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Degradation of Biofilm Formed by Opportunistic Pathogens using Amylase Extracted from *Bacillus tequilensis*

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HIGHLIGHTS

- The goal of this study was to identify an amylase enzyme capable of degrading pathogenic bacterial biofilms. The findings of this research could be applied in the medical field, especially in the area of medical device sanitization. This helps to prevent infection in patients who are exposed to medical devices.
- This is the first study to demonstrate that amylase derived from *Bacillus tequilensis* can degrade microbial biofilms.
- This study sheds light on bacterial biofilm degradation using soil isolate bacteria. The results will aid future research into biofilm degradation using amylase enzymes.

Abstract: Biofilm degradation with amylase is one of the effective ways for controlling bacterial biofilm. Although amylase can be obtained from several sources, microbial amylase is preferred. Information of the new source of amylase and its activity is therefore fundamental for new applications and enzyme technology advancement. In this study, amylase was extracted from bacteria isolated from soil in Nakhon Ratchasima, Thailand. Two different soil isolates AMPB10 and AMPB31 were selected for the purification of amylase; they were identified as *Bacillus tequilensis* and *Bacillus subtilis*, respectively. The efficiencies of purified amylase in degradation of biofilm of *Staphylococcus aureus* TISTR 1466, *Staphylococcus epidermidis* TISTR 518, and *Pseudomonas aeruginosa* TISTRA 781 biofilms were measured. The amylase from AMPB10 and AMPB31 degraded 70.9% and 66.1% of *S. aureus* biofilm, 59.6% and 64.1% of *S. epidermidis* biofilm, and 57.8% and 60.1% of *P. aeruginosa* biofilm, respectively. Amylase produced from AMPB10 had greater biofilm degrading activity on *S. aureus* than AMPB31, while amylase from AMPB31 was more effective against *P.*

aeruginosa and *S. epidermidis* at high concentration. However, AMPB10 amylase showed stronger degrading activity on *P. aeruginosa* at intermediate concentration. To the best of our knowledge, this is the first report demonstrating a successful use of *B. tequilensis* amylase to degrade the bacterial biofilm.

Keywords: Biofilm degradation; Amylase; *Bacillus tequilensis.*

INTRODUCTION

Biofilm is a polymeric matrix containing microorganisms enclosed in polysaccharides, proteins, and extracellular microbial DNA that adheres to biotic or abiotic surfaces. It plays a critical role for microorganisms to survive in diverse environments and protect them from physical, chemical, and biological factors. The role of biofilms includes protecting cells from environmental stresses such as antibiotics, adhesion to surface for the colony formation, and cell to cell communication by Quorum Sensing (QS) molecules [1,2,3]. Biofilm acts as a major threat to industrial equipment, water pipes and medical devices, including contact lenses [4,5]. In fact, it is a major cause of bacterial infections in humans by 65% [6]. Biofilm forming organisms are known to develop multiple infectious diseases. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) causes sepsis and pneumonia [7], *Pseudomonas aeruginosa* has been linked to ventilator-associated pneumonia and burn wound infections, and also hospital-acquired infections [8]. Due to its ability to form biofilms, *P. aeruginosa* has a high tolerance to antibiotics. It has been noted that the efflux pump is more actively generated by cells associated with biofilms than by planktonic cells [9]. *Staphylococcus epidermidis* is the most common cause of medical device infection that can lead to osteomyelitis and acute sepsis [10]. Bacterial attachment and biofilm formation on the host tissues are important steps in the establishment of chronic infection [6].

Exopolysaccharides (EPS) in biofilm is an important component which plays a key role in biofilm formation and antibiotic resistance. Previous reports have shown that mutants that are unable to produce EPS are unable to produce biofilms. Therefore, degradation of EPS is a key aspect of destroying biofilm [11]. The microbial EPS are comprised of either homopolysaccharides or heteropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type, such as D-glucose or L-fructose. Heteropolysaccharides are constituted by repeating units of monosaccharides, including D- glucose, D-galactose, L- fructose, L- rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid [12].

