# BACTERIAL BIOFILM DEGRADATION USING AMYLASE EXTRACTED FROM SOIL MICROORGANISMS

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**A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biomedical Science**

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# การย่อยไบโอฟิล์มที่เกิดจากแบคทีเรียโดยใช้อะไมเลสที่สกัดจากจุลินทรีย์ในดิน

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# BACTERIAL BIOFILM DEGRADATION USING AMYLASE EXTRACTED FROM SOIL MICROORGANISMS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ไบโอฟิล์ม คือ สารเมทริกซ์ที่อยู่ล้อมรอบเซลล์ของแบคทีเรีย เชื้อรา และโปรโตซัว ซึ่งก่อให้เกิดการปนเปื้อนของเชื้อในอุปกรณ์ทางการแพทย์และสายการผลิตในโรงงานอุตสาหกรรม การกำจัดไบโอฟิล์มอย่างสมบูรณ์นั้นทำได้ยากเนื่องจากคุณสมบัติของ exopolysaccharide ที่สามารถก่อตัวขึ้นได้ใหม่จากไบโอฟิล์มที่ตกค้างหลังการบำบัด เอนไซม์อัลฟ่าอะไมเลสถูกนำมาศึกษาเพื่อใช้เป็นสารชีวภาพทางเลือกที่มีศักยภาพในการสลายไบโอฟิล์มที่เป็นมิตรต่อสิ่งแวดล้อม การใช้เอนไซม์อะไมเลสร่วมกับสารบำบัดชนิดอื่น ๆ อาจส่งผลให้การย่อยสลายไบโอฟิล์มเกิดได้ดีขึ้น

การศึกษานี้ได้ทำการสกัดเอนไซม์อะไมเลสโดยใช้วิธีการหมักบนอาหารแข็งจากจุลินทรีย์ที่แยกได้จากดินในจังหวัดนครราชสีมา ประเทศไทย ซึ่งเอนไซม์อะไมเลสจะถูกทำให้บริสุทธิ์โดยใช้การตกตะกอนด้วยแอมโมเนียมซัลเฟต ตามด้วยการประเมินกิจกรรมของอะไมเลสที่ผ่านการทำบริสุทธิ์ การประเมินคุณสมบัติการย่อยไบโอฟิล์มด้วยอะไมเลสทำได้โดยให้เชื้อ *Staphylococcus aureus, Pseudomonas aeruginosa* และ *Staphylococcus epidermidis* สร้างไบโอฟิล์มในจานหลุมขนาด 96 หลุม ตามด้วยการบำบัดด้วยอะไมเลสที่สกัดจากจุลินทรีย์ที่แยกมาจากดิน การจำแนกชนิดของจุลินทรีย์จากดินที่ผลิตเอนไซม์อะไมเลสทำได้โดยการวิเคราะห์ลำดับเบสของยีน 16S rRNA โดยพบว่าจุลินทรีย์ที่แยกได้คือ *Bacillus tequilensis* และ *Bacillus subtilis* ผลการทดลองพบว่าอะไมเลสที่แยกได้จากจุลินทรีย์เหล่านี้สามารถย่อยไบโอฟิล์มได้ในช่วง 60 ถึง 70 เปอร์เซ็นต์ การศึกษาวิจัยในอนาคตควรทำการตรวจสอบอะไมเลสที่สกัดจากจุลินทรีย์ที่แยกมาจากดินแหล่งต่าง ๆ ที่หลากหลายเพื่อให้ได้การย่อยไบโอฟิล์มที่ดีขึ้น

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BIOFILM/AMYLASE/EXTRACTION/ACTIVITY/
SOLID STATE FERMENTATION/DEGRADATION

Biofilm, a matrix that encloses bacteria, fungi, and protozoa are associated with infection of medical devices and production lines of factories. The complete removal of biofilms with current ways of treatments is difficult due to the characteristic of exopolysaccharide. Small residues of biofilm after treatment will allow them for the reformation of biofilm on the attached surface. Alpha-amylase was studied as a candidate for biofilm treating agents from its potential to remove biofilm and also the environmentally friendly characteristic, the combinations of several treating agents with amylase may result in better biofilm degradation.

In this study, amylase was extracted from microorganisms isolated from soil of Nakhon Ratchasima province, Thailand by using solid-state fermentation to evaluate the biofilm degrading activity of amylase extracted from soil microorganisms. The extracted amylases were purified using ammonium sulfate precipitation, and the purified amylases were evaluated for their activities. The biofilm from *S. aureus*, *P. aeruginosa*, and *S. epidermidis* was formed on 96 well microtiter plates and treated with extracted amylase to evaluated the biofilm degrading activity of amylase extracted from soil isolates. The identification of species of the isolates was achieved

by using 16S rRNA sequence analysis. The isolates were revealed to be *Bacillus tequilensis* and *Bacillus subtilis*. As a result of biofilm degradation assay, amylases extracted from soil isolates were successfully reduced biofilm at the range of 60 to 70 percent. Future studies should investigate the extraction of amylase from a variety of soil isolates for better biofilm degradation.



School of Preclinic Student's Signature_____

Academic Year 2019 Advisor's Signature_____

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# LIST OF ABBREVIATIONS

cm = Centimeter

µl = Microliter

ml = Milliliter

mg/ml = Milligram per milliliter

µm = Micrometer

min = minutes

h = hour

PCR = Polymerase Chain Reaction

rpm = Round per minutes

rRNA = Ribosomal ribonucleic acid

TBE = Tris-borate EDTA

SD = Standard deviation

CA = Crude amylase

PA = Purified amylase

# CHAPTER I

# INTRODUCTION

## 1.1 Background/ Problem

Biofilm is an integral component of prokaryotes that cover the cell and allows them to survive in diverse environments. It acts as a major threat to industrial and environmental parts of humans such as medical devices and water pipes (Figure 1) (Craigen, Dashiff, and Kadouri, 2011; Kalpana, Aarthy, and Pandian, 2012). Biofilm forming organisms are known to develop multiple infectious diseases. Furthermore, biofilms have an ability to reduce susceptibility to antibiotics (Mahmood and Mahmood, 2015). Thus, the control of biofilm and biofilm-forming organisms in industrial, environmental, and clinical settings is essential to avoid contaminations.

Pre-existing biofilms are treated by heat, cleaning regimens, low-power laser, sonication, chemical treatments, antibiotics, quorum sensing analogs, lectins, and biological control agents such as invertebrates, protozoa, bacteriophages, predatory bacteria, and enzymes (Craigen et al., 2011). Although there are many ways to treat biofilms, it is still difficult to treat and remove them completely due to exopolysaccharide (EPS). EPS is a component of biofilms, which is a key agent bringing biofilms to have tolerance against the existing biofilm treatment. EPS prevents the penetration of antibiotics and also resists environmental stresses

(Kalpana et al., 2012). Therefore, the importance of EPS degrading agents may increase in the future, rather than other biofilm degrading agents.

To our best knowledge, biofilm degradation by amylase was first tested by Molobela et al. (2010). Craigen et al. (2011) was the first group that focused on biofilm degradation by commercially available α-amylase. Both groups showed degradation of biofilm using amylase. Since then, some bacterial sources of α-amylases have been tested for biofilm degradation (Kalpana et al., 2012; Singh et al., 2015; Vaikundamoorthy, Rajendran, Selvaraju, Moorthy, and Perumal, 2018).

α-Amylase (EC 3.2.1.1) is one of the most intimate enzymes for human beings because it helps digest and gain energy from starch-containing food. Moreover, amylases are used in food, textile, paper, and detergent industries. In other words the life of human beings highly depends on amylase. In fact, approximately 25 % of the world's enzyme market is constructed by amylase (Souza, 2010).

The advantage of using amylase for degrading biofilm relies on its safety towards the environment and ease of obtaining α amylase. The safeness and eco-friendly characteristics of amylase make it a good candidate for biofilm treating agents in many cases.

It was mentioned that the composition of biofilms are diverse and may be difficult to degrade all the biofilm using amylase for now (Craigen et al., 2011). However, we believe that this approach could be used in the future to eradicate the existing biofilm. Therefore this study will focus on screening and isolation of amylase producing bacteria, which degrade biofilms better. Amylase producing microorganisms were sought from the soil environment since soil microorganisms are

mostly still unknown (Meliani, Bensoltane, and Mederbel, 2012). Hence, there are high chances of finding better biofilm degrading amylase producing microorganisms that have not been studied yet. The investigation of the antibiofilm property of amylases produced from bacterial isolates will be conducted.

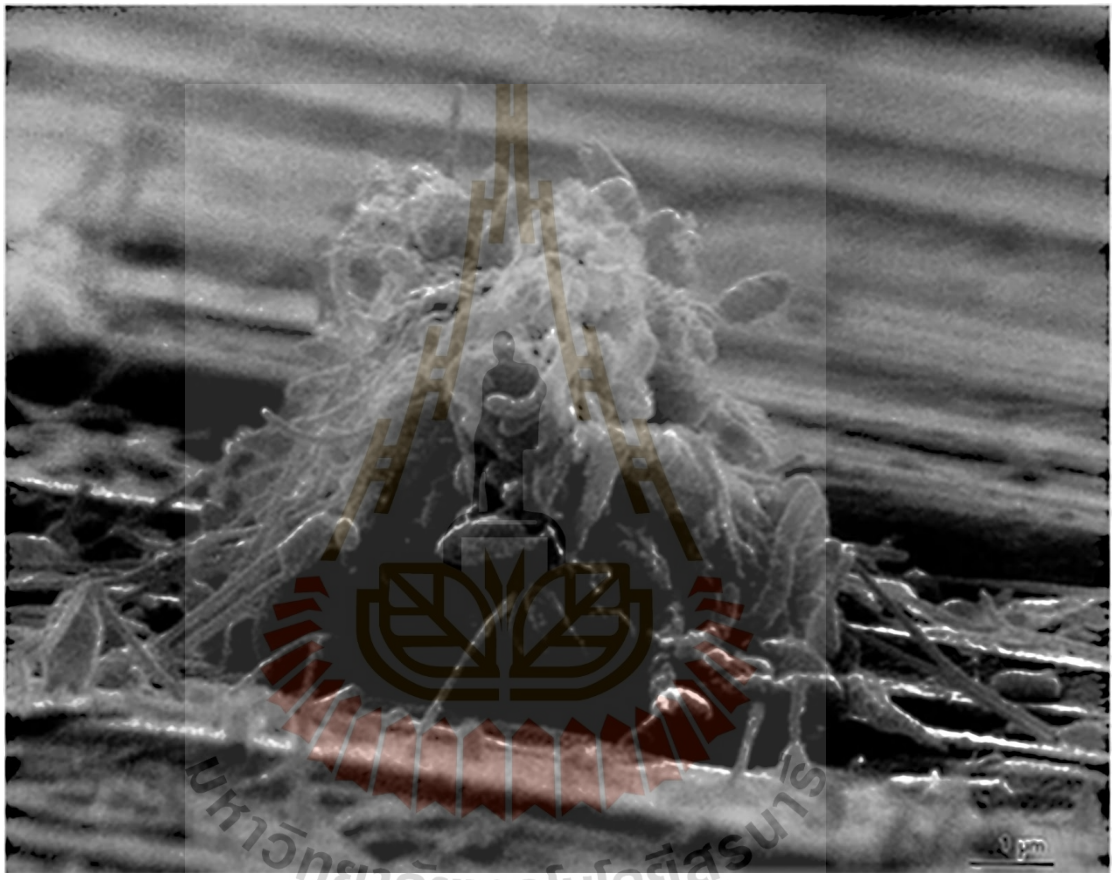


**Figure 1** Scanning electron micrograph of a biofilm on a metal surface from an industrial water system (Donlan and Costerton, 2002).

## 1.2 Research objectives

To isolate amylase producing bacteria from soil, and also to investigate biofilm degradation ability of enzyme amylase of soil isolate.

## 1.3 Research hypothesis

The amylase enzymes extracted from soil isolates degrade biofilms.

## 1.4 Scope and limitations of the study

This work involves the isolation of amylase producing microorganisms from the soil. The isolated strains were cultured and used to extract amylase using solid-state fermentation techniques to test the biofilm degrading ability. The 16S rRNA genes of amylase producing organisms were amplified by PCR technique and the sequences were analyzed to identify their species and also to construct a phylogenetic tree.

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Amylase

Amylase is an enzyme family that digests starch or carbohydrate polymer into sugar by acting on glycosidic bonds between glucose units. Amylases are found in a wide variety of organisms such as microorganisms, plants, and animals (Aiyer, 2005). Amylases are categorized into two groups that are α-1, 4 glucanase and α-1, 6 glucanases. α-1, 4 glucanase includes α-amylase, β-amylase, and glucoamylase or γ-amylase, and α-1, 6 glucanase includes pullulanase (Figure 2). Each amylase has different acting sites and products (El-Enshasy, Abdel Fattah, and Othman, 2013).

### 2.1.1 α-Amylase

α-Amylase is an endoenzyme, that acts upon inner chemical bonds of polysaccharide, it has an activity to catalyze the hydrolysis of random location at internal α-1, 4-glycosidic bonds of the starch molecule and yield oligosaccharides. Most of the α-amylases are metalloenzyme, thus the hydrolysis of carbohydrate polymers are depending on calcium or other divalent cations. The main bacterial sources of these enzymes are *Aspergillus* and *Bacillus*, and their optimal temperature and pH vary from 25 °C to 95 °C, and the pH 1.0 to 11.5 based on the source (El-Enshasy et al., 2013).

### 2.1.2 β-Amylase

β-Amylase is an exoenzyme, that acts at terminal chemical bonds in a polysaccharide, it catalyzes the hydrolysis of α -1, 4-glycosidic linkage on the starch molecule and produces maltose or beta-limit dextrin from the non-reducing end of the polysaccharide. This enzyme is mainly found in plants, fungi, and in some bacteria but not in animal cells. The optimal temperature does not go beyond 60 °C and the pH is mostly neutral to slightly acidic (El-Enshasy et al., 2013).

### 2.1.3 γ-Amylase

γ-Amylase is an exoenzyme that cleaves α -1, 4 or α -1, 6 glycosidic linkage at the nonreducing end, the product will be amylose, amylopectin, and the glucose. The enzyme could be produced by plants, animals, and microorganisms. One of the characteristics of this enzyme is that produced glucose can re-polymerize to form maltose or isomaltose when the concentration of glucose exceeds 30-35 %. This group of amylases are heat tolerant and low optimal pH (El-Enshasy et al., 2013).

