature to separate the supernatant and pellet. Then, the supernatant was thrown and the pellet was washed with 1 mL sodium chloride 0.85%. Afterward, the new solution was separated by centrifuge at 10,000 x *g* for 5 minutes at room temperature and only the pellet was taken. The pellet was re-suspended and mixed with 500 µL of MRS Broth. The broth was supplemented with 500 µL of 20% (*v/v*) Glycerol.

- **Media and Chemicals**

Some media like Skim Milk Growth Powder, *Lactobacillus* MRS Broth, Bovine Serum Albumin (BSA) and Agar Powder Bacteriological Grade were earned from HIMEDIA REF. The casein from bovine milk as a substrate was purchased from SIGMA LIFE. All applied analytical reagent in this study, from Ajax Finechem, were sodium carbonate 4%, sodium chloride 0.85%, hydrochloric acid 37%, trichloroacetic acid (TCA) 10%, sodium phosphate, Folin reagent, citric acid, tyrosine, and Lowry reagent.

Preparation all of media and chemicals were explained below. Sodium chloride 0.85% was prepared by dissolving 1.7 g of it in 200 mL of distilled water. *Lactobacillus* MRS Broth was made by mixing the 16.54 g of *Lactobacillus* MRS Broth powder in 300 mL of distilled water. All of them was autoclaved at 110 $^{\circ}$C for 28 minutes. TCA 10% (*w/v*) was prepared by measuring 10 g of it and mixed then with 100 mL of distilled water. Sodium carbonate 4% (*w/v*) was obtained by mixing 4 g of sodium carbonate in 100 mL of distilled water.

Citrate-phosphate buffer and casein were applied as buffer and substrate for checking the protease activity. Citrate-phosphate buffer pH 5 was made by dissolving 2 g of sodium phosphate and 1.4 g of citric acid in 100 mL of distilled water, followed by measuring the pH with pH meter. Afterward, the buffer was added 1 g of casein as substrate and mixed well with magnetic stirrer for 1 hour. Then, it was cooled it for 5 minutes and as the final step was filtered it with two sheets of filtered paper and then put the solution in refrigerator