Amylase (EC 3.2.1.1) is one of the most intimate enzymes for human beings. It is a digestive enzyme that acts on polysaccharides. The advantage of using amylase for biofilm degradation relies on its environmentally friendly property and the easy process of obtaining the enzyme. Hence, amylase serves as a good biofilm degrading agent candidate. In this study, the amylase enzyme of soil isolate bacteria was purified and characterized. The purified enzyme was investigated for its anti-biofilm activity against biofilm-forming pathogens.

MATERIAL AND METHODS

Isolation of soil bacteria

One gram of soil sample was transferred into the flask containing 99 mL of sterile normal saline. The soil suspension was serially diluted to 10^{-4} and spread on the surface of starch agar (SA) medium (HiMedia Laboratories, India) to enhance the isolation of amylase producing microorganisms. The plate was incubated at 37 ºC for 24 h, the bacterial colonies were later subcultured onto a new SA medium.

Screening of amylase producing bacteria

The bacterial cells were inoculated on SA medium and incubated for 24 h. The plate was then flooded with Lugol's iodine solution (I2KI) (2% KI and 0.2% I), and the clear zone around the colony was measured [13]. The colonies with clear zones are indicated as amylase producers.

Identification of soil isolates

Genomic DNA of soil isolates was extracted using the freeze-thaw technique. A single colony of soil isolate was picked by a sterilized toothpick and suspended in 10 µL of sterile distilled water. The cell suspension was then frozen at -80 °C for a few minutes. The frozen mixture was thawed to induce cell lysis. The freezing and thawing steps were repeated 4 to 6 times. The extracted DNA was used as a template for PCR amplification of the 16S rRNA gene. The PCR amplification was performed by using universal primers, 27F (‘AGAGTTTGATCMTGGCTCAG’) and 1525R (‘AAGGAGGTGATCCAGCC’) [14]. 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 agarose gel in 1X Tris/borate/EDTA (TBE) buffer. An approximate 1500 bp of amplified fragment was cut from agarose gel and purified using FavorPrepTM GEL/PCR Purification Kit (Favorgen, Taiwan) [15]. The purified PCR products were submitted for DNA sequencing at Macrogen, Korea. The obtained sequences were analyzed and compared to the EzBioCloud online gene database (www.ezbiocloud.net). The sequences were aligned with closely related species using multiple sequence alignment program ClustalW. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0.26 with a Neighbor-joining algorithm of 1000 bootstraps.

Solid-state fermentation (SSF) and purification of amylase

The bacterial strains were cultivated overnight in the starch broth containing 1% soluble starch and 0.3% beef extract (pH 7.5). The inoculums were transferred to sterilized oat bran and incubated at 37 ºC under static condition [16]. After 48 h of incubation, a fermented culture was soaked in 20 mM phosphate buffer (PBS) (pH 7) for 30 min at 4 ºC on a rotary shaker. The solid part of the mixture and supernatant containing crude amylase were separated by centrifugation at 4 ºC, 8,000 rpm for 15 min. The purification of amylase from the crude extract was carried out by using ammonium sulfate precipitation [16]. Ammonium sulfate was added until the saturation level reached 20% and incubated at 4 ºC for 30 min on a rotary shaker. The supernatant was transferred to a new tube, and then ammonium sulfate saturation was increased to 60%. The precipitate was recovered by centrifugation at 8,000 rpm for 15 min, and the pellet was suspended in 20 mM PBS [16].

SDS-PAGE and amylase activity staining

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% polyacrylamide gels. Samples were treated with SDS sample buffer and heat-denatured at 90 degrees for 10 min before loading onto the gel. Protein concentration was determined by modified Lowry's method using bovine serum albumin as standard [17]. Whole blue range pre-stained protein ladder, 10 to 240 kDa (Vivantis, Malaysia), was used as a standard molecular weight marker.

