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# Diversity and activity of amylase-producing bacteria isolated from mangrove soil in Thailand

**RATIMA KLINFOONG, CHANUNLAYA THUMMAKASORN, SUNISA UNGWIWATKUL, PARIMA BOONTANOM, AIYA CHANTARASIRI♥**

Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok. Rayong Campus, Rayong 21120, Thailand. Tel./Fax.: +66-38-627000 ext.5446, ♥email: aiya.c@sciee.kmutnb.ac.th

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**Abstract.** *Klinfoong R, Thummakasorn C, Ungwiwatkul S, Boontanom P, Chantarasiri A. 2022. Diversity and activity of amylase-producing bacteria isolated from mangrove soil in Thailand. Biodiversitas 23: 5519-5531.* Mangrove forests are a potential ecosystem for the isolation of various economic enzymes derived from mangrove-associated bacteria. The knowledge of amylase-producing bacteria isolated from mangrove forests in the Southeast Asian region has been scarce. This study aimed to investigate the isolation, genetic identification, and activity characterization of amylase-producing bacteria from mangrove soils in Thailand. The amylase-producing bacteria isolated from mangrove soils in the present were genetically belong to the genera *Bacillus*, *Desulfurella*, *Peribacillus*, *Priestia*, and *Pseudomonas*. Several amylase-producing bacteria such as *Bacillus proteolyticus*, *Desulfurella*, *Pseudomonas entomophila*, and *Pseudomonas putida* found in this study have hardly ever been reported. The *Bacillus paralicheniformis* strain DNP0507 was the most active amylolytic bacterium with 2.395 ± 0.133 U/mg of amylase activity. The optimum temperature and pH for amylolytic activity were determined to be 50°C at a pH of 7.0 with a thermal stability range of 20-60°C at a neutral pH of 7.0-8.0. The enzyme activity was significantly enhanced by $Cu^{2+}$, $Co^{2+}$, and $Pb^{2+}$ and was inhibited considerably by a chelating agent EDTA. Finally, the most active amylolytic *B. paralicheniformis* strain DNP0507 could be applied in baking industries, food industries, and starchy waste valorization.

**Keywords:** Amylase, amylolytic activity, *Bacillus paralicheniformis*, mangrove soil, Thailand

## INTRODUCTION

Mangrove forests are the unique coastal intertidal wetland, which are located in subtropical and tropical coastlines dominated by halophilic plants (Mitsch and Gosselink 2015; Friess 2016). The largest proportion of mangrove forests is found in the Southeast Asian region (Friess 2016); furthermore, mangrove forests and the neighboring coastal environments provide a variety of ecological benefits. They serve as a protective line against coastal erosion (Harefa et al. 2022), a habitat for various fauna (Thomas et al. 2017), a pollution trap, and a contaminant degrading area (Chantarasiri 2021a). In addition, the mangrove environment is a detritus-based ecosystem consisting of numerous microorganisms, especially bacteria, which play an important function in the mineralization of organic matter and recycling of nutrients (Saravanakumar et al. 2016). Therefore, they are considered as a productive ecosystem and a potential source of effective bacteria that produce active enzymes (Pramono et al. 2021). Moreover, their enzymes play a key role in microbial adaptation and evolution associated with different habitats and environments (Alves et al. 2014). Various enzymes produced from the mangrove bacterial species have also been harvested and characterized, such as laccases, lipases, proteases, and polysaccharides. The polysaccharides have gained global attention due to the novel industrial applications (Trivedi et al. 2016). The main enzymes reported include agarases, alginate lyases, carrageenases, cellulases, chitinases, chitosanases, fucoidanases, glucosidases, hemicellulases, pullulanases, and amylases (Saravanakumar et al. 2016; Trivedi et al. 2016).

Amylases are a group of hydrolytic enzymes which hydrolyze the glycosidic linkages of starch and related polysaccharide molecules to produce simpler sugar molecules, such as glucose, maltose, and dextrin (My et al. 2022; Silaban et al. 2020). They are classified into three types comprising α-amylase (1,4-α-D-glucan glucano-hydrolase or glycogenase), β-amylase (1,4-α-D-glucan maltohydrolase, glycogenase, or saccharogen amylase), and γ-amylase (glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase, or 1,4-α-D-glucan glucohydrolase) based on the different mechanisms of the glycosidic linkage hydrolysis (Benjamin et al. 2013; Joshi et al. 2021). Amylases are the enzymes that present industrial interest (Dawood et al. 2015), and the global market of amylases has been estimated to be 320.1 million USD by 2024 (Ashraf et al. 2018). They account for 25-33% of worldwide enzyme production and are widely applied in industrial processes, such as alcohol (from starch), baking, dextrin gum preparation, detergents, distilled beverages, glucose syrup production, and textiles (Alves et al. 2020; Kafilzadeh and Dehdari 2015; Kafilzadeh et al. 2012; Saravanakumar et al. 2016; Sharma and Satyanarayana

2013). Amylases can be produced by a variety of organisms. Microbial amylases are the most common and widely preferred in industrial applications than that of other sources because of their advantages, such as cost-effectiveness, productivity, thermostability, and simple optimization process (Ashwini et al. 2011; Joshi et al. 2021; My et al. 2022). In addition, microbial amylases have successfully replaced the chemical hydrolysis of starch-processing applications (Alves et al. 2014).

Bacteria are the preferred microbe of the enzyme production because they are easy to isolate and have extracellular production properties within a short time (Bhatt et al. 2020). Moreover, bacteria tend to secrete amylases outside the cells to perform extracellular digestion of starch into sugars (Luang-In et al. 2019). To date, many bacteria have been isolated and documented as amylase-producing bacteria, including genera *Arthrobacter*, *Escherichia*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Serratia* and *Streptomyces* (Kafilzadeh et al. 2012; Shafiei et al. 2011). Many bacteria in the genus *Bacillu*s are also capable of producing a high amount of amylase (Gopinath et al. 2017; Joshi et al. 2021; Praney et al. 2019). Some *Bacillus* and *Vibrio* bacteria were isolated from mangrove soil samples in Brazilian mangrove sediment and reported as amylase-producing bacteria (Dias et al. 2009). To improve the knowledge of amylase-producing bacteria and their enzymatic activity, more studies have been conducted.

Little is known about amylase-producing bacteria isolated from the hotspot of mangrove forests of the Southeast Asian region. Therefore, the isolation, identification, and enzymatic characterization of amylase-producing bacteria from mangrove forests in this region would be challenging. Therefore, this study aimed to isolate and screen the amylase-producing bacteria from the mangrove soil in Thailand by using the culture-dependent method. This is a classical method to obtain novel and effective bacteria from environmental samples. All amylase-producing bacteria were genetically identified by the nucleotide analysis of the 16S rRNA gene and phylogenetic tree. Amylases produced from the most active amylolytic bacterium in this study, the *Bacillus paralicheniformis* strain DNP0507, was enzymatically characterized for further biotechnological applications. Finally, the *B. paralicheniformis* strain DNP0507 was applied to the biological degradation on a starchy waste. The results suggested that this amylase-producing bacterium could be considered as a promising microbe in various biotechnological applications.