### 2.1.4 Pullulanase

Pullulanase is a member of amylase that catalyzes the hydrolysis of α-1, 6 linkages in amylopectin. It is widely used in the industry in the process of saccharification together with γ-amylase. The group of amylase that hydrolyze α -1, 6 bonds are called debranching enzymes. The combination of pullulanase and γ-amylase increases in the rate of the saccharification process. Pullulanase is found in mesophilic bacteria such as *Bacillus macerans* and *Bacillus*

*acidopullulyticus*, thermophilic and hyperthermophilic bacteria, and archaea, such as *Bacillus stearothermophilus*, *Clostridium thermosulfurogenes*, and *Rhodothermus marinus* (El-Enshasy et al., 2013).

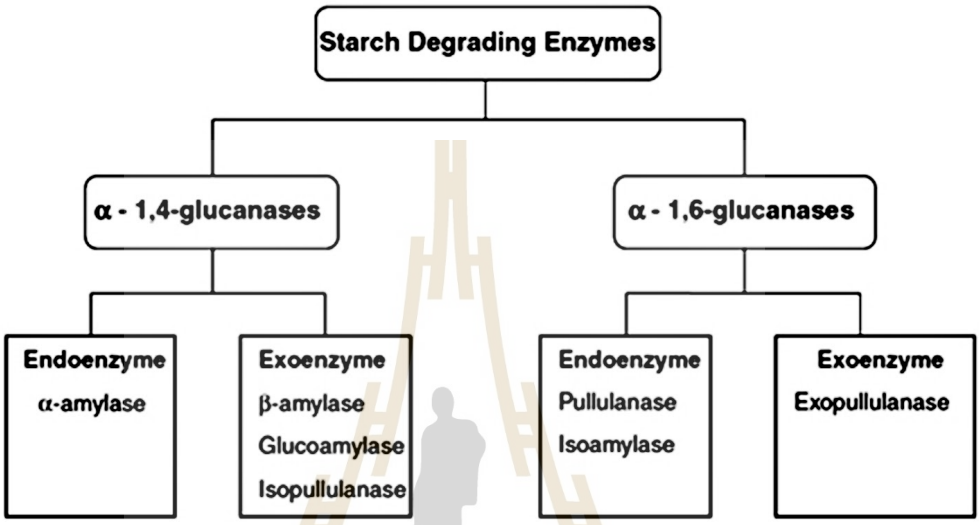



**Figure 2** Classification of starch degrading enzyme based on the type and position of the glycosidic bond they cleave (El-Enshasy et al., 2013).

## 2.2 Biofilm

Biofilm is a form of aggregated bacteria, algae, fungi and protozoa enclosed in a matrix which is composed of extracellular polymeric substance. The function of biofilm includes adhesion to surface, intracellular communication, and protection of microbial community from environmental stresses, thus bacteria can survive in a different environment, such as aquatic, soil, and living tissue (Molobela, Cloete, and Beukes, 2010; Vu, Chen, Crawford, and Ivanova, 2009).

Biofilm formation can be divided into three stages, which are early, intermediate, and mature stages. In the early stage of biofilm formation,

microorganisms attach on the surface and make a contact to form a monolayer of cells. In the intermediate stage, microorganisms bind to the surface and form a colony, concomitantly they form a polymer matrix containing polymeric compound and EPS. The third stage is the formation of a mature community. At this stage, the biofilm structure will be disrupted and microbial cells will be released and disperse (Figure 3) (Taraszkiewicz, Fila, Grinholc, and Nakonieczna, 2013). The dispersal mechanism varies according to the species. The modes of dissemination of microorganisms are detaching from a biofilm by clumping and also fluid-driven and dispersal of biofilms along a surface (Figure 4).

The regulation and formation of biofilms are controlled by quorum sensing (QS). QS allows bacteria to have a cell to cell communication and expression of genes that respond to the population density (Vu et al., 2009). Gram-positive bacteria and gram-negative bacteria use different QS systems. Gram-positive bacteria uses a peptide called auto inducing peptide (AIP) as signaling molecules. The high concentration of AIP allows us to bind to kinase receptors and activate the kinase. The kinase phosphorylates the transcription factor that regulates gene transcription. In the QS system of gram-negative bacteria, they use N-acyl homoserine lactones (AHL) as signaling molecules, which usually bind to transcription factor directory (Rutherford and Bassler, 2012)

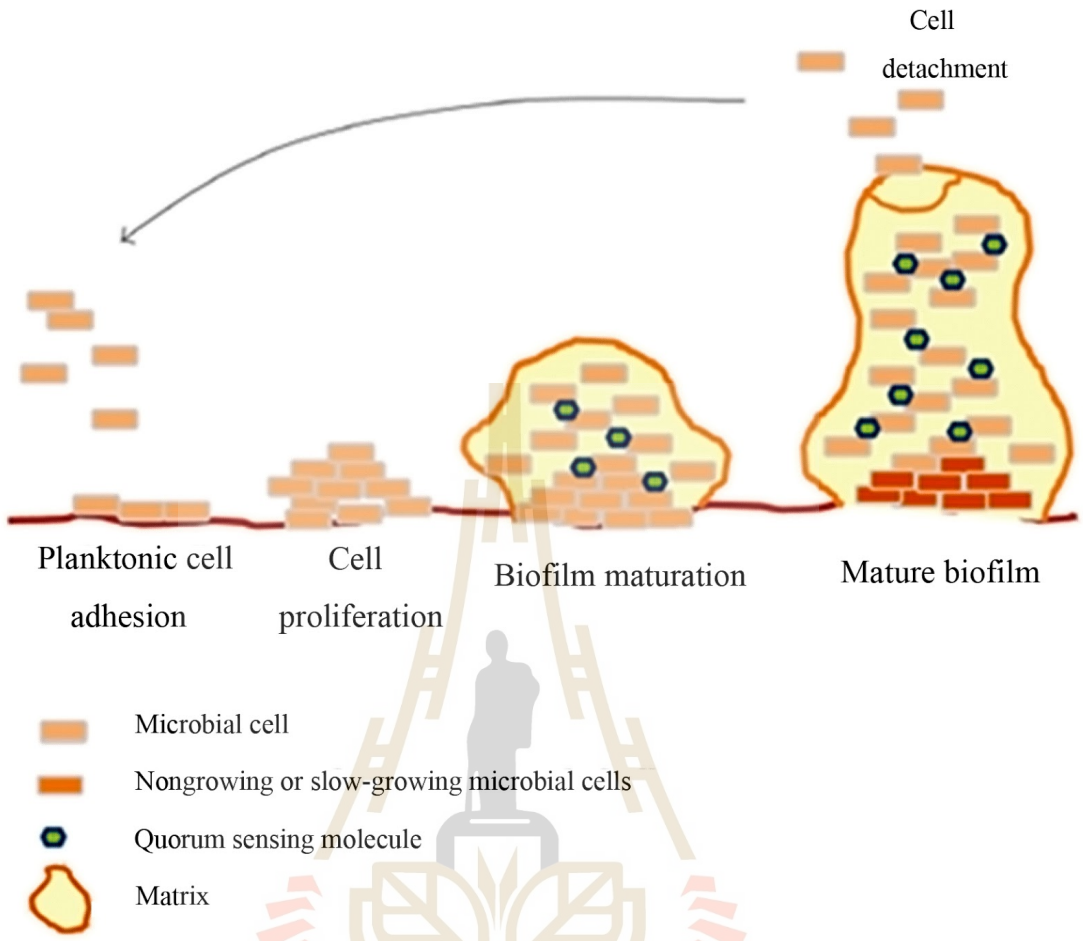



**Figure 3** Biofilm formation cycle (Taraszkiewicz et al., 2013).

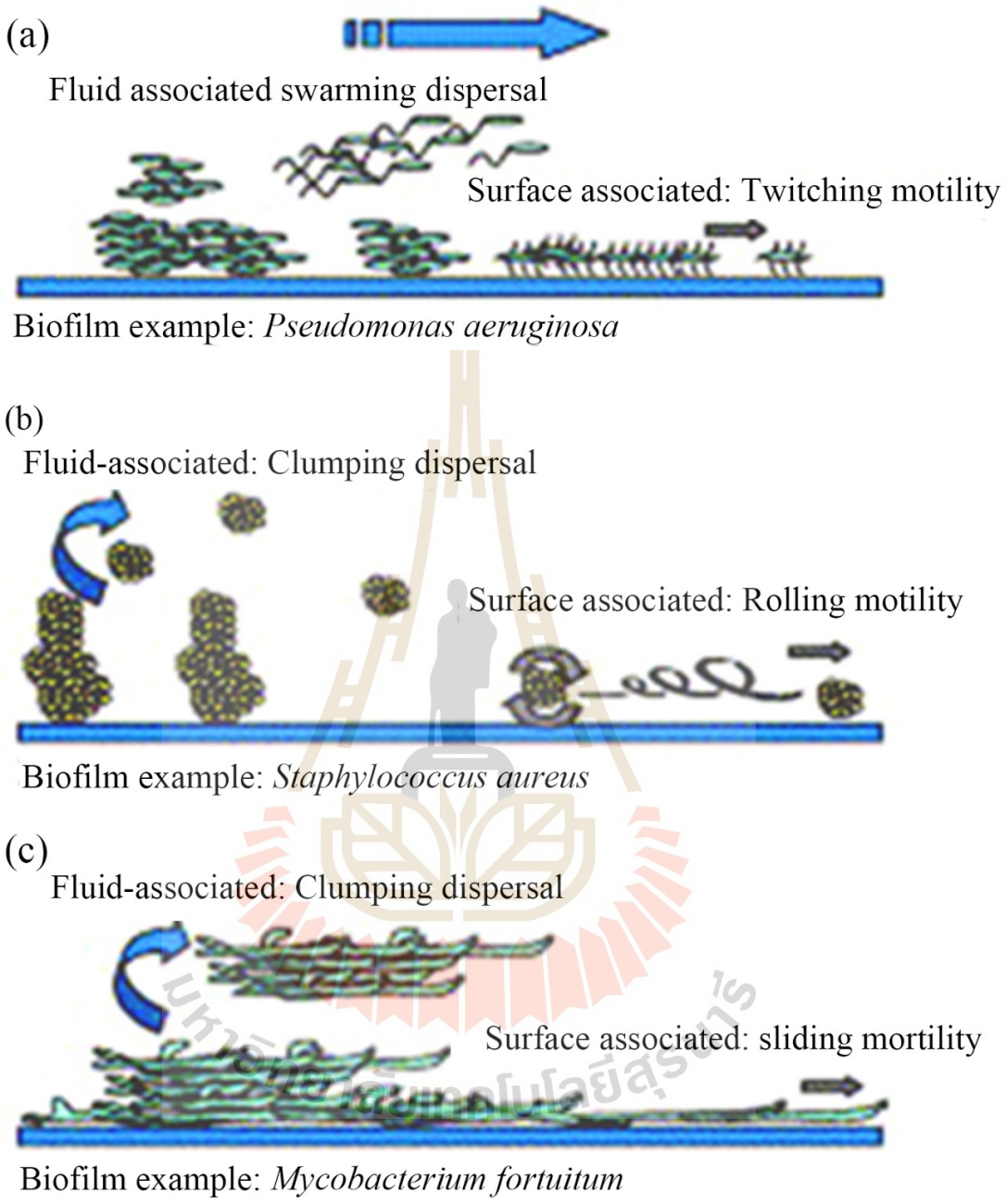



**Figure 4** Various dispersal mechanisms of different bacteria (Hall-Stoodley and Stoodley, 2005).

## 2.3 Extracellular polymeric substance

Extracellular polymeric substances approximately contain 50 to 90 % of organic matter such as polysaccharides (EPS), proteins, nucleic acids, lipids and humic substances (Vu et al., 2009). Extracellular polymeric substances in gram-negative bacteria are neutral to polyanionic and it contains uronic acid. Uronic acid gives extracellular polymeric substance anionic property, allowing the association with divalent cations such as calcium and magnesium, which gives cross-link between polymers and gives greater binding force in developing biofilms. On the other hand, extracellular polymeric substances of gram-positive bacteria are cationic and containing teichoic acid with small quantities of protein (Donlan, 2002).

### 2.3.1 Exopolysaccharide

EPS are high molecular weight polymers that usually consist of monosaccharides and non-secreted carbohydrate substances such as pyruvate, acetate, phosphate and succinate (Figure 5). EPS can be categorized into two groups. Homopolysaccharides give only monosaccharide units such as cellulose, alginate, and dextran when hydrolyzed. Heteropolysaccharides are composed of repeating units of disaccharides to monosaccharides such as xanthan, hyaluronic acid, and heparin, they give more than one type of monosaccharide when hydrolyzed (Hussain et al., 2017).

EPS carries a critical role in forming bacterial biofilms. In fact, a number of studies show that mutants that are unable to produce EPS are unable to produce biofilms (Sutherland, 2001).

Microorganisms produce different polysaccharides in order to form a biofilm. There are several well-studied commercially used polysaccharides described as follows.

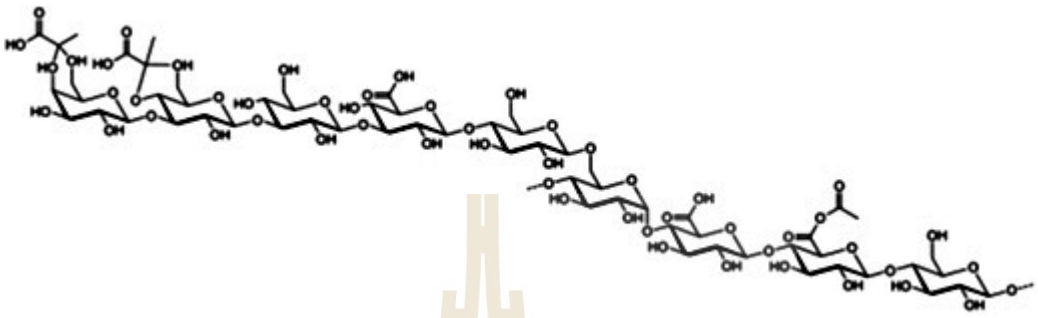


**Figure 5** An example of exopolysaccharide (Hussain et al., 2017).

**Dextran**

Dextran is a high molecular weight polysaccharide of glucose that contains consecutive α-(1, 6)-linkages abundantly in its backbone (Figure 6). There are various structures of dextran which depend on the microbial strain. Dextrans are produced from sucrose using dextransucrase as a catalyst, which is produced by *Leuconostoc*, *Streptococcus* and *Lactobacillus* species. The physical property of dextran is a gel, which can be used to purify separate macromolecules such as proteins, nucleic acids, and polysaccharides. And also this can be used for clinical research and medical application since it can safely be consumed (Vu et al., 2009).