Amylase activity staining was performed with 8% polyacrylamide gel in the presence of 0.5% starch. After electrophoresis, the gel was soaked in 100 mM phosphate buffer containing 2.5% Triton X-100 for 30 min to renature the enzyme. The excess Triton X-100 was removed by washing the gel with distilled water. Then the gel was soaked in 100 mM phosphate buffer and incubated at 55 ºC for 1 h. The amylase activity was developed by staining the gel with I_2KI [18].

Amylase activity assay

The amylase activity was determined based on the method of Xiao et al. (2006) [19]. The reaction mixture contained 0.5 ml of PBS (0.1 M, pH 6.0) and 0.25 mL of 0.1% soluble starch, 25 µL of crude or purified amylase. The mixture was incubated for 10 min at 55 ºC. Then 0.25 mL of 0.1 N HCl was added to stop the reaction. Then I_2KI was added for the development of color. The blue color intensity of the starch-iodine reaction was measured at 690 nm by using a UV-vis spectrophotometer (Thermo Scientific Multiscan Go) [5]. Total activity was calculated using the formula [10, 19]:

$$U/ml = (A_{690}\,control - A_{690}\,sample) / (A_{690}/mg\ starch) / 10\ min / 0.025\ \mu l$$

Thermostability of purified amylase

Amylase was pre-incubated for 30 min at different temperatures (40, 50, and 60 ºC). Then 25 µL of pre-incubated amylase was mixed with the reaction mixture (0.5 mL of phosphate buffer (0.1 M, pH 6.0), and 0.25 mL of 0.1% soluble starch) and incubated for 10 min at 55 ºC. Finally, 0.25 mL of 0.1 N HCl was added to stop the reaction, followed by addition of I_2KI. The blue color intensity was measured at 690 nm using a UV-vis spectrophotometer (Thermo Scientific Multiscan Go) [19].

Biofilm degradation assay

Overnight inoculum of biofilm-forming pathogens, *S. aureus* TISTR 1466*, P. aeruginosa* TISTR 781*,* and *S. epidermidis* TISTR 518, were prepared in Nutrient Broth (NB); 1L contains 3 g of beef extract, 5 g of peptone, and 5 g of sodium chloride, pH 7.0 ± 0.2. One milliliter of an overnight culture was transferred to a

flask containing 99 mL of NB and incubated at 37 ºC under 200 rpm shaking condition for 6 h. Then OD_{600} of cell suspension of bacterial culture was adjusted to 0.5 McFarland standard using NB. A 96-well microplate was filled with 200 µL of 0.5 OD_{600} cell suspension and incubated at 37 ºC for 48 h. The plate was washed with distilled water to remove the free cells, then 200 µL of purified amylase was added to the well and incubated at 37 ºC for 30 min. After incubation, the well was washed with distilled water and allowed to air-dry. The biofilm in a well was stained with 0.5% crystal violet (w/v) for 30 min. The wells were washed with distilled water, and then air-dried. The crystal violet staining biofilm was eluted by 30% acetic acid. The absorbance of crystal violet was measured at 595 nm [4,5].

Data analysis

The statistical analysis of the data was executed using one-way analysis of variance (ANOVA) using IBM SPSS Statistic version 23. To determine the significant difference between groups on ANOVA analysis, Tukey's test was applied with $p < 0.05$.

RESULTS

Isolation, screening, and identification of amylase producing soil bacteria

In this study, soil samples were collected from Nakhon Ratchasima, Thailand. Amylase production was examined by hydrolysis of starch on SA medium using I_2KI. The bacterial isolates that exhibited the highest activity on SA medium were selected and used for biofilm degradation analysis. The isolates used in this study were AMPB10 and AMPB31. The 16s rRNA gene sequence of AMPB10 (GenBank: MT871982) was 100% identical to *Bacillus tequilensis* KCTC 13622 whereas that of AMPB31 (GenBank: MT871983) was 99.93 % identical to *Bacillus subtilis* subsp*. subtilis* NCIB 3610 (Figure 1).