## MATERIALS AND METHODS

### Study area

The study area was the Phra Chedi Klang Nam Mangrove Forest in Rayong Province, which is located on the east region of Thailand (12° 39′ 58″ N, 101° 14′ 30″ E). This mangrove forest is located on the Rayong River Estuary that is a major urban area with a dense human population (Chantarasiri 2021a). It is mostly overgrown by halophilic plants, including *Avicennia alba* Blume., *Bruguiera gymnorrhiza* (L.) Lamk., and *Rhizophora apiculata* Blume (Chantarasiri 2021a). This area was demonstrated as the identical area in the previous studies of Chantarasiri (2015; 2021a; 2021b) which reported the isolation, genetic identification, and enzymatic characterization of many cellulolytic, phenanthrene-degrading, and ligninolytic bacteria, respectively. Therefore, this mangrove forest was considered as a biodiversity hotspot for a bacterial isolation study in the east region of Thailand. The location of the Phra Chedi Klang Nam Mangrove Forest is shown on an aerial photograph map in Chantarasiri (2015).

### Procedures

*Sampling of the mangrove soil samples*

The Phra Chedi Klang Nam Mangrove Forest plays a significant role as a natural waste trap of Rayong Province. The mangrove soil receives the municipal contaminants and contains household starchy substrates. The mangrove soil samples were collected from the study area during the late rainy season in September 2017. Thirty samples were randomly collected from a depth of 0-5 cm to obtain primary aerobic bacteria. All samples were kept in sterilized plastic containers at a temperature of 4°C and taken to the laboratory for bacterial isolation within 24 h of collection. The soil temperatures were measured *in situ* by a needle probe thermometer (Extech Instruments, USA). The soil pH values were determined from 1 g of soil samples suspended in 10 mL of neutral distilled water using a digital pH meter (Ohaus, USA).

*Isolation, colony purification, and screening of the amylase-producing bacteria from the mangrove soil samples*

One gram of each mangrove soil sample was suspended in 10 mL of 0.85% (w/v) sterilized NaCl solution (Sigma-Aldrich, Germany). The suspensions were serially diluted with 0.85% (w/v) of sterilized NaCl solution to obtain 1: 100,000 dilutions and spread plated on tryptone soya agar (HiMedia, India) at a pH of 6.26 (the average pH of the collected samples). All plates were incubated in a KB720 incubator (Binder, Germany) at a temperature of 30.01°C (the average soil temperature) for 24 h. The isolated bacteria were selected based on their colony's morphological dissimilarities and streak plated on tryptone soya agar to obtain a pure isolate. Finally, each pure isolate was named for the isolation numbers.

The screening of the amylase-producing bacteria was conducted from Kafilzadeh and Dehdari (2015) with minor modifications using a starch agar and iodine solution staining method. The starch agar contained 0.6 g of bacteriological peptone (HiMedia, India), 0.05 g of $MgSO_4.7H_2O$ (Ajax Finechem, Australia), 0.05 g of KCl (Ajax Finechem, Australia), 2.5 g of soluble starch (HiMedia, India), 1.5 g of agar powder (HiMedia, India), and 0.05 mg of nystatin (Alfa Aesar, UK) in 100 mL of distilled water. The pH value of the starch agar was adjusted to 6.26. Nystatin was added to the medium as the fungicidal for inhibiting any growth of fungi and yeasts. Five microliters of overnight growth culture in the tryptone soya broth (HiMedia, India) of each bacterial isolate was

spotted on the starch agar plates. All spotted plates were incubated at a temperature of 30.01°C for 24 h in a KB720 incubator and then flood-plated with an iodine solution for 10 min. The iodine solution contained 0.33 g of $I_2$ (Ajax Finechem, Australia) and 0.67 g of KI (Ajax Finechem, Australia) in 100 mL of distilled water (Chantarasiri 2021c). The amylase-producing isolates were detected by the amylolytic zone around their colonies on the starch agar after being stained with iodine solution. The hydrolysis capacity (HC) value that determined the amylolytic performance of each bacterial isolate was calculated by the ratio between the diameter of the amylolytic zone and the diameter of the bacterial colony. All experiments were performed in triplicate.

*Genetic identification and phylogenetic analysis of the amylase-producing bacteria*

The isolated amylase-producing bacteria were genetically identified by the nucleotide analysis of the 16S rRNA gene. The genomic DNA of each isolated bacteria was extracted by a genomic DNA isolation kit (Bio-Helix, Taiwan), following the standard protocol proposed by Bio-Helix. The 16S rRNA genes from the extracted genomic DNA were amplified by a polymerase chain reaction (PCR) using the OnePCR reaction mixture (Bio-Helix, Taiwan) with a universal 27F primer of 5′-AGAGTTTGATCMTGGCTCAG-3′ and a universal 1492R primer of 5′-TACGGYTACCTTGTTACGACTT-3′ (Macrogen Inc., South Korea). The PCR conditions were conducted according to Ferbiyanto et al. (2015) and Boontanom and Chantarasiri (2020) for 35 amplification cycles in a Mastercycler Nexus Gradient thermal cycler (Eppendorf, Germany). The PCR conditions were initialized by a preheating step at a temperature of 94°C for 4 min. The amplification cycles were followed by a denaturation step at a temperature of 94°C for 40 sec, an annealing step at a temperature of 55°C for 60 sec, and an extension step at a temperature of 72°C for 1 min 10 sec. The final extension step of the PCR conditions was performed at a temperature of 72°C for 10 min. All PCR products were held at a temperature of 4°C till further analysis. The approximately 1,500-bp PCR products were electrophoresed on 1.0% (w/v) of OmniPur agarose gel (Merck, Germany) and visualized by the Novel Juice DNA staining reagent (Bio-Helix, Taiwan). The PCR products were nucleotide sequenced using the services of Macrogen Inc. (South Korea). Nucleotide sequence similarity analysis of the PCR products was aligned by the BLASTn program of the National Center for Biotechnology Information (NCBI). The phylogenetic tree was generated by the distance method with the BioNJ algorithm and phylogenetic bootstrapping of 100,000 replications using the SeaView program version 4.6.4 (Gouy et al. 2010). The tree was annotated by the FigTree program version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

All nucleotide sequences of the 16S rRNA genes obtained from this study were deposited in the GenBank database of NCBI under the accession numbers of OP136071, OP136074, OP136077, OP136083, OP136136, OP136147, OP136148, OP136153, OP136154, OP136970, and OP137143.

*Preparation of the partially purified amylases*

The isolated amylase-producing bacteria were initially cultured in 3 mL of tryptone soya broth at a pH of 6.26 and incubated at a temperature of 30.01°C for 16 h in a KB720 incubator. Subsequently, 1% (v/v) of each bacterial culture was inoculated in 100 mL of starch broth at a pH of 6.26 and incubated at a temperature of 30.01°C for 24 h with shaking at 150 rpm in a LSI-3016R orbital shaking incubator (Daihan Labtech, South Korea). The starch broth contained 0.6 g of bacteriological peptone (HiMedia, India), 0.05 g of $MgSO_4.7H_2O$ (Ajax Finechem, Australia), 0.05 g of KCl (Ajax Finechem, Australia), and 2.5 g of soluble starch (HiMedia, India) in 100 mL of distilled water. The cultured broths were centrifuged by Digicen 21 R centrifugation (Ortoalresa, Spain) at 3,000× g at a temperature of 4°C for 20 min to obtain cell-free supernatants.