**Kefiran**

Kefiran is a capsular polysaccharide that has gel-like characteristics (Figure 7). *Lactobacillus* species such as *L. rhamnosus*, *L. kefir* and *L. kefiranofasciens* produce kifferan. It has antibacterial, antifungal, and antitumor activity, and this has been traditionally consumed as a dairy product (Vu et al., 2009).

**Cellulose**

Cellulose is a polysaccharide that has a simple structure which constructs of monosaccharide glucose with a 1-4 β-glycosidic bond (Figure 8). It is produced by genera *Acetobacter*, *Agrobacterium*, *Pseudomonas* and *Rhizobium* (Vu et al., 2009).

**Gellan**

Gellan produced by non-pathogenic bacterium *Sphingomonas elodea* ATCC 3146, formed of linear polysaccharide that has tetra polysaccharide repeating unit of D-glucose, D-glucuronic acid and L-rhamnose (Figure 9). It forms an elastic gel in solution and this can be used as a gelling agent of food (Vu et al., 2009).

**Curdlan**

Curdlan, the polysaccharide that is made up of linear β-1, 3-linked glucose residues is a water-soluble polysaccharide that has a low molecular weight (Figure 10). Bacteria that produce curdlan are *Alcaligenes faecalis* and *Agrobacterium*. Curdlan has the ability to form an elastic gel when the aqueous solution heated above 55 ºC. The characteristic of curdlan has been used for food and pharmaceutical industries (Vu et al., 2009).

**Xanthan**

Xanthan is a heteropolysaccharide that has a high molecular weight, the chemical conformation of xanthan contains glucose units with the side chain of trisaccharide of D-mannose with an acetyl group, β-D-glucuronic acid and a terminal β-D-mannose unit linked to a pyruvate group (Figure 11). Xanthan is produced by *Xanthomonas campestris* and used widely in food, cosmetic, and pharmaceutical industries (Vu et al., 2009).

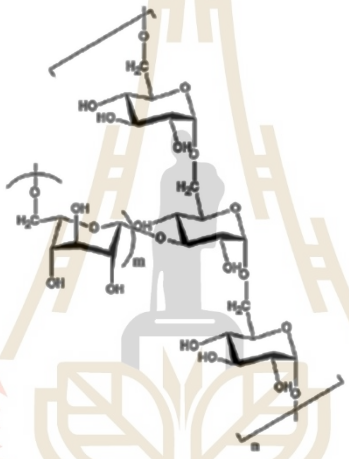


**Figure 6** The structure of dextran with branching at C3 (Vu et al., 2009).

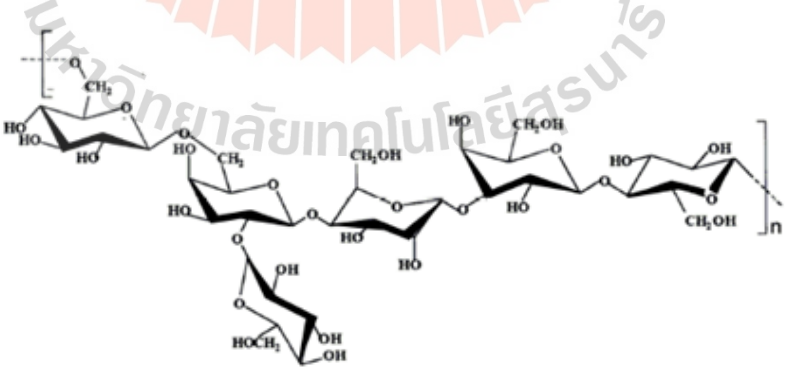


**Figure 7** Structure of Kefiran (Vu et al., 2009).

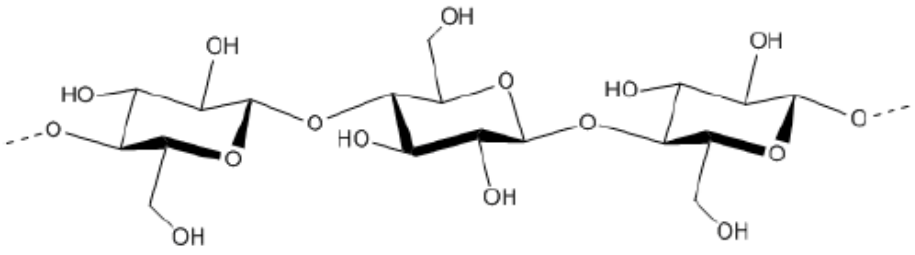


**Figure 8** Structure of bacterial cellulose (Vu et al., 2009).

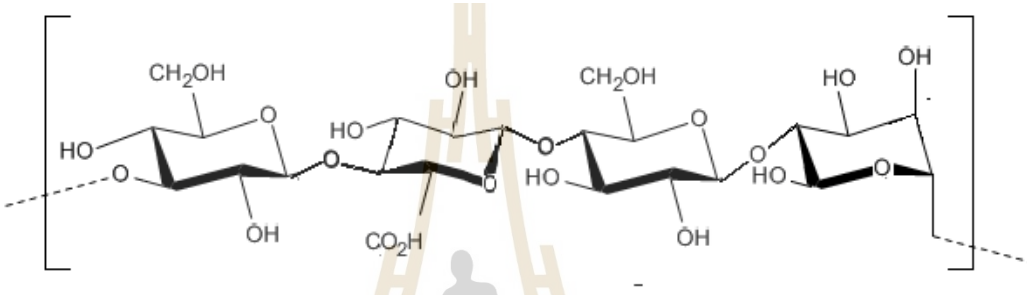


**Figure 9** Structure of gellan gum (Vu et al., 2009).

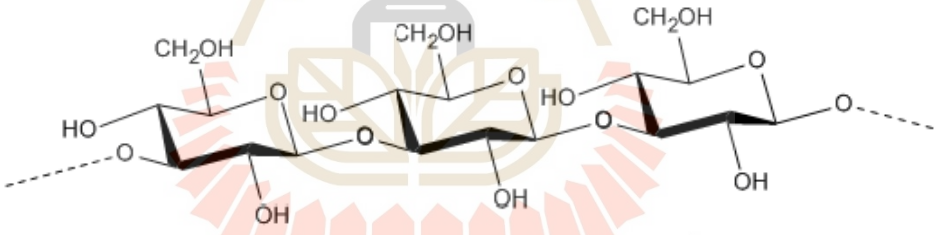


**Figure 10** Chemical structure of curdlan (Vu et al., 2009).

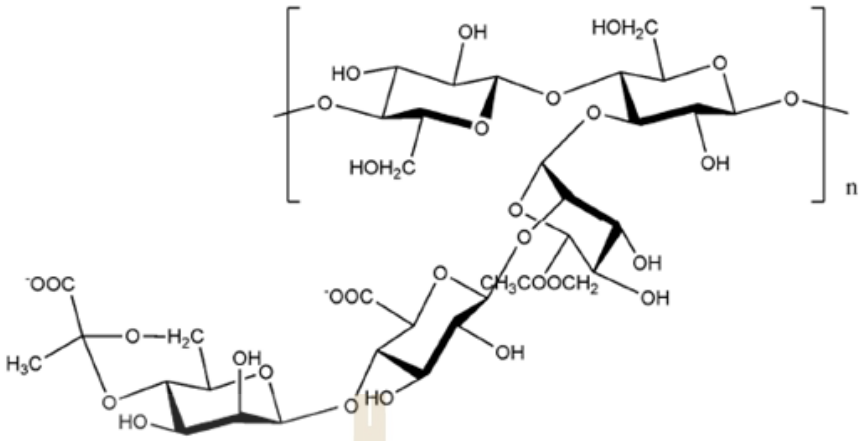


**Figure 11** Chemical structure of Xanthan (Vu et al., 2009).

## 2.4 Biofilm forming Bacteria

There are a numerous number of biofilm-forming bacteria. Some common pathogenic biofilm-forming bacteria are described as follows.

### 2.4.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that is ubiquitous in the environment due to their ability to survive in different niches, they have an ability to utilize many organic substances into their energy sources. The first human infection by *P. aeruginosa* was reported in 1862, the infected site shows blue-green pus on some infection. Most infections due to *P. aeruginosa are associated* with the compromise of host defense, such as infection in AIDS patients or neutropenic patients undergoing chemotherapy (Lyczak, Cannon, and Pier, 2000). *P. aeruginosa* is also known as a difficult organism to treat with antibiotics because of its antimicrobial-resistant character (Figure 12). The general resistance comes from the combination of several factors including; the

low permeability of the cell wall, genetic capacity for expressing mechanism to resist antibiotics and obtain resistance genes from other organisms (Lambert, 2002). Figure 12 shows the antibiotic resistance of *P. aeruginosa* biofilm that is exposed to an antibacterial agent.

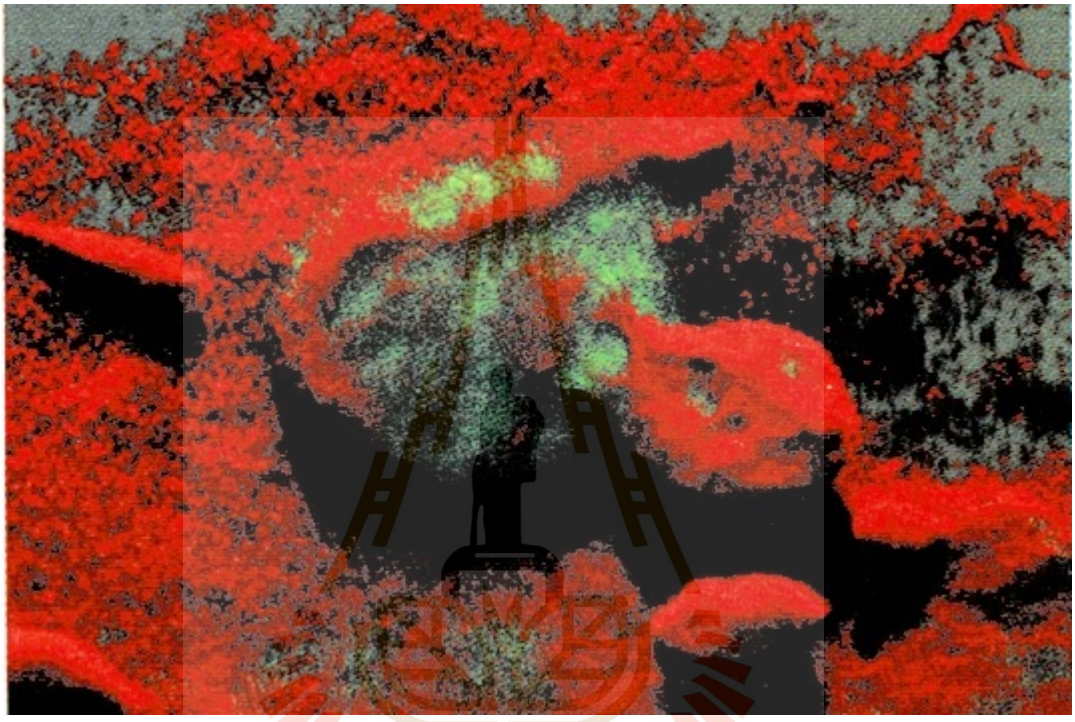


**Figure 12** Three dimensional reconstructions of *P. aeruginosa* biofilm that was exposed to an antibacterial agent and stained with dyes. Living cells shows in green color and red for the dead cells (Willey, Sherwood, and Woolverton, 2011).

### 2.4.2 *Staphylococcus aureus*

*Staphylococcus aureus* is an opportunistic gram-positive bacteria that has been recognized as an important pathogen because they have an antibiotic-resistant strain called methicillin-resistant *Staphylococcus aureus*(MRSA) which are often multi-resistant against antibiotics (Kluytmans, Van Belkum, and Verbrugh, 1997).

### 2.4.3 *Staphylococcus epidermidis*

*Staphylococcus epidermidis* is an opportunistic pathogen that is a member of coagulase-negative staphylococci. They are causes of nosocomial infections and medical device infections such as intravascular catheters, joint prostheses, and artificial heart valves (Rohde et al., 2005).

## 2.5 Medical impact of biofilm-forming microorganisms

The biofilm-forming microorganisms has a high impact since it reduces susceptibility to antibiotics and it allows the bacteria to develop antibiotic resistance. The biofilm-forming microorganisms are problematic to the human body since they cause multiple infections at different sites (Table 1) (Mahmood and Mahmood, 2015). Some common diseases caused by biofilm-forming bacteria are described below.

### 2.5.1 Native valve endocarditis

Native valve endocarditis (NVE) is generally infection at the mitral, aortic, tricuspid, and pulmonic valve of the heart by fungi or bacteria that circulate in the bloodstream. The infection generally does not happen unless the site is damaged, since microorganisms poorly interact with endothelium. However, once the damage has developed such as non-bacterial thrombotic endocarditis (NBTE), which is the development of thrombus, NVE easily occurs when the pathogen has inoculated into blood (Donlan and Costerton, 2002).

### 2.5.2 Otitis Media

Otitis Media is an infectious disease of the middle ear that has symptoms of inflammation of mucoperiosteal lining. Microorganisms causing Otitis media are *Streptococcus pneumoniae, Haemophilus influenzae*, and other microorganisms or a mixture of two or more different microorganisms. This disease is a common childhood disease that can be acute or chronic (Donlan and Costerton, 2002).

### 2.5.3 Chronic Bacterial Prostatitis

Chronic Bacterial Prostatitis is a condition that has an infected prostate gland. The organisms which infect the prostate gland are commonly *Escheria coli*. infection happens when the microorganisms ascended from urethra or reflex of urine containing infective organisms into prostatic ducts (Donlan and Costerton, 2002).

### 2.5.4 Cystic fibrosis

Cystic fibrosis (CF) is a chronic disease of the lower respiratory system that is a common inherited disease. The patients with CF have a deficiency in the mucociliary clearance system that cleanses inhaled particles in the bronchopulmonary epithelium due to the reduction of secretion and increase in the absorption of electrolytes, that lead to dehydration and thickening of secretion covers the respiratory epithelium (Donlan and Costerton, 2002).