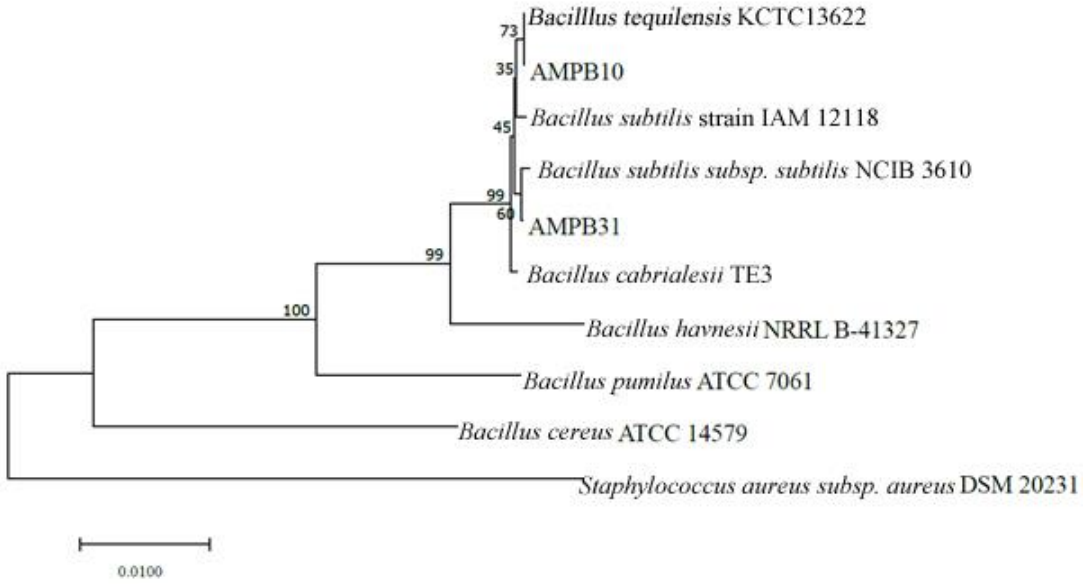

Figure 1. Phylogenetic tree reconstructed using neighbor-joining method based on 16s rRNA gene sequence of AMPB10, AMPB31, and closely related species. *Staphylococcus aureus* subsp. *aureus* DSM 20231 was used as an outgroup.

SDS-PAGE and Activity Staining

The crude amylase enzyme presents in the fermented oat bran supernatant and the partially purified amylase enzyme precipitated with ammonium sulfate were characterized in 12% polyacrylamide gel. After purification, the band with 50 kDa corresponding to amylase was observed from the crude enzyme (Figure 2a).

The molecular weight of amylases was identified based on the result of activity staining. The activity staining is a technique based on SDS-PAGE, which contains starch in the polyacrylamide gel. After electrophoresis, the gel was soaked in Triton X-100 to renature amylase. As a result, amylase begins to break down the starch in the gel, and the activity was revealed by adding I2KI [6]. The interaction of starch with triiodide anion results in a vivid blue-black color. Thus, the areas with amylolytic activity show achromatic bands (Figure 2b). As a result, the amylase band was estimated to be 50 kDa.