Amylases were separated by following the method described by Wu et al. (2018) with modifications. The collected supernatants were purified by ammonium sulfate precipitation at 30-80% saturation. The enzyme pellet was collected by Digicen 21 R centrifugation at 12,000× g at a temperature of 4°C for 20 min and desalted by 30-kDa Amicon ultra centrifugal filter units (Millipore, Ireland). Partially purified amylases were kept in 20 mM of sodium phosphate buffer (pH of 7.0) at a temperature of 4°C in Protein LoBind tubes (Eppendorf, Germany) until use.

*Determination of the amylolytic activity of the partially purified amylases*

The amylolytic activity of the partially purified amylases was determined as the methods described by Mishra and Behera (2008) with minor modifications. The activity was assayed by incubating 0.1 mL of partially purified amylases with 1.0% (w/v) of soluble strach as a substrate in 1.0 mL of 20 mM of sodium phosphate buffer (pH of 7.0) at a temperature of 50°C for 10 min. The enzyme activity was spectrophotometrically assayed using the DNS method by determinating the amount of glucose released from the amylolytic reaction at 540 nm (Miller 1959). The enzyme activity values were calculated by a glucose standard curve. The enzyme content was determined by the Bradford method (Bradford 1976) using Quick Start Bradford Protein Assay (BIO-RAD, USA). One unit (U) of amylolytic activity was defined as the amount of enzyme that liberated 1 μmol of glucose per minute under the assay conditions (Kizhakedathil and Chandrasekaran 2018). The amylolytic activity was quoted in unit per milligram (U/mg). All experiments were performed in triplicate.

*Enzymatic characterization of the amylases from the most active amylolytic bacterium*

Enzymatic characterization was determined on the partially purified amylases produced from the most active amylolytic bacterium, *B. paralicheniformis* strain DNP0507. The study focused on three parameters that

affected the amylolytic activity consisting of temperature, pH, and certain chemical additives. The amylolytic activity was measured accordingly, as mentioned in the previous experiments. All experiments were performed in triplicate.

The optimum temperature of the amylolytic activity was determined at temperatures ranging from 20°C to 70°C in 20 mM of sodium phosphate buffer at a pH of 7.0. Thermal stability was measured by pre-incubating the partially purified amylases at a temperature ranging from 20°C to 70°C for 1 h. The relative activity of the amylases was monitored afterwards.

The effect of the pH on the amylolytic activity and pH stability was performed in the pH-varied buffers, including 20 mM of citrate buffer (pH of 4.0-6.0), 20 mM of sodium phosphate buffer (pH of 6.0-8.0), and 20 mM of glycine-NaOH buffer (pH of 8.0-11.0). The amylolytic activity was determined in the buffers at a temperature of 50°C. The pH stability was measured by pre-incubating the enzyme in the abovementioned buffer at a temperature of 50°C for 1 h. The relative activity of the amylases was monitored afterwards.

The effect of some chemical additives was determined accordingly as mentioned by Wu et al. (2018). Partially purified amylases were pre-incubated in 20 mM of sodium phosphate buffer at a pH of 7.0 supplemented with chemical additives at a temperature of 50°C for 1 h. The residual amylolytic activity was monitored after being incubated with various additives. There were 11 metal ions used in this experiment comprising $Ba^{2+}$ (as $BaCl_2$), $Ca^{2+}$ (as $CaCl_2$), $Co^{2+}$ (as $CoCl_2$), $Cu^{2+}$ (as $CuCl_2$), $Fe^{2+}$ (as $FeCl_2$), $K^+$ (as KCl), $Mg^{2+}$ (as $MgCl_2$), $Mn^{2+}$ (as $MnCl_2$), $Na^+$ (as $NaCl_2$), $Pb^{2+}$ (as $PbCl_2$), and $Zn^{2+}$ (as $ZnCl_2$) (Ajax Finechem, Australia). The effects of the surfactant agent and chelating agent on the amylolytic activity were investigated using sodium dodecyl sulfate (SDS) (Ajax Finechem, Australia) and ethylene diamine tetraacetic acid (EDTA) disodium salt (Calbiochem, Germany). All chemical additives were supplemented with a final concentration of 5 mM.

*Application of the amylases from* Bacillus paralicheniformis *strain DNP0507 on the hydrolysis of starchy waste*

Starchy wastes affect many ecosystems and can cause serious environmental problems. Therefore, it would be crucial to convert these wastes economically into valuable products. Fried starch residues from fried chicken processes were considered as the starchy waste in this experiment. The fried starch residues were kindly granted from a food factory in Ayutthaya Province, Thailand. The hydrolysis reaction was performed by incubating 1.0 U/mg of partially purified amylases from the *B. paralicheniformis* strain DNP0507 with 1.0% (w/v) of fried starch residues in 1.0 mL of 20 mM of sodium phosphate buffer (pH of 7.0) supplemented with 5 mM of $Cu^{2+}$ (as $CuCl_2$) at a temperature of 50°C for 10 min. The amount of glucose released from the amylolytic reaction was spectrophotometrically assayed using the DNS method as abovementioned. The control was the hydrolysis reaction of commercial amylases by incubating 1.0 U/mg of iKnowZyme HTAA (ReachBiotechnology, Thailand) with 1.0% (w/v) of fried starch residues under similar experimental conditions. All experiments were performed in triplicate.

**Data analysis**

The results obtained in this study are shown as a mean ± standard deviation (S.D.) value. Data analysis was statistically investigated by one-way ANOVA multiple comparison analysis followed by Tukey's test with a 95% confidence interval using R software version 4.2.1 (R Foundation for Statistical Computing, Austria).

## RESULTS AND DISCUSSION

**Description of the collected mangrove soil samples**

Thirty mangrove soil samples were collected from the Phra Chedi Klang Nam Mangrove Forest. The texture collected soil samples was sandy and muddy clays according to the previous study of Chantarasiri (2021b). The temperature measured *in situ* by a needle probe thermometer ranged from 28.9°C to 32.0°C, and the average temperature was 30.01 ± 1.61°C. The pH of the collected soil sample ranged from 5.08 to 7.05, and the average pH of the collected soil samples was 6.26 ± 0.46. The average temperature and pH values were used as the growth conditions for the following bacterial isolation and cultivation procedures in this study.

**Isolation, colony purification, and screening of amylase-producing bacteria from the mangrove soil samples**

A total of 123 bacterial isolates were isolated from the mangrove soil samples and subsequently colony purified using tryptone soya agar. Finally, all purified bacteria were named for the isolation numbers. A starch agar and iodine solution staining method was used to evaluate the primary enzymatic activity of the active amylolytic bacteria. The results showed 11 isolates that were defined as axenic isolates of amylase-producing bacteria. Their HC values ranged from 1.44 ± 0.03 to 3.79 ± 0.05. The amylase-producing bacterium isolate DNP2804 had a significant maximum HC of 3.79 ± 0.05 ($p < 0.05$). The isolates DNP0803 and DNP2704 had the secondary ahe tertiary HC values of 3.74 ± 0.10 and 3.66 ± 0.10, respectively. The amylolytic zone around the bacterial colonies on the starch agar plates after being stained with iodine solution is shown in Figure 1. The HC values and colony morphology of all the isolated amylase-producing bacteria are shown in Table 1.