### 2.5.5 Periodontitis

Periodontitis is a disease associated with the infection of supporting tissue of teeth. There are reversible mild infections that have inflammation on the gingiva to severe infection that destroys the periodontal tissues such as gingiva,

periodontal ligament, and alveolar bone. Chronic periodontitis causes teeth to exfoliate from gingiva and deepen the periodontal pocket. The organisms found from the patients with periodontitis are *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Eubacterium timidum*, *Eubacterium brachy*, *Lactobacillus* sp., *Actinomyces naeslundii*, *Pseudomonas anaerobius*, *Eubacterium* sp. strain D8, *Bacteroides intermedius*, *Fusobacterium* sp., *Selenomonas sputigena*, *Eubacterium* sp. strain D6, *Bacteroides pneumosintes*, and *Haemophilus aphrophilus,* all of them are positively correlated to gingivitis (Donlan and Costerton, 2002).

**2.5.6 Colonization of biofilm forming microorganisms in medical devices**

It was reported that 99 % of the microorganisms produce biofilms, unfortunately, they have the great capacity to contaminate medical devices. The studies of biofilm on medical devices have been studied for 20 years, and devices such as prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, intrauterine devices, and dental unit waterlines could be the site of colonization. It is resulting in an increase in the mortality rate of about 4.6 % (Donlan and Costerton, 2002; Mahmood and Mahmood, 2015).

**Table 1** Site of infection and List of diseases related to some common biofilm-forming bacterial species (Mahmood and Mahmood, 2015).

| Site | Name of Diseases | Common bacterial species involved in biofilm formation |
|---|---|---|
| Musculoskeletal | Osteomyelitis<br>Cellulitis<br>Bursitis<br>Septic arthritis | *Staphylococcus aureus*<br>*Pseudomonas aeruginosa*<br>*Salmonella species*<br>*Streptococcus pyogenes*<br>*Streptococcus pneumonia* |
| Cardiovascular | Endocarditis | *Streptococcus viridans*<br>*Streptococcus gordoni*<br>*Staphylococcus aureus*<br>*Staphylococcus epidermidis*<br>*Haemophilus species*<br>*Cardiobacterium hominis*<br>*Actinobacillus actinomycetemcomitans* |
| Respiratory | Otitis media<br>Tonsillitis<br>Cystic fibrosis<br>Legionnaire's disease | *Streptococcus pneumoniae*<br>*Haemophilus influenza*<br>*Moraxella catarrhalis*<br>*Klebsiella penumoniae*<br>*Pseudomonas aeruginosa*<br>*Burkholderia cepacia*<br>*Legionella pneumonia* |
| Gastrointestinal | H. Pylori infection<br>Biliary Tract Infection<br>Peritonitis | *Helicobacter pylori*<br>*Escherichia coli*<br>*Bacteroides fragilis*<br>*Fusobacterium species* |

**Table 1** (Continued).

| Site | Name of Diseases | Common bacterial species involved in biofilm formation |
|---|---|---|
| Genito-Urinary | Prostatitis Cystitis<br>Urethritis<br>Pyelonephritis | *Escherichia coli*<br>*Proteus mirabilis*<br>*Staphylococcus epidermidis*<br>*Enterococcus faecalis*<br>*Pseudomonas aeruginosa* |
| Skin or soft Tissue | Necrotizing fasciitis<br>wounds | *Streptococcus pyogens*<br>*Staphylococcus aureus*<br>*Vibrio vulmificus*<br>*Clostridium pefringens*<br>*Bacteroides fragilis* |

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Culture media

- Starch agar (SA) contained (per 1 liter) 3 g of beef extract, 10 g of soluble starch, and 12 g of agar. the final pH was adjusted to 7.5 ± 0.2.
- Starch liquid medium contained (per 1 liter) 3 g of beef extract and 10 g of soluble starch. the final pH was adjusted to 7.5 ± 0.2.
- Nutrient broth (NB) contained (per 1 liter) 3 g of beef extract, 5 g of peptone, and 5 grams of sodium chloride. the final pH was adjusted to 7.0 ± 0.2.
- Nutrient agar (NA) contained (per 1 liter) 3 g of beef extract, 5 g of peptone, 5 grams of sodium chloride and 15 g of agar. The final pH was adjusted to 7.0 ± 0.2.
- The sterilization of the medium was performed by autoclaving at 121 ºC, 15 p.s.i for 15 min.

### 3.1.2 Buffer and solutions

- 20mM phosphate buffer solution (PBS) pH 7.0 contained (per 1 liter) 1.64g of Monosodium phosphate ($Na_2HPO_4$), and 1.16 g of Disodium phosphate, NaH2PO4.

- 100mM phosphate buffer solution (PBS) pH 6.0 contained (per 1 liter) 1.70g of Monosodium phosphate ($Na_2HPO_4$), and 12.11 g of Disodium phosphate, NaH2PO4.
- 10X TBE buffer for electrophoresis contained (per 1 liter) 108 g of Tris-HCL, 55 g of boric acid and 40 ml of 0.5 M EDTA pH 8.0.
- 0.1N Hydrochloric acid was prepared by adding 3.7 ml of hydrochloric acid in 95.3 ml of distilled water
- 30 % glacial acetic acid was prepared by adding 30 ml of hydrochloric acid in 70 ml of sterile distilled water
- 0.5 % Crystal violet was prepared by dissolving 0.5 g of crystal violet in 20 ml of 95 % ethyl alcohol, followed by adding 0.2 g of ammonium oxalate monohydrate dissolved in 80 ml of sterile distilled water.

**3.1.3 SDS-PAGE and Native-PAGE reagents**

- 0.5 M Tris-HCl, pH6.8 was prepared by dissolving 3 g of Tris in 40 ml of sterile distilled water and then the pH was adjusted to 6.8. Sterile distilled water will be filled up to 50ml.
- 1.5 M Tris-HCl, pH 8.8 was prepared by dissolving 36.3 g of Tris in 150 ml of sterile distilled water and then the pH was adjusted to 8.8. Sterile distilled water will be filled up to 200 ml.
- 10 % SDS was prepared by dissolving 1 g of SDS in 10 ml of sterile distilled water.
- 10 % Ammonium persulfate (APS) was prepared by dissolving 1 g of SDS in 10 ml of sterile distilled water.

- Native PAGE sample buffer contained 7ml of 0.5 M Tris-HCl, pH6.8, 3ml of glycerol, and 1.2 mg of bromophenol blue.
- SDS-PAGE sample buffer contained (total volume of 9.5ml) 3.55 ml of sterile distilled water, 1.25ml of 2.5 ml of glycerol, 2.0 ml of 10 % SDS, and 0.5 g of 0.5 % bromophenol blue.
- SDS-PAGE running buffer contained (per 1 liter) 3.02 g tris base, 14.4 g of Glycine, and 1 g of SDS.
- Native-PAGE running buffer contained (per 1 liter) 3.02 g tris base, 14.4 g of Glycine.
- Coomassie blue contained (per 1 liter) 0.25 g of coomassie blue R250, 400 ml of methanol, and 70 ml of glacial acetic acid.
- Destain solution contained (per 1 liter) 400 ml of methanol and 70 ml of Glacial acetic acid.

**3.1.4 Research kits**

- FavorPrep$^{TM}$ GEL/PCR Purification Mini Kit purchased from Favorgen, Taiwan.

**3.1.5 Primers used for PCR amplification**

- The name of the oligonucleotides and their sequences are listed in Table 2.

**Table 2** List of primers used in this study.

| Name | Sequences (5′-3′) | Sources |
|---|---|---|
| 27F | AGAGTTTGATCMTGGCTCAG | (Wawrik, Kerkhof, Zylstra, and Kukor, 2005) |
| 1525R | AAGGAGGTGATCCAGCC | (Wawrik, Kerkhof, Zylstra, and Kukor, 2005) |

### 3.1.6 Miscellaneous materials

- GoTaq®Green master mix used for PCR amplification was purchased from Promega, USA.
- One hundred bp DNA Ladder. The marker was purchased from HiMedia Laboratories, India
- Acrylamide/Bis-acrylamide solution 30 %, purchased from HiMedia Laboratories, India
- TEMED was purchased from PanReac Applichem, US
- Bovine serum albumin was purchased from BIO-RAD, California
- Protein assay dye reagent concentrate was purchased from BIO-RAD, California
- Prestained protein ladder, 10 to 180kDa, purchased from Thermo Fisher Scientific, US.

### 3.1.7 Equipment

All the instruments used in this study are listed in Table 3.

**Table 3** List of instruments used in this study.

| Name | Source |
|---|---|
| Autoclave | Tomy, USA |
| Hot Air Oven | Memmert, Schwabach |
| Shaking incubator | N-biotek, Korea |
| Spectrophotometer | PG Instrument, UK |
| Nanodrop Spectrophotometer | Thermo Fisher Scientific, US |
| omniPAGE Mini Vertical Protein Electrophoresis System | Cleaver scientific, UK |
| Fluorescence-inverted microscope | Olympus, Japan |
| Centrifuge machine | Hettich, Germany |
| Microcentrifuge | Denville, Canada |
| pH meter | METTLER-TOLEDO, USA |
| Laminar flow | Esco, Switzerland |
| Vortex mixer | FINEPCR, Korea |
| Submarine Agarose Gel Unit | Hoefer, USA |
| Submarine Agarose Gel Unit | Hoefer, USA |
| Power Supply | Wealtec, UK |
| UltraBright LED Transilluminator | Maestrogen, Taiwan |
| Thermal cycler | Thermo Fisher Scientific, US |

## 3.2 Methods

### 3.2.1 Sample collection

Soil samples were collected with a shovel and polystyrene bag using aseptic techniques. The surface of the soil was removed about 10 cm and a few grams of soil samples were collected into polystyrene bags.

### 3.2.2 Screening of amylase producing microorganisms and isolation

One gram of collected soil samples were suspended into 250 ml of Erlenmeyer flask containing 99 ml of sterile normal saline solution. The solution was serially diluted to $10^{-3}$ dilution using sterile normal saline water. Diluted samples were plated on starch agar (SA), and incubated for 24-48 hrs or until the bacterial colony appeared. Bacterial colonies were transferred to new SA to isolate the colonies and incubated at 37 for 24-48 hrs. Production of amylases were observed by staining starch agar by iodine solution. Colonies surrounded with clear zone were recognized as amylase producing microorganisms (Gebreyohannes, 2015).

### 3.2.3 16S rRNA gene amplification

Genomic DNA of selected organisms were extracted using the freeze-thaw method. Ten µl of sterilized distilled water was transferred into 1.5 ml eppendorf, and bacterial colony was picked up with a sterilized toothpick and suspended in it. The bacterial suspension was frozen at -80 °C and thawed 8 times and used as a DNA template for PCR amplification of 16S rRNA genes. PCR amplification of 16S rRNA genes was performed using universal primers 27F and 1525R (Wawrik, 2005).

The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 90 s, extension at 72 °C for 90 s, and a final elongation at 72 °C for 7 min.

The PCR products were verified on 0.7 to 1.5 % (w/v) agarose gel in 1X Tris/borate/EDTA (TBE) buffer. Amplified fragment length of 1500 bp was cut from agarose gel and the band was purified by using FavorPrep$^{TM}$ GEL/PCR Purification Kit (Favorgen, Taiwan). The purified PCR product checked the concentration with a nanodrop spectrophotometer to check if there were enough concentration for sequencing.

**3.2.4 16S rRNA gene sequencing**

16S rRNA genes after purification with the Purification kit were submitted for sequencing at Macrogen, Korea. The similarities of obtained sequences were detected between a query sequence and database sequences in the GenBank using BLAST (Basic local alignment search tool) (Altschul et al., 1997).

**3.2.5 Construction of phylogenetic tree**

The sequences of 16S rRNA genes were compared with known sequences from the NCBI database. The sequences were aligned with closely related strains found in the database by using ClustalW. The phylogenetic tree was constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis software version 7.0 (MEGA 7.0). The tree topologies were evaluated by using bootstrap analysis (500 replications).

### 3.2.6 Extraction of amylase enzyme

Amylase producing isolate was inoculated to Starch broth (SB) and incubated overnight at 200 rpm shaking condition. One ml of overnight inoculums were transferred 250 ml Erlenmeyer flask containing 99ml SB and incubated for 6 hrs at 200 rpm shaking condition. After 6 hrs, Optical density (OD) at 600 nm was measured and adjusted to OD600 to 0.5 with SB. Six ml of OD adjusted inoculums to four grams of oat bran are transferred into a 250 ml Erlenmeyer and incubated.

Fifteen ml of phosphate buffer solution (PBS) to four grams of oat bran was added to the fermented oat bran and kept on a rotary shaker with ice for 30 min with shaking condition of 150 rpm. The fermented oat bran with PBS was transferred into 50 ml conical tubes and centrifuged for 15 min at 4 °C with 8000 rpm to remove the solid part of the mixture. The supernatant that contains crude protein was transferred into new 50ml conical tubes. The steps were referred from Raul, Biswas, Mukhopadhyay, Kumar Das, and Gupta, 2014 and called Solid State Fermentation.

The supernatant was transferred into a 250 ml Erlenmeyer flask and ammonium sulfate was added for precipitating the amylase enzyme, and it was incubated on ice for 30 min to precipitate protein on a rotary shaker. The solution was transferred into a 50 ml conical tube and centrifuged for 15 min at 4 °C with 8000 rpm. A Pellet obtained after centrifugation was suspended with 20mM PBS (Raul et al., 2014) and centrifuged again to obtain a clear solution that contains partially purified amylase.

### 3.2.7 Determination of protein concentration

The concentration of partially purified amylases was measured by protein assay using Bradford reagent. One part of Protein assay dye reagent was diluted with four parts of deionized (DDI) water and filtered by Whatman #1 filter to remove particulates. Five different dilutions; 0, 25, 50, 75, and 100 %, of 2 mg/ml bovine serum albumin (BSA) solution was prepared to be standard. Ten µl of Samples and BSA solutions were placed into a microtiter well plate. 190 µl of diluted protein assay dye reagent was added to each well and mixed well. The plate was incubated for five min and absorbance was measured with Spectrophotometer at $A_{max}$ = 595 nm. The concentration of partially purified amylase was calculated based on a quadratic equation obtained from the BSA concentration curve.