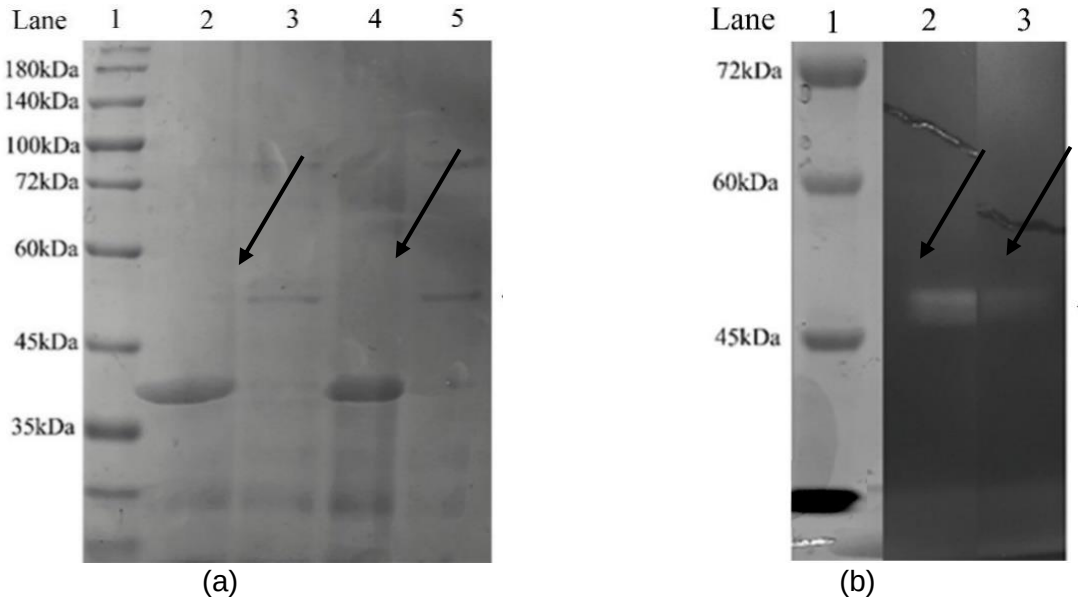

Figure 2. a) SDS-PAGE analysis of supernatant and purified amylase from AMPB10 and AMPB31. Lane 1: Whole blue range prestained protein ladder, 10 to 240 kDa (Vivantis, Malaysia.) Lane 2: 1st precipitate of amylase from AMPB10, Lane 3: 2nd precipitate of amylase from AMPB10. Lane 4: 1st precipitate of amylase from AMPB31. Lane 5: 2nd precipitate of amylase from AMPB31. b) Activity staining of purified amylase AMPB10 and AMPB31. Lane 1: Whole blue range prestained protein ladder, 10 to 240 kDa (Vivantis, Malaysia.) Lane 2: purified amylase of AMPB10. Lane 3: purified amylase of AMPB31. The arrows on Figure (a) and (b) are the position of the amylase band.

Amylase activity of a purified enzyme

Amylase enzymes produced by strain AMPB10 and AMPB31 were extracted and purified, then used for an evaluation of the amylolytic activity. In order to confirm the presence of amylase enzyme in the extract, an amylase activity assay was performed. The amylase enzyme activities of strains AMPB10 and AMPB31were determined based on the measurement of blue color derived from starch iodine reaction. The spectroscopic measurement was held at 690 nm according to the protocol of Xiao et.al. (2006) [19]. The protein concentration and specific activity of crude and purified amylase were measured to establish the efficiency of purification (Table 1). As shown in Table 1, the protein concentration of AMPB10 and AMPB31 increased by 6.4-fold and 8.5-fold, and specific activity increased by 13.9-fold and 13.4-fold, respectively.

Table 1. Protein concentration and the activity of purified amylase and amylase of crude extract of isolates AMPB10 and AMPB31.

Isolates	Total activity (U mL^{-1})*	Total protein (mg/mL)	Specific activity (U mL^{-1})
AMPB10 Crude	0.24±0.03	0.63±0.02	3.86±0.49
AMPB10 1st precipitate	1.22±0.01	3.51±0.44	27.81 ±0.25
AMPB10 2nd precipitate	1.336±0.06	4.06±0.05	53.74±2.17
AMPB31 Crude	0.23±0.01	0.59±0.01	3.94 ±0.21
AMPB31 1st precipitate	1.15±0.04	4.45±0.01	26.13±0.69
AMPB31 2nd precipitate	1.64±0.06	5.00±0.01	52.29 ±1.88

The experiment was performed in triplicate.