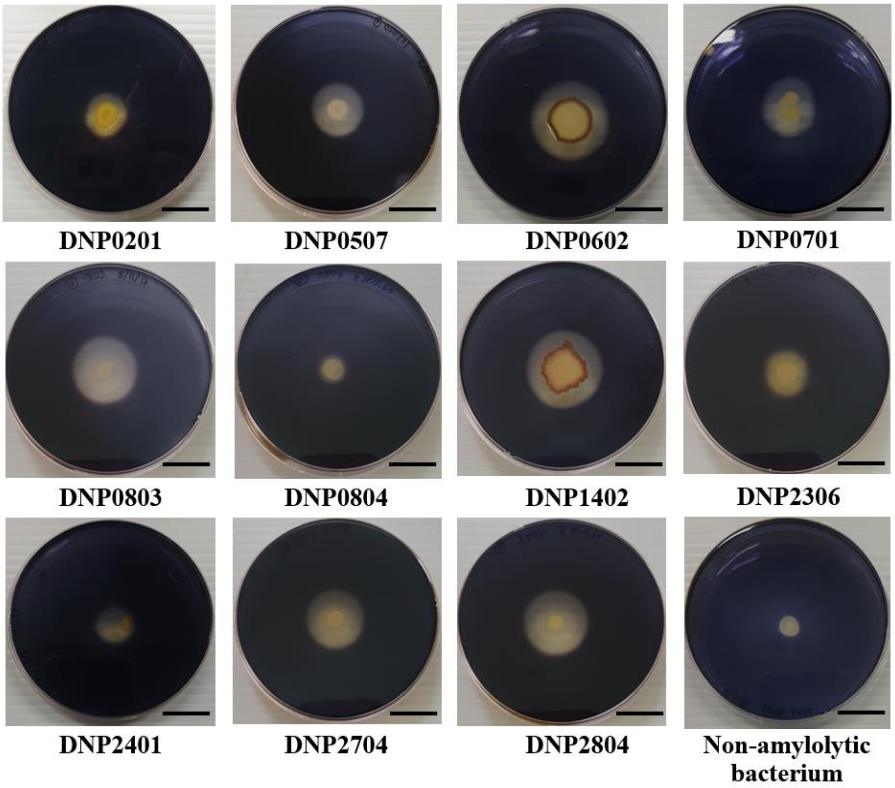


**Figure 1.** The amylolytic zone around the bacterial colonies of the amylase-producing bacteria on the starch agar plates after being stained with iodine solution. The negative control was non-amylolytic bacterium inoculated on the starch agar. The bar represents two centimeters

**Table 1.** Colony morphology and HC values of the 11 isolated amylolytic bacteria from the mangrove soil samples

| Bacterial isolate | Pigmentation | Shape | Margin | Elevation | HC value |
|---|---|---|---|---|---|
| DNP0201 | Opaque cream | Circular | Entire | Flat | $1.81 \pm 0.01^{bc}$ |
| DNP0507 | Opaque white | Circular | Undulate | Umbonate | $2.25 \pm 0.21^{d}$ |
| DNP0602 | Opaque white | Circular | Undulate | Flat | $1.87 \pm 0.09^{c}$ |
| DNP0701 | Opaque white | Circular | Entire | Raised | $1.67 \pm 0.02^{b}$ |
| DNP0803 | Opaque white | Circular | Entire | Raised | $3.74 \pm 0.10^{f}$ |
| DNP0804 | Opaque pale yellow | Circular | Entire | Convex | $1.44 \pm 0.03^{a}$ |
| DNP1402 | Opaque white | Circular | Undulate | Raised | $1.65 \pm 0.02^{b}$ |
| DNP2306 | Opaque white | Circular | Entire | Convex | $2.55 \pm 0.10^{e}$ |
| DNP2401 | Opaque pale yellow | Circular | Entire | Raised | $1.65 \pm 0.09^{bc}$ |
| DNP2704 | Opaque cream | Circular | Entire | Flat | $3.66 \pm 0.10^{f}$ |
| DNP2804 | Opaque cream | Circular | Entire | Flat | $3.79 \pm 0.05^{f}$ |

Note: The mean values of the HC value followed by the same letter were not significantly different according to the one-way ANOVA with Tukey's test ($p < 0.05$) analyzed by R software version 4.2.1. The experiments were performed in triplicate

**Genetic identification and phylogenetic analysis of amylase-producing bacteria**

The amylase-producing bacteria were genetically identified by the PCR amplification and analysis of their 16S rRNA gene sequences. Nucleotide alignment of the 16S rRNA genes using the BLASTn program of the NCBI revealed that the 11 amylase-producing bacteria belonged to five genera comprising genus *Bacillus* of the Phylum *Bacillota* (*Firmicutes*), genus *Desulfurella* of the Phylum *Campylobacterota*, genus *Peribacillus* of the Phylum *Bacillota*, genus *Priestia* of the Phylum *Bacillota*, and genus *Pseudomonas* of the Phylum *Pseudomonadota* (*Proteobacteria*) with 97-99% identity. All resulting E values of the BLASTn alignments were zero. The five isolates of amylase-producing bacteria (DNP0507, DNP0701, DNP0803, DNP1402, and DNP2306) were closely similar to various species of *Bacillus* involving *Bacillus* sp., *B. paralicheniformis*, *B. proteolyticus*, and *B. tequilensis*. Three bacterial isolates (DNP0201, DNP2704, and DNP2804) were closely similar to *Pseudomonas* species, including *Pseudomonas* sp., *P. entomophila*, and *P. putida*. The three others (DNP0602, DNP0804, and

DNP2401) were closely similar to *Desulfurella propionica*, *Peribacillus simplex*, and *Priestia flexa*. The results of the nucleotide alignment of the 16S rRNA genes are shown in Table 2.

The phylogenetic tree of the amylase-producing bacteria was analyzed and generated by the distance method with BioNJ algorithm and phylogenetic bootstrapping of 100,000 replications. The result showed that the 11 amylase-producing bacteria and other closely related bacteria were phylogenetically clustered into eight clades. The bacterial isolates DNP0507, DNP0803, DNP2306, DNP0701, and DNP1402 fell into the *Bacillus* clades (clades I, II, V, and VI) with bootstrap values ranging from 78 to 100. The isolates DNP2401 and DNP0804 were grouped in the *Priestia* clade (clade III) and the *Bacillus* and *Peribacillus* clade (clade IV) with the bootstrap values of 100 and 94, respectively. These clades shared a common ancestor with other bacteria of the Phylum *Bacillota*. The other clades of this phylogenetic tree were *Pseudomonas* clade of Phylum *Pseudomonadota* (clade VII) and *Desulfurella* clade of the Phylum *Campylobacterota* (clade VIII). The bacterial isolates DNP0201, DNP2704, and DNP 2804 fell into the *Pseudomonas* clade with bootstrap values ranging from 76 to 100. Lastly, the bacterial isolate DNP0602 was clustered in the *Desulfurella* clade with a bootstrap value of 50, which was considered as a unique clade of this phylogenetic tree. A circular phylogenetic tree is shown in Figure 2.