### 3.2.8 Activity assay

The activity of amylase was determined by the starch iodine method, which measures the blue color intensity developed from starch iodine reaction. The reaction mixture contains 0.5 ml of 100mM PBS, pH6, and 0.1 % soluble starch. The reaction mixture was incubated at 40, 50, 55, 60, and 70 °C for 5 min in dry bath incubator, then 25ul of crude amylase and diluted partially purified amylase was added to the mixture and incubated at 40, 50, 55, 60, and 70 °C for 10 min. The reaction was stopped by adding 0.25 ml of 0.1 N HCl and color was developed by adding 0.25 ml of iodine solution (2 % KI and 0.2 % I).

The absorbance was measured at 690 nm using a UV-vis spectrophotometer (Kalpana et al., 2012). One unit of enzyme activity was defined as the disappearance of an average of 1 mg of iodine binding starch material per minute in the assay reaction. U/ml was calculated using the formula: U/ml = (A690 control -A690 sample) ÷ A690/mg starch ÷ 10 min ÷ 0.025 ml, A690/mg was estimated from the measurement of different concentration of starch reacted with iodine (Xiao, Storms, and Tsang, 2006;Kalpana et al., 2012).

**3.2.9 Amylase stability assay**

The stability of purified amylase was tested using the starch-iodine method. Hundred mM PBS was mixed with purified amylase and incubated at 40, 50, 60, and 70 °C for 30 min. After the incubation, 0.25 ml of 0.1 % starch was added into it. The mixture was incubated for 10 min at 55 °C and color was developed with iodine solution. Blue color intensity was measured at 690 nm and the specific activity was calculated to obtain the percentage of remaining activity after 30 min of incubation at different temperatures.

**3.2.10 SDS-PAGE and Native-PAGE**

Amylase enzyme size in kilodalton (kDa) was estimated by SDS-PAGE (Laemmli 1970). The purified enzyme was heat-denatured and ran through a 14 % concentration of polyacrylamide gel. The gel was stained with coomassie blue. The molecular weight was predicted from the protein ladder.

Native-PAGE (Zymography) was obtained with the following method. All the reagents of SDS-PAGE were prepared without SDS, and amylase enzyme solution was loaded without heat denaturation. The gel after electrophoresis was soaked in

a 1 % starch solution for 30 min, and the iodine solution was added to the gel. The enzyme activity was developed as a clear zone.(Ahmed, Morishima, Babiker, and Mori, 2009).

**3.2.11 Biofilm formation assay**

Biofilm forming ability was evaluated by the following steps. Bacteria were inoculated into a 50 ml conical tube containing 3 ml of nutrient broth (NB) and incubated overnight at 37 °C at 200 rpm shaking condition. One ml of overnight inoculums were transferred into a flask containing 99 ml of NB and incubated for 6 hrs with 200 rpm shaking condition. The OD after incubation was measured and adjusted to OD600 = 0.5. The solution was loaded onto a microtiter plate and incubated. After the incubation, the plate was washed with distilled water and air-dried. The biofilm was fixed using 95 % ethanol and dried again. The biofilm was stained using 0.4 % crystal violet for 10 min. The wells are washed again to remove the excess crystal violet and the wells were dried and observed under a microscope (Kalpana et al., 2012).

**3.2.12 Biofilm degradation assay**

The biofilm-forming strains were incubated overnight in NB. 0.5 ml of overnight inoculums were transferred to a flask containing 250 ml of sterilized NB and incubated for 6 hrs. The optical density of the incubated pathogens were measured using a spectrophotometer at OD = 600. $OD_{600}$ of each pathogen was adjusted to 0.4 - 0.5 with NB, and 200 µl was placed on each well of microtiter plates. The plates were incubated at 37 °C for 48 hrs statically (Kalpana et al., 2012).

The medium in the microtiter plates were discarded and 100 ng/ml, 250 ng/ml, 500 ng/ml, and 1000 ng/ml of partially purified amylase was added to each well. The plates were incubated at room temperature, and 37 °C for 30 min and washed twice with distilled water. The wells were stained as described above in the biofilm formation assay. The wells were observed with a microscope, and crystal violet was eluted with 30 % acetic acid to measure with spectrophotometer with absorbance at 595 nm.

# CHAPTER IV

# RESULTS

## 4.1 Selection of amylase producing isolates

A total of 17 soil samples were collected from the Nakhon Ratchasima area, Thailand in October 2017. Soil samples from Suranaree University of Technology (SUT), Nakhon Ratchasima College (NMC), and Sakaerat environmental research station (SERS) were sampled 4 samples, 3 samples, and 10 samples respectively.

The GPS location of the collection sites were listed below (Table 4). The soil samples were transferred to the polyethylene bag and carried to the laboratory and kept at 4 °C to preserve the microorganisms in the collected soils.

**Table 4** GPS location of the place where soil had sampled.

| **Name of the Place** | **state of the place** | **GPS location** |
|---|---|---|
| SUT | Cassava field | 14°52'39.5"N 102°00'21.7"E |
| SUT | sugar cane field | 14°52'38.8"N 102°00'22.6"E |
| SUT | Bushes | 14°52'45.3"N 102°01'32.4"E |
| NMC | Bushes | 14°57'07.1"N 102°00'56.6"E (2 samples) |
| NMC | Bushes | 14°57'06.1"N 102°00'56.5"E |

**Table 4** (Continued).

| Name of the Place | state of the place | GPS location |
|---|---|---|
| NMC | Bushes | 14°57'05.4"N 102°00'56.6"E |
| SERS | forest | 14°30'36.6"N 101°55'54.2"E (10 samples) |

From the 17 different soil samples, one gram of soil samples were serially diluted to spread on the Starch agar (SA). The colony formed on starch agar was isolated to a new SA plate and 141 colonies were isolated as a result. The amylase productions were observed on the starch agar when the iodine solution was added onto the plate. Figure 13 shows a clear zone that represents the amylase activity on SA. The clear zone, colony size of each isolate were measured and recorded (data not shown). As a result, 76 isolates from 141 isolates were classified as amylase producing isolate. The ratio between colony size and the clear zone was calculated for each microorganism, and that data was used to narrow down the number of isolates that were used in further experiments.

The isolates which had better ratings in amylase production were evaluated to have amylase production ability using solid-state fermentation as it is described in section 3.2.6. Six-teen best amylase producers were extracted the amylase using solid-state fermentation (result not shown), and AMPB 10, AMPB 31, and AMPB 43 were selected to be used in the further experiments as a result.

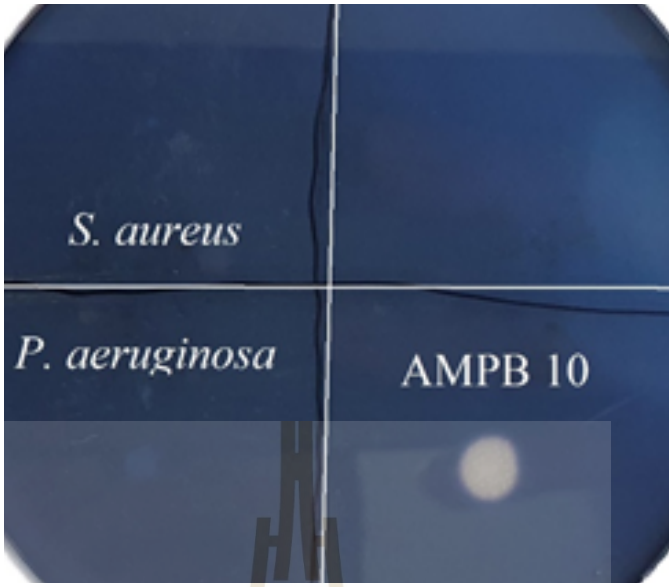



**Figure 13** Clear zone developed on the starch agar. There were *P. aeruginosa*, *S. aureus*, and AMPB 10, which is an amylase producing microorganisms isolated in this study, were inoculated. All of *S.aureus*, *P.aeruginosa, and* AMPB 10 colony grew on the plate. However, a clear zone that indicates the amylase activity appeared only around the colony of AMPB 10.

## 4.2 Identification of species

The sequenced similarity of AMPB 10, and AMPB 31 and AMPB 43 were 100 % identical to *Bacillus tequilensis* KCTC 13622, and 99.93 % identical to *Bacillus subtilis subsp*. *Subtilis* NCIB 3610 respectively.

The phylogenetic tree was constructed as described in section 3.2.5 to determine the phylogenetic relationship of 8 16S rRNA gene sequences (Figure 14). The phylogenetic tree includes four *Bacillus* sp. and *Staphylococcus aureus* as our group.

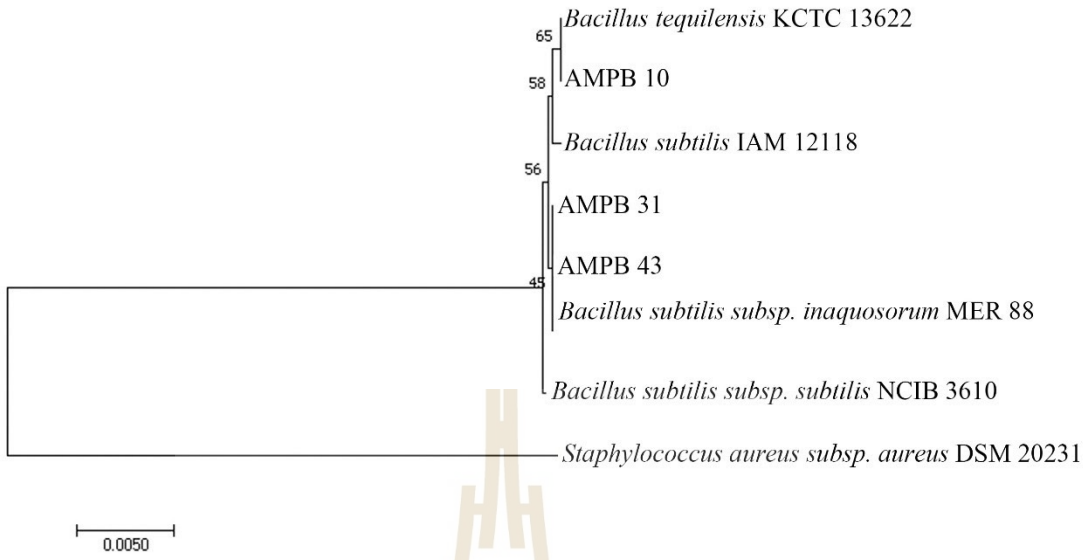



**Figure 14** Phylogenetic tree constructed from 16S rRNA gene sequences using the neighbor-joining method. The phylogenetic tree of *Bacillus subtilis* sp. and its closest homologs based on the 16S rRNA sequence using MEGA7 software by using neighbor-joining method with bootstrap values based on 500 replicates are included at the branch nodes.

## 4.3 Determination of extracted protein concentration

The amylase producing isolates selected in section 4.1, AMPB 10, AMPB 31, and AMPB 43 were incubated overnight for extraction of amylase. Simultaneously, *Bacillus amyloliquefaciens* was incubated overnight to be a control of biofilm degradation.

The amount of oat bran and ammonium sulfate concentration used for the extraction and purification were estimated from preliminary experiments (data not shown). Thirty-six ml of OD adjusted Starch broth (SB) culture was poured on to 24 g of sterile oat bran to obtain sufficient amounts of amylase and incubated. Ninety ml

of PBS was added to extract amylase from fermented oat bran. The supernatant containing amylase was successfully separated from the fermented oat bran by centrifugation. The concentration of the supernatant was measured and listed in Table 5 as before purification.

Ninety ml of supernatant from AMPB 10, AMPB 31, and AMPB 43 and *B. amyloliquefaciens* were portioned out and 47.95 g of ammonium sulfate that is equivalent to 80 % saturation against 90 ml of supernatant was added to the supernatant. The precipitate obtained from the supernatant by centrifugation was suspended with 4 ml of PBS and centrifuged again to remove the excess precipitate. The concentration after purification was estimated with the Bradford protein concentration assay (Table 5). As a result of the purification step, the concentration of protein in the purified solution increased eightfold compared to non-purified supernatant with a protein recovery rate of 44.5 ± 4.5 %.

**Table 5** Protein concentration estimated from protein assay.

| | AMPB 10 (mg/ml) | AMPB 31 (mg/ml) | AMPB 41 (mg/ml) | *B. amyloliquefaciens (mg/ml)* |
|---|---|---|---|---|
| Before purification | 0.2655 ± 0.01 | 0.3031 ± 0.01 | 0.232 ± 0.00 | 0.2335 ± 0.01 |
| After purification | 1.835 ± 0.04 | 2.315 ± 0.07 | 1.875 ± 0.20 | 2.19 ± 0.013 |
| Recovery rate | 38.4 % | 42.4 % | 44.9 % | 52.1 % |

## 4.4 Evaluation of extracted amylase

The crude amylase (CA) and purified amylase (PA) activities from extracted from selected isolates AMPB10, AMPB 31, AMPB 43, and *B. amyloliquefaciens* were measured at 55 °C for the comparison of activity between CA and PA. The information was used to calculate total activity and specific activity of CA and PA extracted from AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefaciens* to inspect the degree of success of the amylase purification step. The CA and PA had an average specific activity of 14.33 ± 2.10, and 33.00 ± 2.35 respectively, and relative activity between non-purified and purified amylase was 2.34 ± 0.33 on average (Table 6).

The PA form selected isolates AMPB10, AMPB 31, AMPB 43, and *B. amyloliquefaciens* were measured at 6 different points of temperatures to estimate the optimum temperature. The result indicated that AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefaciens* has moderate activity at the range of 30 to 40 °C, the highest activity was at the range of 50 to 60 °C and it mostly lost activity above 70 °C. The result specified the optimum temperature of all the amylases were at the rage of 50 to 60 °C. (Figure 15).