Characterization of the purified enzyme

To evaluate the optimum temperature and pH for amylase of AMPB10 and AMPB31, enzyme assay was performed at various temperature (30, 40, 50, 55, 60, 70 °C) and pH (3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9) conditions. The results showed that the purified amylase of both AMPB10 and AMPB31 exhibited optimum

activity at a temperature of approximately 55 °C (Figure 3). However, the amylase activity of AMPB10 was somewhat better than AMPB31 at all temperatures. While the maximum activity of purified amylase of AMPB10 and AMPB 31 were at pH 7 and pH 6.5, respectively (Figure 4). The activity of amylase of AMPB10 and AMPB31 was strongly decreased below pH 6. The purified enzyme of both strains seemed to favor alkaline condition over acidic condition.

The enzyme stability test was performed to evaluate the thermostability of purified amylases by pre-incubating the purified enzyme at various temperatures before measuring enzyme activity. The result showed that amylase activity gradually decreased when incubating temperature increased from 40 °C to 50 °C, and dramatically decreased when incubating at 60 ºC for both AMPB10 and AMPB31 (Figure 5).

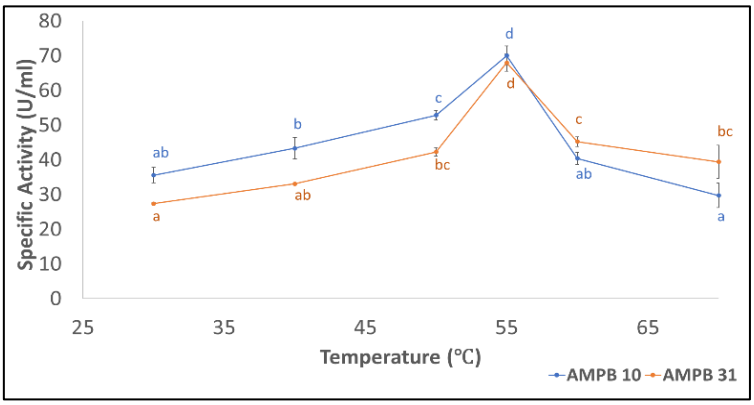

Figure 3. Activity of partially purified amylase measured at various temperatures (30-70 °C).

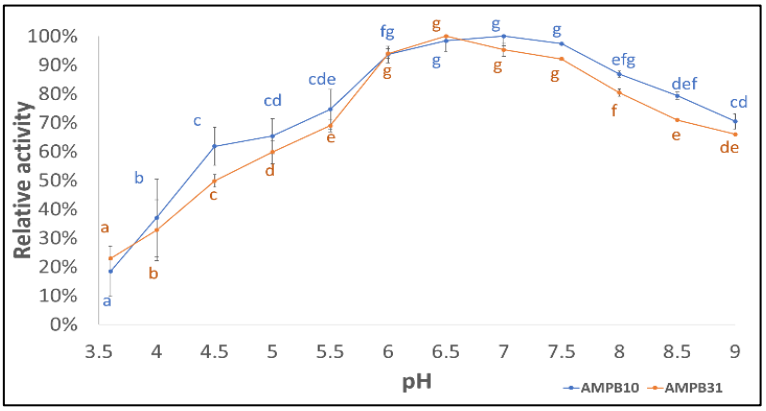

Figure 4. Relative activity of AMPB10 and AMPB31 amylase at different pH (pH3.5-9)

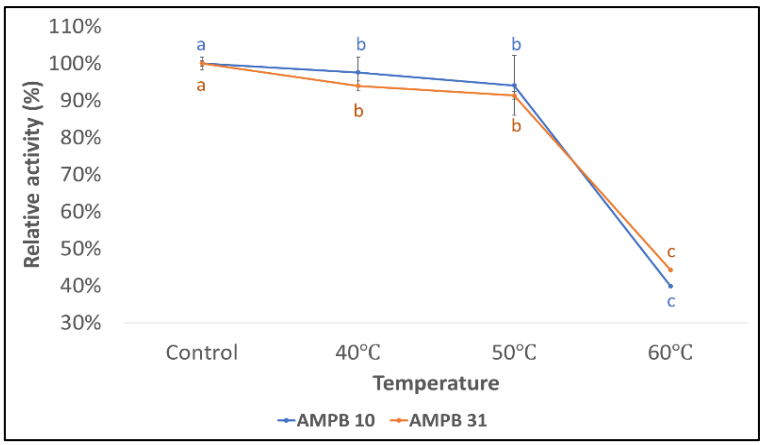

Figure 5. Stability of purified amylase. The activity of amylase extracted from AMPB10 and AMPB31, which was pre-incubated at three different temperatures (40, 50 and 60 °C) followed by amylase activity assay. The control was measured without pre-incubation.