In this study, the amylase-producing bacteria were found to be closely related based on the sequence alignment results and bootstrap values of the 16S rRNA gene when the identity was more than 97%, and the bootstrap value was more than 75%, such as *B. paralicheniformis* strain DNP0507. The ones which were lower than a 97% identity or a 75% bootstrap value were presented at the genus level, such as *Desulfurella* sp. strain DNP0602. The genetic identification of the 11 amylase-producing bacteria is shown in Table 3. Therefore, the predominant amylase-producing bacteria in this study were the group of *Bacillus*.

All nucleotide sequences of 16S rRNA gene obtained from this study were deposited in the GenBank database of NCBI under the accession numbers OP136071, OP136074, OP136077, OP136083, OP136136, OP136147, OP136148, OP136153, OP136154, OP136970, and OP137143. All amylase-producing bacteria were stored as frozen stocks in 20% (v/v) of glycerol and kept at the King Mongkut's University of Technology North Bangkok (KMUTNB), Thailand.

**Table 2.** Identity percentages of 16S rRNA gene sequences of the 11 amylase-producing bacteria with closely related bacteria

| Bacterial isolate | Closely related bacteria | GenBank accession No. (database) | Query cover (%) | Identity (%) | E value |
|---|---|---|---|---|---|
| DNP0201 | *Pseudomonas* sp. strain Ammsd-1 | LC208794.1 | 100 | 97.67 | 0.0 |
| DNP0507 | *Bacillus paralicheniformis* strain P5 | MH488999.1 | 99 | 98.39 | 0.0 |
| DNP0602 | *Desulfurella propionica* strain U-8 | NR_026460.1 | 99 | 97.23 | 0.0 |
| DNP0701 | *Bacillus* sp. strain PTP1 | KY910137.1 | 98 | 98.05 | 0.0 |
| DNP0803 | *Bacillus tequilensis* strain JAAKPT | MN049471.1 | 100 | 97.10 | 0.0 |
| DNP0804 | *Peribacillus simplex* strain YNC-4 | MF977326.1 | 100 | 98.44 | 0.0 |
| DNP1402 | *Bacillus* sp. strain PTP1 | KY910137.1 | 98 | 98.11 | 0.0 |
| DNP2306 | *Bacillus proteolyticus* strain SUF05.1LB | MT052664.1 | 99 | 99.67 | 0.0 |
| DNP2401 | *Priestia flexa* strain SBMP3 | NR_118382.1 | 100 | 98.87 | 0.0 |
| DNP2704 | *Pseudomonas entomophila* strain TL9 | MN493076.1 | 100 | 98.22 | 0.0 |
| DNP2804 | *Pseudomonas putida* strain ATCC 12633 | NR_114479.1 | 100 | 98.50 | 0.0 |

Note: The identity results were analyzed on June 30, 2022.

**Table 3.** Genetic identification of the 11 amylase-producing bacteria and their GenBank accession numbers

| Bacterial isolate | Identified species | GenBank accession no. (deposited) |
|---|---|---|
| DNP0201 | *Pseudomonas* sp. strain DNP0201 | OP136071 |
| DNP0507 | *Bacillus paralicheniformis* strain DNP0507 | OP136074 |
| DNP0602 | *Desulfurella* sp. strain DNP0602 | OP136077 |
| DNP0701 | *Bacillus* sp. strain DNP0701 | OP136970 |
| DNP0803 | *Bacillus tequilensis* strain DNP0803 | OP136083 |
| DNP0804 | *Peribacillus simplex* strain DNP0804 | OP137143 |
| DNP1402 | *Bacillus* sp. strain DNP1402 | OP136136 |
| DNP2306 | *Bacillus proteolyticus* strain DNP2306 | OP136147 |
| DNP2401 | *Priestia flexa* strain DNP2401 | OP136148 |
| DNP2704 | *Pseudomonas entomophila* strain DNP2704 | OP136154 |
| DNP2804 | *Pseudomonas putida* strain DNP2804 | OP136153 |

Note: The nucleotide sequences of the 16S rRNA gene were deposited in the GenBank database of NCBI on August 3, 2022.

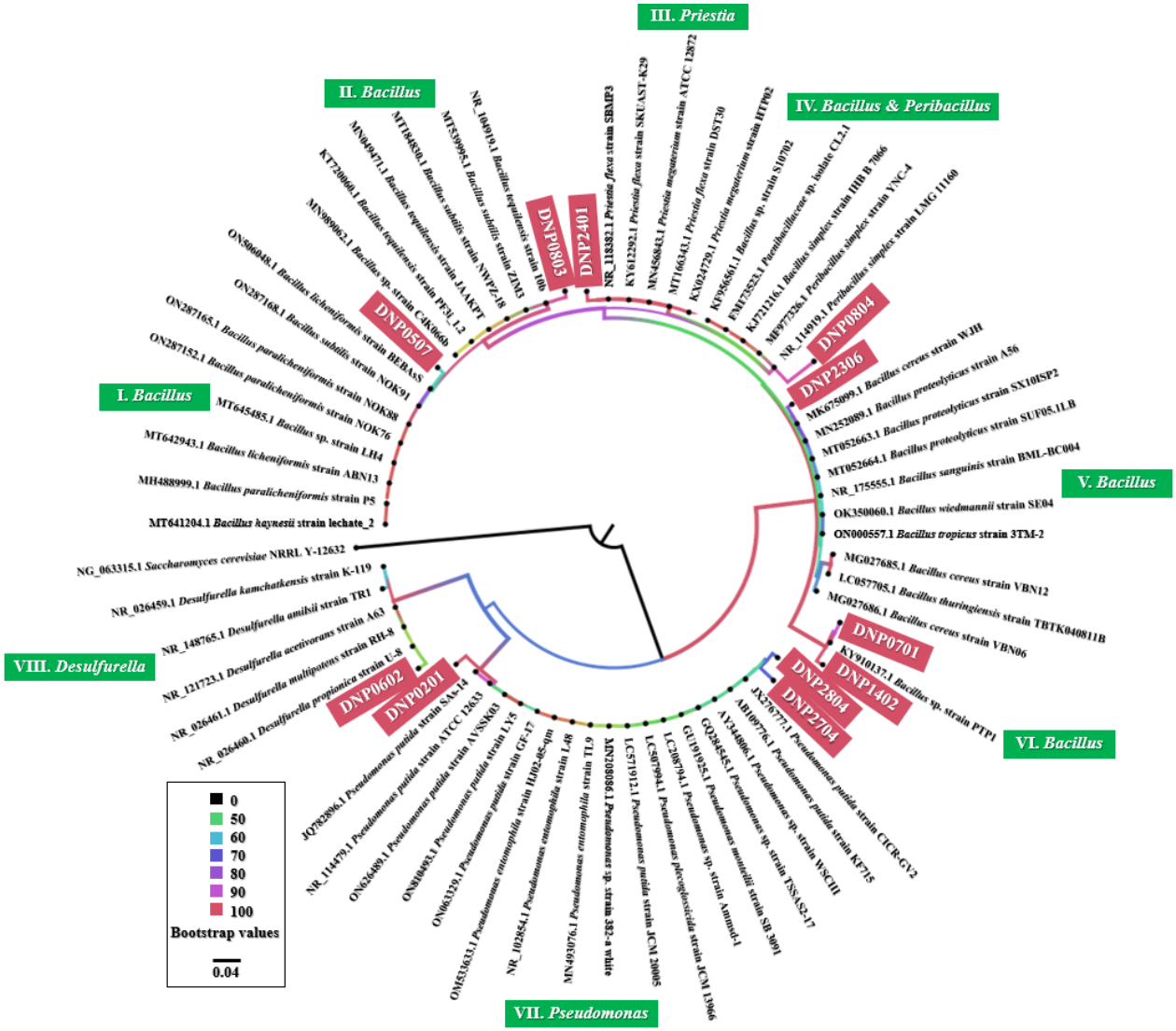


**Figure 2.** The circular phylogenetic tree of the amylase-producing bacteria is shown using the distance method with the BioNJ algorithm with 100,000 bootstrap replications. The phylogenetic tree was generated by SeaView software version 4.6.4 and visualized by FigTree software version 1.4.4