**Table 6** Result of activity assay at 55 °C.

| **Sample name** | **Total activity (U/ml)*** | **Specific activity (U/mg)**** | **Relative activity** |
|---|---|---|---|
| AMPB 10 CA*** | 3.66 ± 0.04 | 14.63 ± 0.15 | |
| AMPB 10 PA**** | 67.89 ± 0.11 | 37.00 ± 0.06 | 269 % |
| AMPB 31 CA | 3.41 ± 0.08 | 11.38 ± 0.29 | |
| AMPB 31 PA | 71.83 ± 0.29 | 31.03 ± 0.13 | 276 % |
| AMPB 43 CA | 3.22 ± 0.03 | 14.01 ± 0.13 | |
| AMPB43 PA | 57.61 ± 1.03 | 30.73 ± 0.55 | 221 % |
| *B. amyloliquefaciens* CA | 3.98 ± 0.05 | 17.30 ± 0.21 | |
| *B. amyloliquefaciens* PA | 70.62 ± 0.49 | 32.25 ± 0.22 | 189 % |

*U/ml = (A690 control -A690 sample) ÷ A690/mg starch ÷ 10 min ÷ 0.025 ml

**U/mg= protein concentration ÷ U/ml

*** CA = Crude amylase

**** PA = Purified amylase

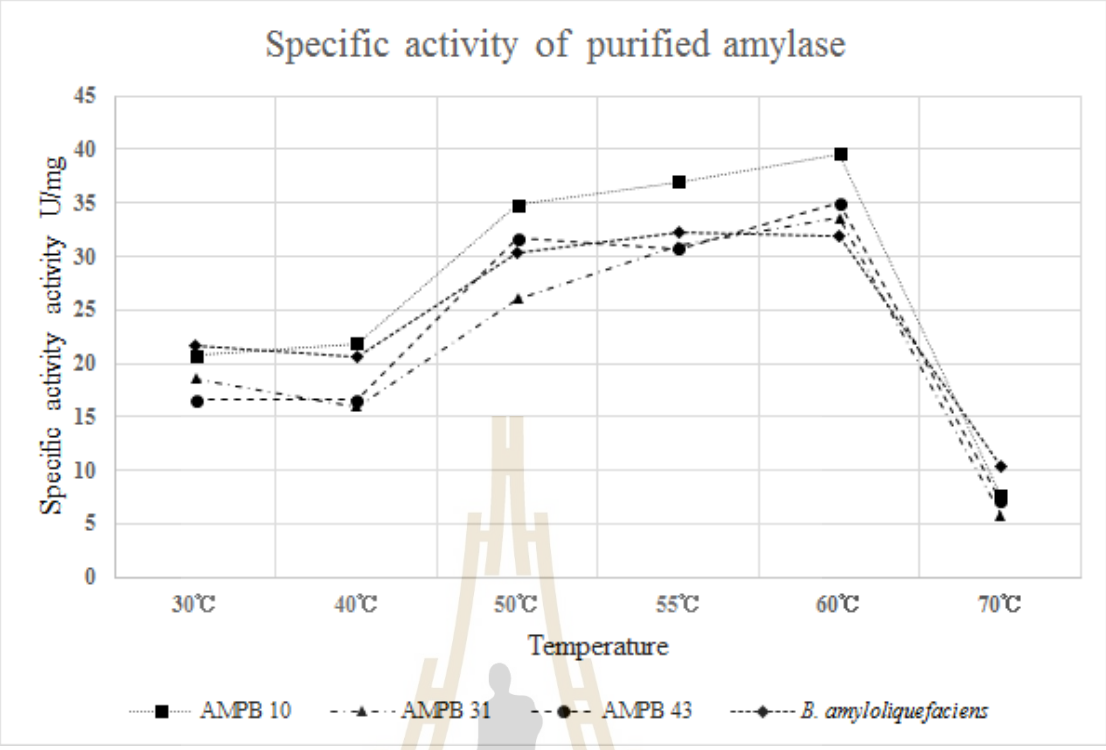



**Figure 15** Graph of the specific activity obtained from extracted amylase. The graph shows the activity curve of the amylases extracted from AMPB10, AMPB 31, AMPB 43 and *B. amyloliquefaciens.*

The stability of Purified amylases from the selected isolates AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefaciens* were measured to deepen the understanding of extracted amylase according to section 3.2.9. This assay measured the activity of amylase after 30 minutes of incubation at different temperatures to estimate the remaining activity, which indicates the stability of the enzyme. The incubation was held at 40 °C, 50 °C, and 60 °C and relative activity reduced for an average of 14.2 ± 6.8 %, 18.2 ± 5.2 %, and 84.6 ± 9.6 % respectively. The stability of amylase at 40 to 50 °C showed a mild reduction of activity however, the result at 60 °C showed a dramatic reduction in the specific activity (Figure 16).

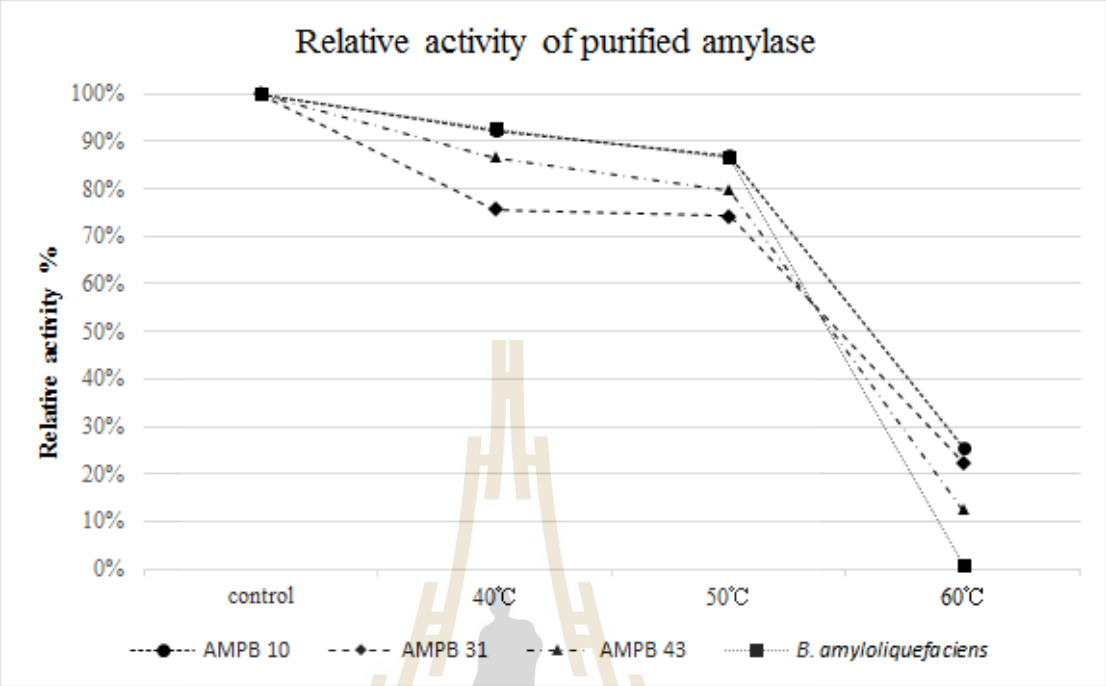



**Figure 16** The graph of Stability assay obtained from extracted amylase. This graph represents the percentage of remaining activity after 30 min of incubation at different temperatures. The control is the activity of amylase that was not incubated.

SDS-PAGE and native-PAGE were held to obtain the physical information of amylases extracted from selected isolates AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefaciens.* According to the result of zymography, the activity of all the amylases extracted from AMPB10, AMPB 31, and AMPB 43 was observed at the same level as *B. amyloliquefaciens* (Figure 17a). The result of SDS-PAGE (Figure 17 b) showed 6 common sizes of bands among the purified amylases from selected isolates and *B. amyloliquefaciens*.

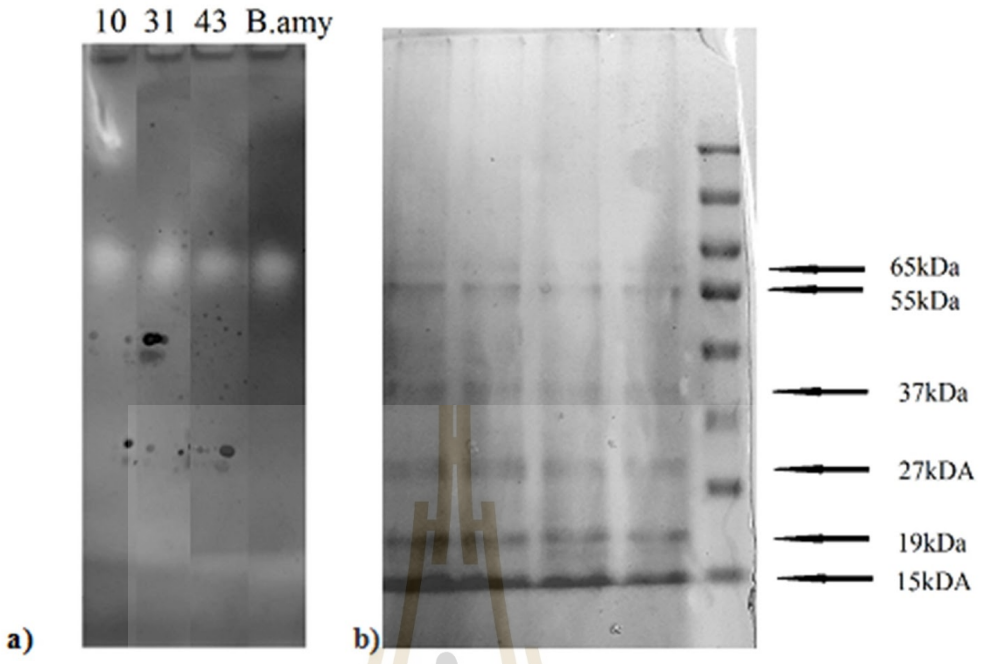



**Figure 17** Result of PAGE with the sample AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefacieans*. a) Result from Non denaturing PAGE. b) Result from SDS-PAGE with prestained protein ladder, 10 to 180kDa. a) has false positive in the lane of AMPB 10 at the upper part. The false-positive came from the technical error.

## 4.5 Evaluation of biofilm degrading activity

Biofilm forming ability was tested with five clinical isolates, which are S*taphylococcus aureus* DMST 20654 (MRSA), *Staphylococcus aureus* TISTR 1466, *Pseudomonas aeruginosa* TISTR781, *Escheria coli* 2037, and *Staphylococcus epidermidis* TISTR 518. Three strains out of five strains, *S. aureus* TISTR 1466, *P. aeruginosa* TISTR781, and *S. epidermidis* TISTR 518, produced biofilm on 96 well plates (data not shown). Thus, *S. aureus* TISTR 1466, *P. aeruginosa* TISTR781, and *S. epidermidis* TISTR 518 were selected to be used for biofilm degradation assay.

The biofilms from *S. aureus* TISTR 1466, *P. aeruginosa* TISTR781, and *S. epidermidis* TISTR 518 were formed on 96 well plate as it is described in section 3.2.12. Each biofilm was treated with different concentrations of amylase extracted from selected isolates AMPB 10, AMPB 31, AMPB 43, and *B. amyloliquefacieans*. The result of the biofilm degradation assay was observed under the microscope and the photo was taken (Figure 18, 19, and 20). The biofilm produced by *S. aureus*, *P. aeruginosa*, and *S. epidermidis* were successfully degraded in maximum rate of 60 to 70 % with purified amylase. Rate of degradation increased when the treating temperature increased, and also the amount of enzyme added to the well increased, except for *S. aureus*. For the biofilm of *S. aureus*, the degradation rate decreases when the concentration of amylase increased (Figure 21, 22, and 23).

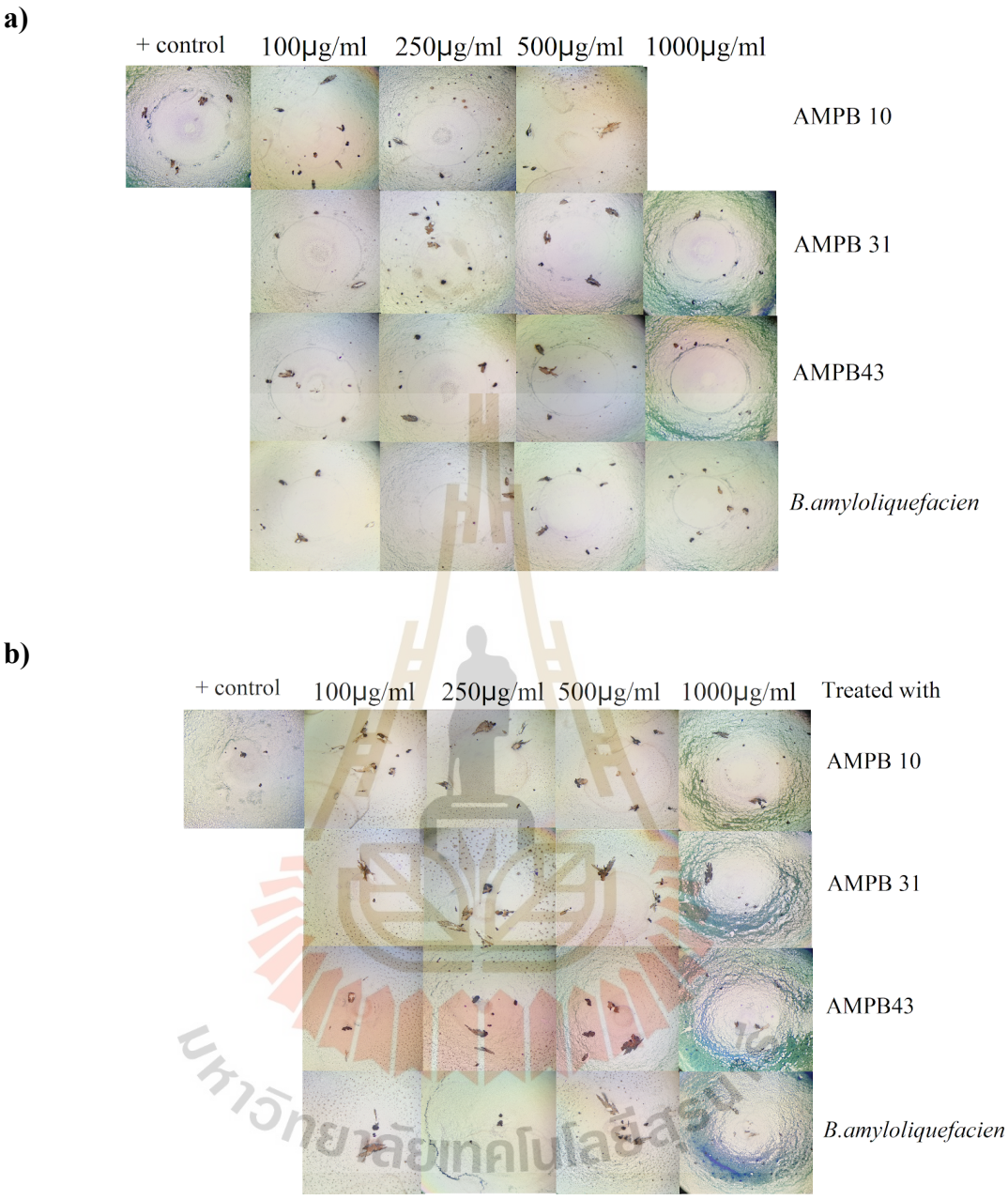



**Figure 18** Biofilm from *S. aureus* treated with amylase extracted from 4 microorganisms. a) Treated at room temperature (25 °C). b) Treated at 37 °C. a) is a lack of a picture of 1000 µl of treatment with AMPB 10, due to its unacceptable error.