Biofilm degradation by amylase of AMPB10 and AMPB31

Biofilm degradation assay was performed by treating a pre-formed biofilm of test pathogens, *S. aureus* TISTR 1466, *P. aeruginosa* TISTR781 and *S. epidermidis* TISTR 518, with amylase. The biofilms were treated with different concentrations of purified amylase as described in materials and methods. The correlation between amylase concentration and biofilm degrading activity was established (Table 2). The AMPB10 amylase degraded 70.9% of the preformed bacterial biofilm of *S. aureus*, which was the best degradation observed. The effectiveness of AMPB10 against *P. aeruginosa* and *S. epidermidis* was 57.8% and 59.6%, respectively. AMPB31 degraded 66.1%, 60.1% and 64.1% of *S. aureus, P. aeruginosa* and *S. epidermidis* biofilm, respectively. There is no statistically significant difference between the degradation of AMPB10 and AMPB31 against the biofilms formed. However, the biofilm degradation efficacy of AMPB10 against *S. aureus* was approximately 5% higher than that of AMPB31.

Table 2. Reduction of biofilms produced by *S. aureus*, *P. aeruginosa* and *S. epidermidis* as treated with amylase of isolates AMPB10 and AMPB31.

Concentration (ug/mL)	Biofilm reduction (%)					
	S.aureus		*S. epidermidis*		*P. aeruginosa*	
	AMPB10	AMPB13	AMPB10	AMPB13	AMPB10	AMPB13
25	23.15 ± 0.04[a]	19.26 ± 0.00[a]	13.75 ± 0.02[a]	14.77 ± 0.02[a]	10.58 ± 0.02[a]	11.64 ± 0.02[a]
50	39.81 ± 0.03[a]	40.80 ± 0.01[a]	29.00 ± 0.01[a]	28.43 ±0.03[a]	28.21 ± 0.02[a]	32.99 ± 0.01[a]
150	51.48 ± 0.09[a]	50.31 ± 0.10[a]	41.07 ± 0.02[a]	43.99 ± 0.04[a]	50.88 ± 0.01[a]	41.93 ± 0.04[a]
250	63.25 ± 0.02[a]	57.46 ± 0.03[a]	52.65 ± 0.03[a]	55.05 ± 0.01[a]	55.26 ± 0.00[a]	50.47 ± 0.04[a]
500	70.85 ± 0.04[a]	66.06 ± 0.01[a]	59.62 ± 0.03[a]	64.13 ± 0.02[a]	57.81 ± 0.03[a]	60.11 ± 0.01[a]

The experiment was performed in triplicate. No significant differences in biofilm reduction were noted between AMPB10 and AMPB31.

DISCUSSION

This study investigated the bacterial biofilm degradation with amylase produced from soil bacterial strains, AMPB10 and AMPB31. The strains were isolated from agricultural soil collected from Northeast of Thailand. Based on 16S rRNA gene sequence, strains AMPB10 and AMPB31 were classified as *B. tequilensis* and *B. subtilis*, respectively.

Bacillus tequilensis is a Gram-positive, spore-forming bacillus. It was first isolated from a sample of an approximately 2,000-year-old shaft-tomb located in the Mexican state of Jalisco, near the city of Tequila [21]. It has been shown that an amylase enzyme of *B. tequilensis* RG-01 is thermo-tolerant and solvent stable [18]. The amylase of RG-01 is also active at both acidic and alkaline pH (5.0 to 9.0) [20]. According to the 16S rRNA analysis, *B. tequilensis* isolated from the tomb was a closely relative to *B. subtilis* [21]. The phylogenetic relationship between AMPB10 and *B. subtilis* also exhibited a close relation (Figure 1). *Bacillus subtilis* is a rod-shaped, spore forming Gram-positive bacterium. Alpha-amylase from *B. subtilis* is one of the most used amylases and it has important industrial applications [22].