**Table 4.** Amylolytic activity of the 11 amylase-producing bacteria

| Amylase-producing bacteria | Amylolytic activity (U/mg) |
|---|---|
| *Pseudomonas* sp. strain DNP0201 | 1.009 ± 0.103 $^{de}$ |
| *B. paralicheniformis* strain DNP0507 | 2.395 ± 0.133 $^{f}$ |
| *Desulfurella* sp. strain DNP0602 | 0.560 ± 0.056 $^{c}$ |
| *Bacillus* sp. strain DNP0701 | 0.552 ± 0.077 $^{c}$ |
| *B. tequilensis* strain DNP0803 | 0.817 ± 0.056 $^{d}$ |
| *P. simplex* strain DNP0804 | 1.084 ± 0.181 $^{e}$ |
| *Bacillus* sp. strain DNP1402 | 0.063 ± 0.008 $^{a}$ |
| *B. proteolyticus* strain DNP2306 | 0.476 ± 0.032 $^{bc}$ |
| *P. flexa* strain DNP2401 | 1.026 ± 0.088 $^{de}$ |
| *P. entomophila* strain DNP2704 | 0.121 ± 0.008 $^{a}$ |
| *P. putida* strain DNP2804 | 0.249 ± 0.019 $^{ab}$ |

Note: The mean values of the amylolytic activity followed by the same letter were not significantly different according to the one-way ANOVA with Tukey's test ($p < 0.05$) analyzed by R software version 4.2.1. The experiments were performed in triplicate

**Amylolytic activity of the partially purified amylases**

The amylase-producing bacteria were examined for the amylolytic activity assay. The crude amylases were harvested, partially purified, and used for the examination of the amylolytic activity. The amylolytic activity assays showed that they could produce partially purified amylases with 0.063 ± 0.008 U/mg to 2.395 ± 0.133 U/mg. The amylase-producing bacteria of the Phylum *Bacillota* satisfactorily produced amylolytic activity, such as *B. paralicheniformis* strain DNP0507, *P. simplex* strain DNP0804, and *P. flexa* strain DNP2401. The bacterium *B. paralicheniformis* strain DNP0507 was considered the most active amylolytic bacterium with significant amylolytic activity among the 11 bacterial strains ($p<0.05$). This significantly produced a high activity of amylases by 2.395 ± 0.133 U/mg. Therefore, it was selected for the subsequent experiments. Their amylolytic activities are shown in Table 4. Interestingly, the *B. paralicheniformis* strain DNP0507 was not the most active amylolytic bacterium based on the

HC value determination on the starch agar (Figure 1 and Table 1).

**Enzymatic characterization of the amylases from the *Bacillus paralicheniformis* strain DNP0507**

The partially purifeid amylases from the *B. paralicheniformis* strain DNP0507 were characterized for the amylolytic activity under different conditions, such as temperatures, pH values, and chemical additives. The optimum temperature and pH for the amylolytic activity are shown in Figures 3A and 4A, respectively. The optimum temperature and pH for the amylolytic activity were a moderate temperature of 50°C ($p$<0.05) with a pH of 7.0 of 20 mM of sodium phosphate buffer ($p$<0.05). The buffer types did not significantly affect the amylolytic activity at the same pH value as shown in Figure 4A.

The enzyme activity remained stable up to a temperature of 60°C ($p$<0.05), while the activity was decreased to 53% of the relative amylolytic activity after incubation at a temperature of 70°C for 1 h in 20 mM of sodium phosphate buffer at a pH of 7.0. The enzyme was stable by above 97% of the relative amylolytic activity ($p$<0.05) at a neutral pH ranging from 7.0 to 8.0 after incubation for 1 h in 20 mM of sodium phosphate buffer. The temperature and pH for the enzyme stability are shown in Figures 3B and 4B, respectively. The buffer types significantly affected the stability of the enzyme at a pH of 8.0 as shown in Figure 4B.

The effects of the various chemical additives on the amylolytic activity from the amylases of *B. paralicheniformis* strain DNP0507 are shown in Table 5. The amylase enzyme was significantly enhanced by $Cu^{2+}$, $Co^{2+}$, and $Pb^{2+}$ at a final concentration of 5 mM with 112.34%, 109.82%, and 107.17% of the residual amylolytic activity, respectively ($p$<0.05). The surfactant agent, SDS, did not significantly inhibit the amylolytic activity of the enzyme under the experimental conditions. However, the metal ion chelating agent, EDTA, could significantly inhibit the amylolytic activity with 65.55% of residual activity remaining ($p$<0.05).

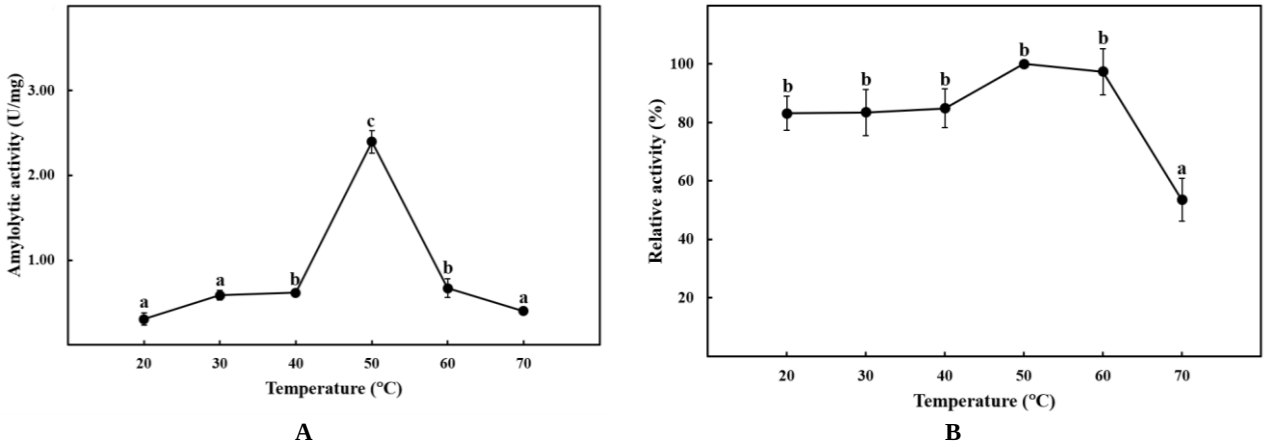


**Figure 3.** Effect of the temperature on the amylolytic activity (A) and stability (B) from the amylases of the *Bacillus paralicheniformis* strain DNP0507. The error bars represent the standard deviation of the triplicate. The mean values followed by the same letter were not significantly different according to Tukey's test ($p$ < 0.05) that were analyzed by R software version 4.2.1. The experiments were performed in triplicate