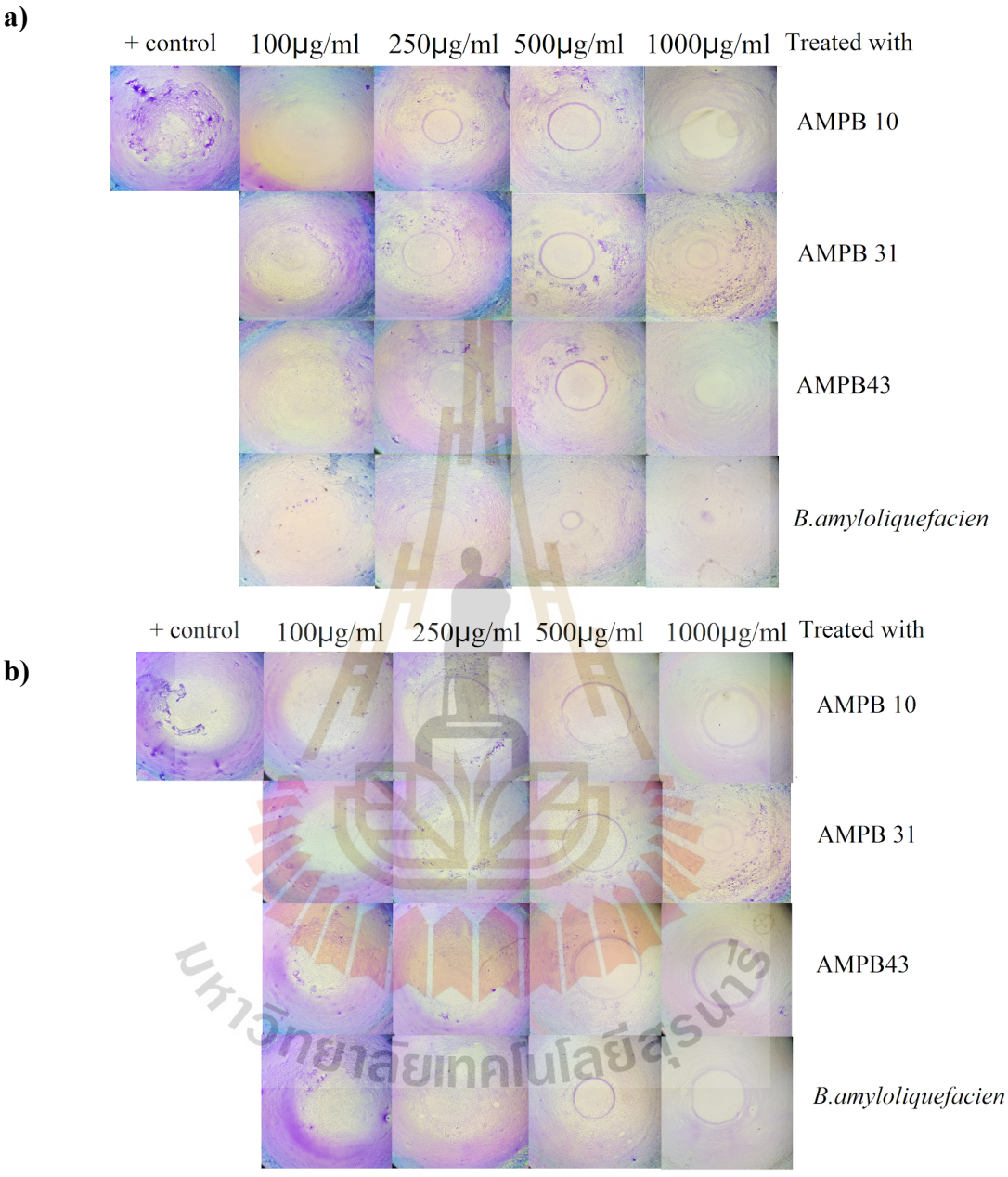



**Figure 19** Biofilm from *P. aeruginosa* treated with amylase extracted from 4 microorganisms. a) Treated at room temperature (25 °C). b) Treated at 37 °C.

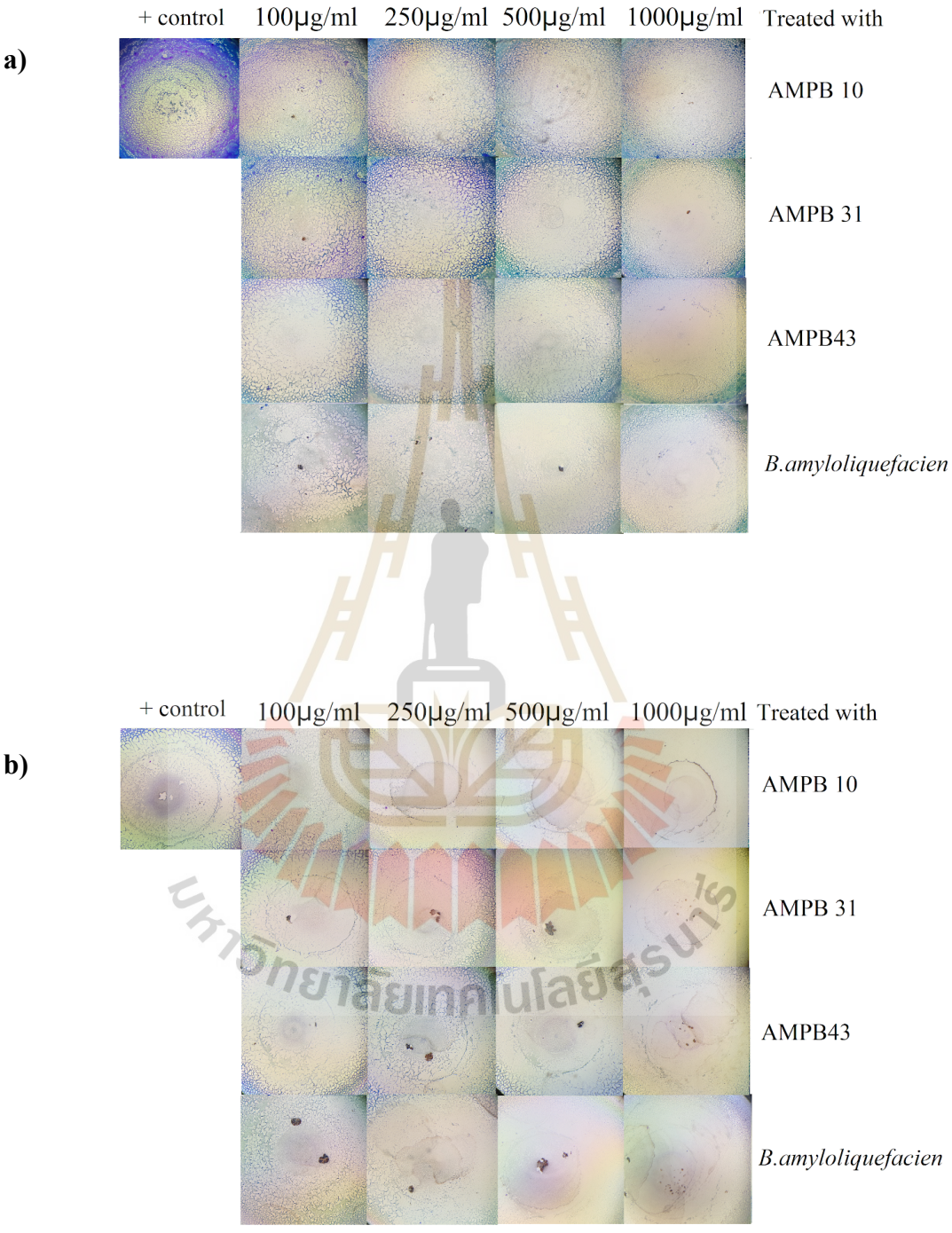
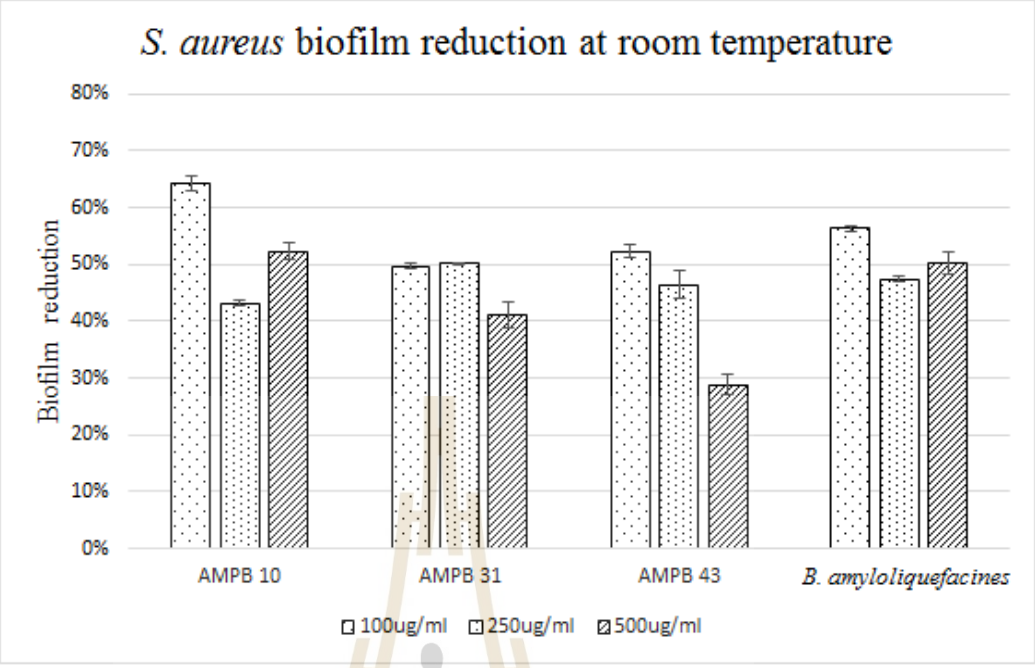



**Figure 20** Biofilm from *S. epidermidis* treated with amylase extracted from 4 microorganisms. a) Treated at room temperature (25 °C). b) treated at 37 °C.

**a)**




**b)**

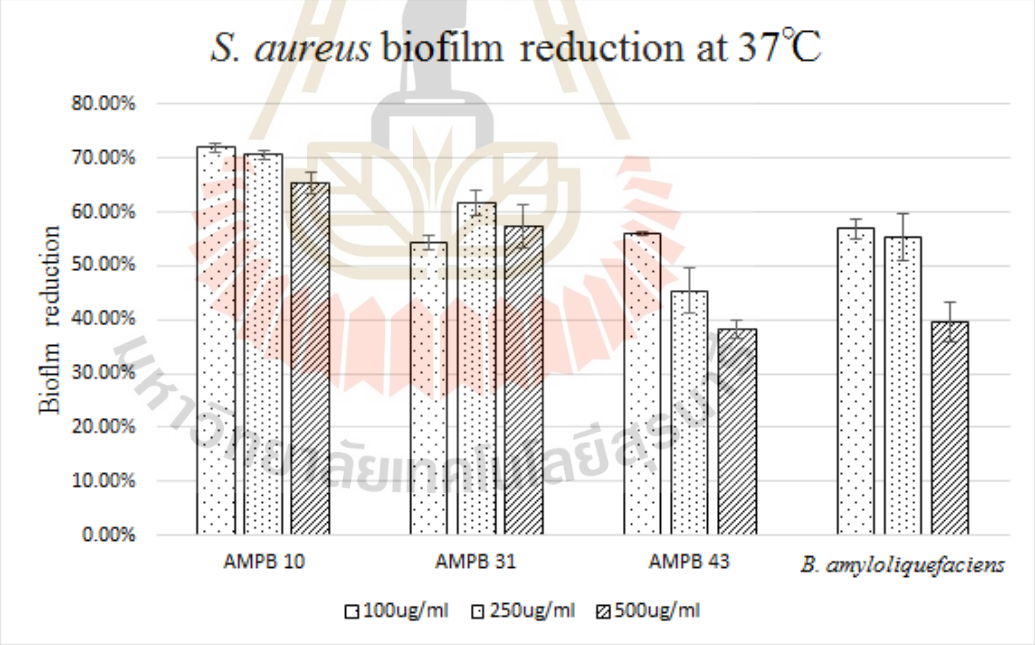
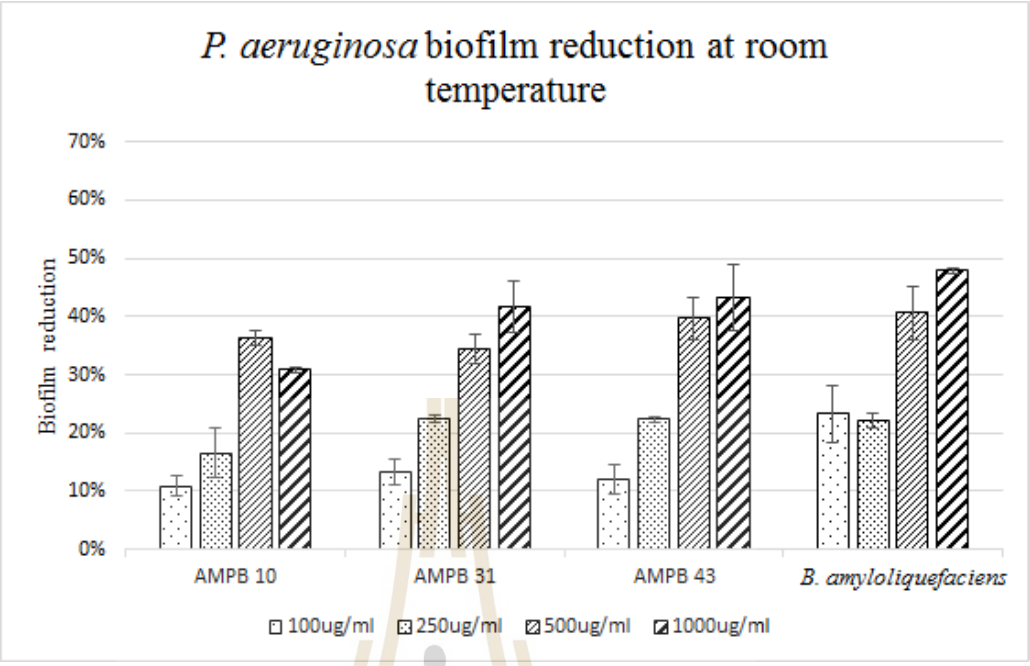



**Figure 21** Percentage of *S. aureus* biofilm reduction treated with amylase extracted from 4 microorganisms. Treatment with 1000 µl was not shown since there were large errors.