In this study, an amylase enzyme was purified from strains AMPB10 and AMPB31 by ammonium sulfate precipitation. After purification, the total protein concentration of AMPB10 and AMPB31 increased 6.4-fold and 8.5-fold, respectively. When compared to crude enzymes, the specific activity of purified AMPB10 and AMPB31 enzymes increased 13.9-fold and 13.4-fold, respectively. These results indicated that amylase of AMPB10 and AMPB31 were successfully recovered after purification.

It has been shown that enzyme amylase produced from *Bacillus* species ranges from 50-60 kDa except for the amylase extracted from *Bacillus licheniformis* (31 kDa) [16]. The activity staining revealed that enzyme amylase of AMPB10 and AMPB31 was approximately 50 kDa (Figure 2b).

Purified amylase extracted from AMPB10 and AMPB31 exhibited a reduction of biofilm of *S. aureus, P. aeruginosa* and *S. epidermidis*. In all the cases of biofilm degradation, it increased as the concentration of amylase increased. The result of biofilm degradation indicated that *S. aureus* biofilm was much easier to be degraded by AMPB10 and AMPB31 amylase than *P. aeruginosa* and *S. epidermidis* biofilm. It is possible that this is due to structural variations amongst bacterial biofilms. A biofilm of *S. aureus* may be more sensitive to AMPB10 and AMPB31 amylase than those of *P. aeruginosa* and *S. epidermidis*.

The *S. aureus* and *S. epidermidis* are considered to produce a homopolysaccharide biofilm matrix. A major component of *S. aureus* and *S. epidermidis* biofilm matrix is polysaccharide intercellular adhesion (PIA), also called Poly-β-1,6-*N*-acetyl-glucosamine (PNAG) [23]. It has been suggested that *S. epidermidis* biofilm degradation mechanism by amylase enzyme is due to a weakening of physical integrity of PNAG rich biofilm

[4]. Since the composition of biofilm from *S. epidermidis* and *S. aureus* are similar, biofilm of *S. aureus* is assumed to degrade in the same manner with *S. epidermidis*. However, there was a report suggesting that PNAG may not always be the main component of *S. aureus* biofilm matrix. Some strains of *S. aureus* contained a lower amount of PNAG in the biofilm [24]. As a result, the possibility of *S. aureus* and *S. epidermidis* biofilm degradation occurring in a separate manner cannot be ruled out. On the other hand, the biofilm of *P. aeruginosa* was made up of heteropolysaccharide, the matrix with complex polysaccharide structure. Hence it would not be easy to degrade by most amylase [24]. In this study, biofilm degradation of *P. aeruginosa* was more difficult compared to *S. aureus* and *S. epidermidis*. However, AMPB10 and AMPB31 amylases demonstrated sufficient degradation activity to *P. aeruginosa* biofilm approximately by 60% (Figure 6)

Although there have been several studies on biofilm degradation with amylase, no prior studies have examined the biofilm degradation ability of *B. tequilensis* amylase. The amylase of *B. tequilensis* AMPB10 is capable of degrading biofilm of *S. aureus*, *S. epidermidis* and *P. aeruginosa*, with the best activity against *S. aureus*. Thus, this is the first study to employ amylase extracted from *B. tequilensis* to degrade biofilms. In comparison to amylase of *B. subtilis* AMPB31, *B. tequilensis* AMPB10 amylase had better pH tolerance at low and high pH but less heat tolerance. In 2014, Tiwari et.al. mentioned that the enzyme from this species tolerates high temperatures and solvents [20]; therefore, amylase of *B. tequilensis* AMPB10 might be able to apply for biofilm degradation under crucial conditions.

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