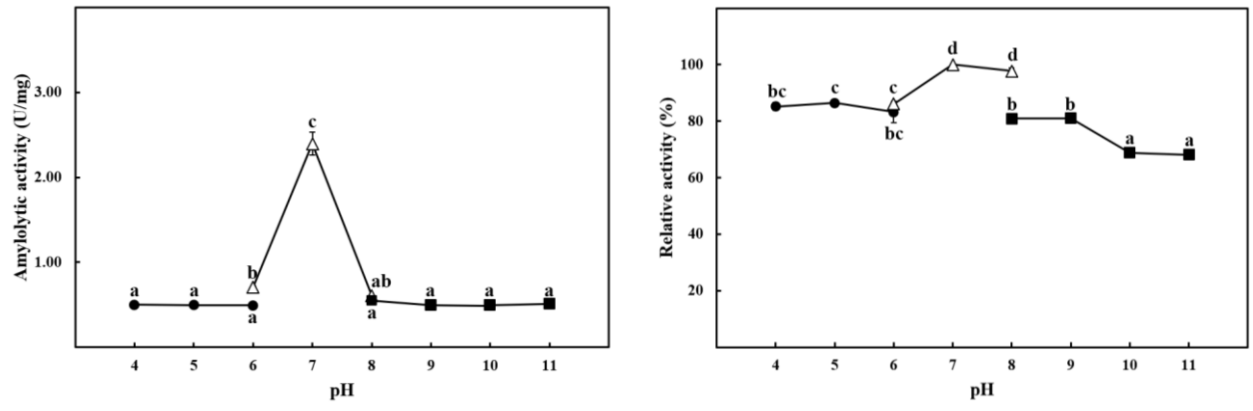


**Figure 4.** Effect of pH on the (A) amylolytic activity and (B) stability from the amylases of the *Bacillus paralicheniformis* strain DNP0507. The activity of the enzyme was determined in a citrate buffer (●), sodium phosphate buffer (Δ), and glycine-NaOH buffer (■). The error bars represent the standard deviation of the triplicate. The mean values followed by the same letter were not significantly different according to Tukey's test ($p$ < 0.05) that were analyzed by R software version 4.2.1. The experiments were performed in triplicate

**Table 5.** Effect of the various chemical additives on the amylolytic activity from the amylases of the *Bacillus paralicheniformis* strain DNP0507

| Chemical additives | Residual amylolytic activity (%) |
|---|---|
| $Ba^{2+}$ | 93.53 ± 0.069 [ab] |
| $Ca^{2+}$ | 96.84 ± 0.025 [ab] |
| $Co^{2+}$ | 109.82 ± 0.035 [b] |
| $Cu^{2+}$ | 112.34 ± 0.021 [b] |
| $Fe^{2+}$ | 100.55 ± 0.099 [ab] |
| $K^{+}$ | 93.66 ± 0.039 [ab] |
| $Mg^{2+}$ | 88.89 ± 0.061 [ab] |
| $Mn^{2+}$ | 100.81 ± 0.015 [ab] |
| $Na^{+}$ | 90.35 ± 0.061 [ab] |
| $Pb^{2+}$ | 107.17 ± 0.035 [b] |
| $Zn^{2+}$ | 93.92 ± 0.063 [ab] |
| SDS | 84.07 ± 0.054 [ab] |
| EDTA | 65.55 ± 0.121 [a] |

Note: The mean values of the amylolytic activity followed by the same letter were not significantly different according to the one-way ANOVA with Tukey's test ($p < 0.05$) that were analyzed by R software version 4.2.1. The experiments were performed in triplicate

### Application of the amylases from the *Bacillus paralicheniformis* strain DNP0507 on the hydrolysis of starchy waste

Fried starch residues from fried chicken processes were utilized for the starchy waste in this experiment. The starchy waste was subsequently hydrolyzed by the partially purified amylases from the *B. paralicheniformis* strain DNP0507 to obtain simpler sugar molecules. The amount of sugar released from the fried starch residues was measured after enzymatic hydrolysis using the spectrophotometric DNS method. Interestingly, the hydrolysis result by the partially purified amylases from the *B. paralicheniformis* strain DNP0507 was able to provide a sugar yield of 0.309 ± 0.006 mg/mL, while the commercial amylases yielded a sugar of 0.310 ± 0.021 mg/mL. The amount of resulting sugar obtained from the *B. paralicheniformis* amylases was almost equal to that of the commercial amylases under similar experimental conditions.

## Discussion

Amylases have accounted for more than 25% of the enzyme market in the world. The use of amylases appears as a promising technology in many foods, starch hydrolysis, and fermentation industries. These industries demand for novel, low-cost, effective, and versatile amylases. Mangrove soils are the habitat of various bacteria that some individuals are considered as the potential sources of industrial enzymes. Studies on amylase-producing bacteria isolated from mangrove soils have been scarce and are not well understood. Therefore, amylase-producing bacteria remains largely unexplored and many potential bacteria may await discovery.

In this study, there were 123 bacterial isolates isolated from the mangrove soil samples of the Phra Chedi Klang Nam Mangrove Forest in Rayong Province, Thailand by using the culture-dependent method. The screening of amylolytic bacteria using the starch agar method showed 11 bacterial isolates (8.94% of the isolated bacteria), which were defined as amylase-producing bacteria. A previous study found that 192 bacterial isolates were obtained from the soil samples of the Muthupettai Mangrove Forest in Tamil Nadu, India (Kanimozhi et al. 2014). Eleven isolates (5.73% of the isolated bacteria) were considered as amylase-producing bacteria. Moreover, a recent study showed that three bacterial isolates were isolated from the mangrove forest soils of the Mangrove Wonorejo in Surabaya, Indonesia (Khiftiyah et al. 2018). The results exhibited that there was only one bacterial isolate considered as amylase-producing bacteria. It was believed that the active amylase-producing bacteria were rarely isolated from mangrove soils. The HC values were the presumptive method for the screening of amylase-producing bacteria. The most active amylase-producing bacteria based on the HC values in this study was the bacterium isolate DNP2804 with the HC value of 3.79. It was higher than that of previous reported amylase-producing bacteria from other soil samples. The amylase-producing bacteria from soil samples in the Nasinuan Community Forest, Thailand showed the HC values ranging from 1.18 to 1.71 (Luang-In et al. 2019). Amylase-producing *Bacillus* species, isolated from various soil samples in Pakistan, showed the HC values ranging from 1.05 to 2.11 (Rehman and Saeed, 2015).