**a)**




**b)**

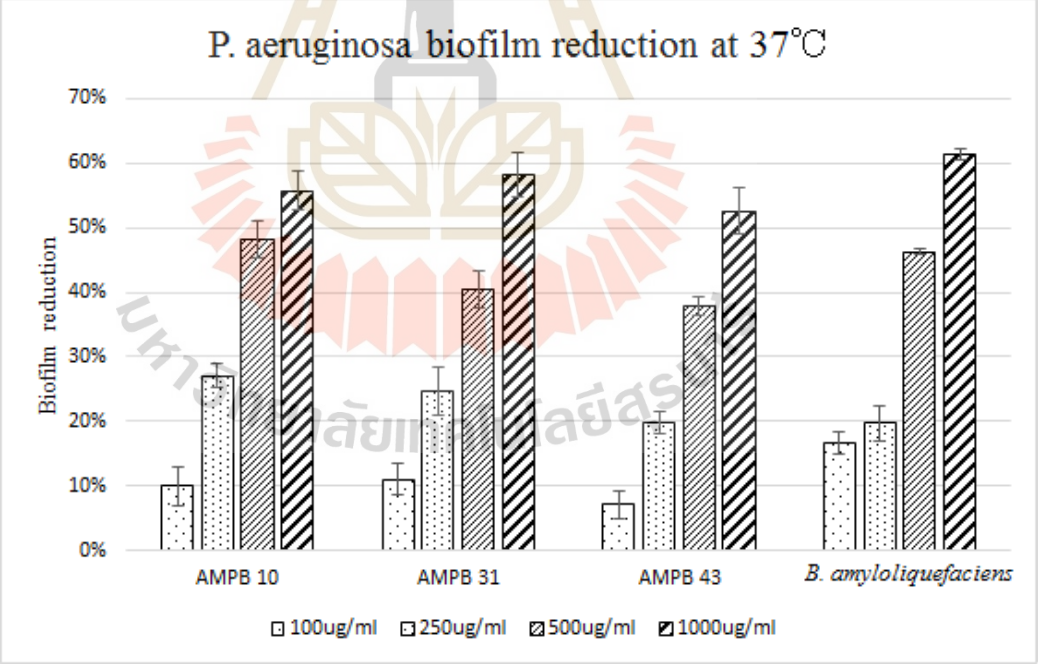
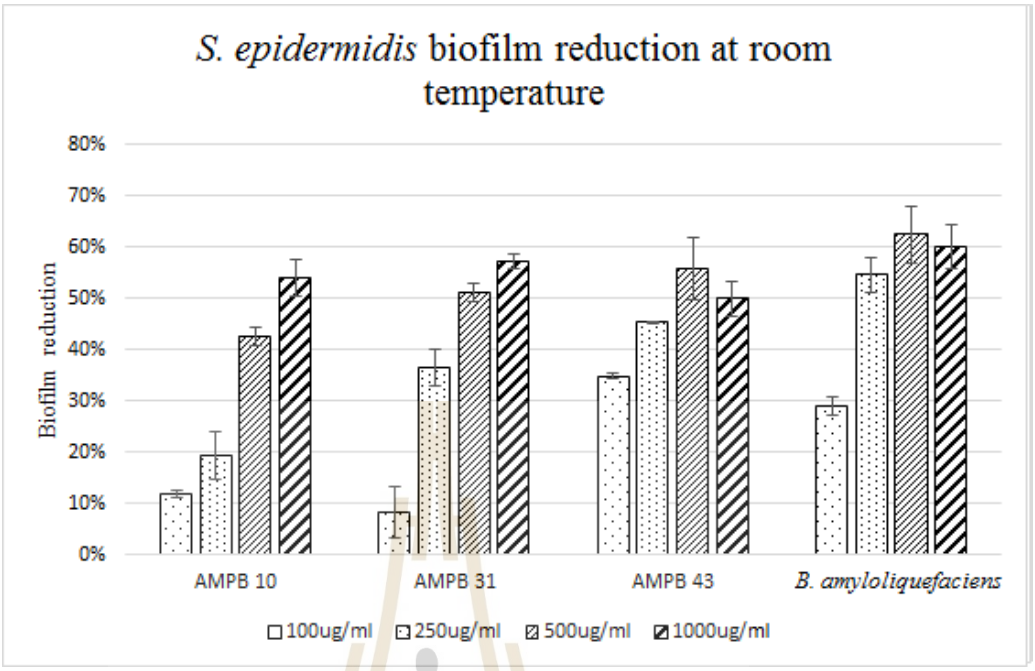



**Figure 22** Percentage of *P. aeruginosa* biofilm reduction treated with amylase extracted from 4 microorganisms.

**a)**




**b)**

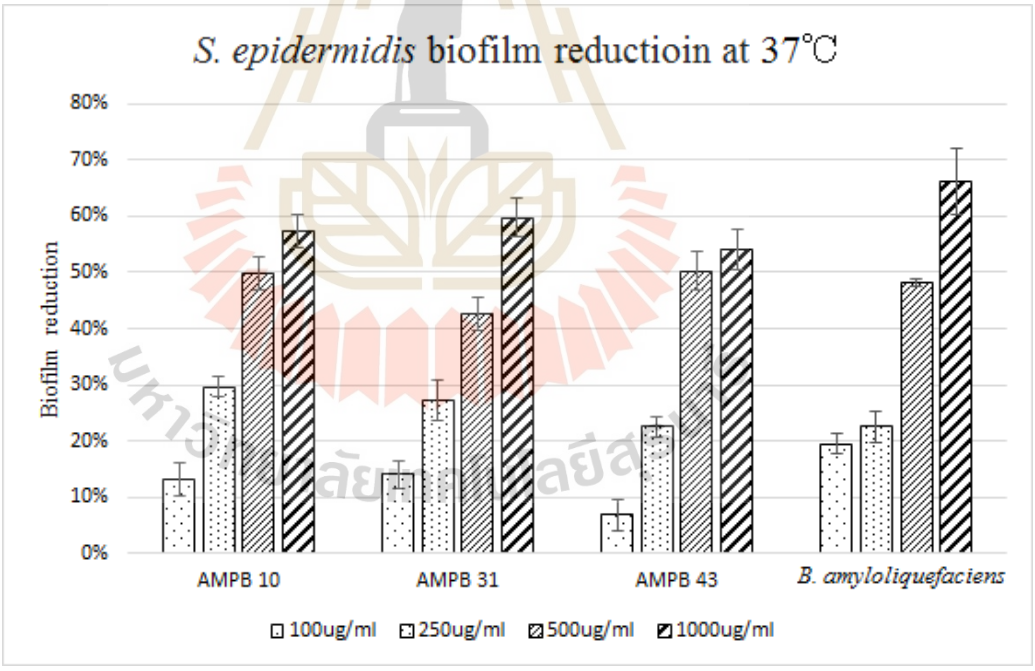



**Figure 23** Percentage of *S. epidermidis* biofilm reduction treated with amylase extracted from 4 microorganisms.

# CHAPTER V

# DISCUSSION AND CONCLUSION

As a result of 16S rRNA sequencing, the 16S rRNA sequence of AMPB31, and AMPB 43 was 99.92 % identical to *Bacillus subtilis* sp. and 16S rRNA showed 100 % identical to *Bacillus tequilensis.* As it is generally known, *Bacillus* species are one of the main amylase sources that are used in the industrial production of α-amylase (El-Enshasy et al, 2013).

16S rRNA from AMPB 10 was analyzed as strain *B. tequilensis,* and this strain was reported to have closely related strain *B.subtilis* according to the 16S rRNA analysis (Gatson, Benz, Chandrasekaran, Satomi, Venkateswaran, and Hart, 2006). *B. tequilensis* RG-01was reported producing α-amylase, which is a thermotolerant solvent stable amylase. The optimum temperature and optimum pH of *B. tequilensis* α-amylase are 55 °C and pH 5.5 respectively. The total activity of α-amylase derived from *B. tequilensis* RG-01 is approximately 2500 U/ml with the glucose determination with arsenomolybdate. The α-amylase is stable at the range of pH 5.0 to 10.0, which indicates excellent buffering property (Tiwari, Shukla, Mishra, and Gaur, 2014)

The possible explanation AMPB 10 had the best activity among all the tested may be related to the amylase enzyme extracted *B. tequilensis* RG-01. However the

information of amylase from *B. tequilensis* KCTC 13622 was not found currently to our knowledge.

As a result of zymography, the activity observed on the zymography showed at the same level as *B. amyloliquefaciens*. The native-PAGE is also known as non-denaturing PAGE, which indicates the protein run in the gel is still at their folded state. Electrophoresis of denatured protein is depending on its charge and mass however, non-denatured protein moves in the gel according to their charge, mass, physical shape, and size of the protein. Thus, it was estimated the morphology of amylase extracted from *B. amyloliquefaciens* and AMPB 10, AMPB 31, and AMPB 43 shares similar characteristics, since all the amylases extracted from selected isolates and amylase from *B. amyloliquefaciens* showed activity at the same level (Figure 18 a). *B. amyloliquefaciens* is known to produce α-amylase, and protein size of α-amylase from *B. amyloliquefaciens* has a size of 58 kDa (Gangadharan, Nampoothiri, Sivaramakrishnan, and Pandey, 2009), and the band around 58kDa were observed on SDS-PAGE (Figure 18 b) and amylase from AMPB 10, AMPB 31, AMPB 43 as well. Regarding the result of activity, stability, zymography, and SDS-PAGE, amylase from AMPB 10, AMPB 31, and AMPB 43 have a high possibility to be α-amylase.

The size estimation of amylase extracted from AMPB 10, AMPB 31, and AMPB 43 could be estimated using zymography obtained by SDS-PAGE. The polyacrylamide gel containing starch could be prepared and the extracted amylase

will be loaded without denaturing. After electrophoresis, polyacrylamide gel will be soaked in the Triton X-100 to remove SDS in the polyacrylamide gel, hence the amylase regains its activity. The Amylase activity will degrade the starch in the polyacrylamide gel and it gives clear color when the gel stained by the iodine solution (Maria de Lourdes, Peixoto-Nogueira, da Silva, Maller, and Cabral, 2012) This method will make sure the size of extracted amylase and be able to identify the extracted amylase by comparing the known size of amylase.

The optimum temperature of purified amylase obtained from *B. amyloliquefaciens* have an optimum temperature at 55 °C and this optimum temperature matched with the result of optimum temperature derived from *B. amyloliquefaciens* free amylase enzyme (Demirkan, Dincbas, Sevinc, and Ertan, 2011)

The best biofilm degradation rate of *S.aureus, P.aeruginosa,* and *S. epidermidis* were 71.94 %, 61.5 %, and 66.24 % respectively in this study. The obtained results from this study are similar to the research from Kalpana et al., the group was able to reduce biofilm in maximum 63.89 % of MRSA and *P. aeruginosa* with α-amylase enzyme from *B. subtilis* obtained in the form of lyophilized powder (Sigma Aldrich, USA, cat no. 10069).

The results obtained in these experiments give insights towards the biofilm degradation that amylase activity itself does not correlate directly to the biofilm degradation in this study. Strain AMPB 10 and *B. amyloliquefaciens* showed better

activity compared to the AMPB31 and AMPB 43 (Figure 15). However, degradation of biofilm by AMPB 31 was better in the case of *P. aeruginosa* and *S. epidermidis* biofilm compared to AMPB 10. The phenomena that high starch degrading activity does not guarantee high degradation possibly explained by the content of biofilm composition. Biofilm was formed by different saccharides and protein as mentioned in the literature review. Hence the composition of biofilm may affect the degradation of the biofilm by amylase.

The result of biofilm degradation in which biofilm formed by *S. aureus* showed the irregular degradation compared to the degradation results from *P. aeruginosa* and *S. epidermidis,* the degradation result of *S. aureus* was inversely proportional to the concentration of amylase. The result possibly explained by the presence of inhibitors in the amylase solution that act on biofilm to protect from degradation. According to Zevallos et al., 2017, wheat products contain amylase trypsin inhibitor (ATI) and oat bran also has that. Wheat ATI is a family of compact, highly disulfide-linked and protease-resistant protein. ATI is a strong activator of dendritic cells, macrophages, monocytes, and that causes intestinal inflammation in humans. ATI in the oat bran possibly extracted together with the amylase extraction step and inhibit the biofilm degradation *S. aureus* biofilm.

As a result of this study, amylase producing organisms were successfully isolated and extracted amylase. And the extracted amylases were successfully degraded biofilm.

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# REFERENCES


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# APPENDIX

# APPENDIX

# SEQUENCES OF SOIL ISOLATED MICROORGANISMS

**AMPB 10**

CCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCA

CCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAA

GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATT

CCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGAT

TTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG

TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC

CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAA

TGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC

ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCC

CGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTA

AGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGG

GCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCG

GAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACA

CTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCG

CTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTT

CGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAAT

TCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCG

GTT

GAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTAC

GCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTG

GCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCT

ATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAA

CCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAA

GATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG

TGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCC

ATTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCC

GAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTA

GCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGT

TACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGC

**AMPB 31**

CCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCA

CCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAA

GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATT

CCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGAT

TTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG

TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC

CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAA

TGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC

ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCC

CGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTA

AG

GTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC

CCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGA

GTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTT

AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTC

CCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGC

CACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCC

ACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTT

GAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTAC

GCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTG

GCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCT

ATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAA

CCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAA

GATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG

TGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCC

GTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGC

CGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATT

AGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTG

TTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGC

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**AMPB 43**

CCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCA

CCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAA

GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATT

CCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGAT

TTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG

TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC

CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAA

TGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC

ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCC

CGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTA

AGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGG

GCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCG

GAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACA

CTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCG

CTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTT

CGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAAT

TCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCG

GTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTT

TACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGC

TGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCC

CTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAA
ACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGA
AGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGC
CGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAG
CCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTAT
TAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGT
GTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCG
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