Many microbiological studies in mangrove ecosystems have been reported in recent years (Liu et al. 2019). A study on bacterial diversity in the Bhitarkanika mangrove soil, India by using the culture-dependent method showed the predominance of bacterial genera, such as *Bacillus*, *Pseudomonas*, *Desulfotomaculum*, *Desulfovibrio*, *Desulfomonas*, *Methylococcus*, *Vibrio*, *Micrococcus*, *Klebsiella*, and *Azotobacter* (Mishra et al. 2012). In this study, the genetic identification and phylogenetic analysis of the 16S rRNA gene sequences revealed that the amylase-producing bacteria belonged to various genera, including *Bacillus*, *Desulfurella*, *Peribacillus*, *Priestia*, and *Pseudomonas*.

The *Bacillus* species were ubiquitously distributed in various samples and environments. A previous study reported that *Bacillus* was found in the sediment samples of the Phra Chedi Klang Nam Mangrove Forest (Chantarasiri 2015). The amylase-producing *Bacillus* species in this study were genetically identified as the *Bacillus* sp. strain DNP0701, *Bacillus* sp. strain DNP1402, *B. paralicheniformis* strain DNP0507, *B. proteolyticus* strain DNP2306, and *B. tequilensis* strain DNP0803. The genus *Bacillus* is a major producer of extracellular amylases, which is generally preferred for industrial production (Pranay et al. 2019), such as *B. amyloliquefaciens* (Deb et al. 2013; My et al. 2022), *B. licheniformis* (Abdel-Fattah et al. 2013; Ashraf et al. 2018; Wu et al. 2018), *B. subtilis* (Salem et al. 2020), and *B. velezensis* (Bhatt et al. 2020). *Bacillus paralicheniformis* was isolated from a fermented soybean paste (Cheonggukjang), identified, and characterized in 2015 (Dunlap et al. 2015). This bacterium was reported to have an α-amylase gene in its genome and was able to produce extracellular α-amylase (Ghazouani et

al. 2020). The amylases from *B. paralicheniformis* were proven to be a highly efficient raw starch digesting enzyme (Božić et al. 2020). Therefore, it had the attempts to optimize the production of amylases from this novel bacterium (Bekler et al. 2019). *B. proteolyticus* was reported as a protease producing bacteria (Bhaskar et al. 2007). This bacterium could be isolated from marine environments (Liu et al. 2017). There was almost no report about amylase-producing *B. proteolyticus*. Therefore, this study showed that the *B. proteolyticus* strain DNP2306 could be designated as an amylase-producing bacterium. *B. tequilensis* was first isolated from a Mexican shaft tomb (Gatson et al. 2006). Previous reports showed that amylase-producing *B. tequilensis* could be isolated from soil samples in Uttar Pradesh, India (Tiwari et al. 2014) and mud samples from the coastal environment in Tamil Nadu, India (Manohar et al. 2017).

*Pseudomonas* bacteria are commonly found in mangrove soil samples of the Phra Chedi Klang Nam Mangrove Forest as reported in Chantarasiri (2021a). The bacterial genus *Pseudomonas* is a prolific producer of a number of extracellular amylases (Khannous et al. 2014), such as *P. aeruginosa* (Raju and Divakar 2013), *P. stutzeri* (Maalej et al. 2013), *P. luteola* (Khannous et al. 2014), and *P. balearica* (Kizhakedathil and Chandrasekaran, 2018). The amylase-producing *Pseudomonas* isolated and identified in this study were the *Pseudomonas* sp. strain DNP0201, *P. entomophila* strain DNP2704, and *P. putida* strain DNP2804. *P. entomophila* was isolated from a female specimen of the fruit fly *Drosophila melanogaster* (Mulet et al. 2012). It was initially characterized as a natural pathogen of *Drosophila* (Vallet-Gely et al. 2010). *P. putida is* frequently isolated from human, water, plant, soil, and polluted sites (Weimer et al. 2020). There is almost no report about amylase-producing *P. entomophila* and *P. putida*. Importantly, this study showed that the *P. entomophila* strain DNP2704 and *P. putida* strain DNP2804 were designated as amylase-producing bacteria.

*Desulfurella* is a genus of sulfur-reducing bacteria using elemental sulfur as the electron acceptor with high acid-tolerant ability (Florentino et al. 2016). The *Desulfurella* sp. strain DNP0602 was isolated, genetically identified, and designated as an amylase-producing bacterium in this study. At present, there is no clarified report on the amylase from the *Desulfurella* species. Only one evidence related to amylase from *Desulfurella* is the glycoside hydrolase sequence of the *D. acetivorans* strain A63, which has been published in the GenBank database with accession number of AHF97462.

*Peribacillus simplex* (previously *Bacillus simplex*) is an endophytic bacterium that could be isolated from plant root (Martínez-Hidalgo et al. 2021). The *P. simplex* strain DNP0804 was isolated and designated as an amylase-producing bacterium in this study. This bacterium was also isolated from the mountain soil obtained from Turkey and defined as amylase-producing bacterium (Ortakaya et al. 2017).

*Priestia flexa* (previously *Bacillus flexus*) could be isolated from soil (Divyashree and Shamala 2010) and marine sediment (Al Farraj et al. 2020) samples. In this study, the *P. flexa* strain DNP2401 was found to be an amylase-producing bacterium. The species *P. flexa* has been previously reported by Zhao et al. (2008) and Elechi et al. (2022) to be an amylase-producing bacterium. A previous study showed that the *P. flexa* strain U8 was isolated from the rhizospheric soil of paddy plants in India and was reported as a starch hydrolyser (Roy et al. 2020).

The most active form of amylase-producing bacteria based on enzyme activity was the *B. paralicheniformis* strain DNP0507. It showed the most significant amylolytic activity by 2.395 ± 0.133 U/mg. It was higher than that of a previous reported amylase-producing *Bacillus* from other soil samples (Luang-In et al. 2019). The amylases from the *B. paralicheniformis* strain DNP0507 could be possible as α-amylases because theses enzymes were usually produced by bacteria belonging to the genus *Bacillus* (Sajedi et al. 2005). Moreover, the studies of Bekler et al. (2019), Božić et al. (2020), and Ghazouani et al. (2020) have identified the amylases from *B. paralicheniformis* as α-amylases. However, the identification of the *B. paralicheniformis* strain DNP0507 amylases should be further verified by molecular weight and amino acid sequencing analyses.

The *B. paralicheniformis* strain DNP0507 was not the most active form of amylase-producing bacteria based on the HC value in the screening procedure. It exhibited only 2.25 ± 0.21, which was less than that of the *P. putida* strain DNP2804 (HC value of 3.79 ± 0.05), *B. tequilensis* strain DNP0803 (HC value of 3.74 ± 0.10), *P. entomophila* strain DNP2704 (HC value of 3.66 ± 0.10) and *B. proteolyticus* strain DNP2306 (HC value of 2.55 ± 0.10). The agar plate-screening method often exhibited the conflicting correlation between the resulting HC value and enzyme activity. Furthermore, Luang-In et al. (2019) had studied the isolation of amylase-producing bacteria from soil and also found this conflict in their results. This conflicting result could be due to the fluctuations in some experimental factors, which affected the production of bacterial extracellular enzyme (Chantarasiri 2021c).

The partially purified amylases from the *B. paralicheniformis* strain DNP0507 were enzymatic characterized. The amylases from the *B. paralicheniformis* strain DNP0507 were active and stable under the mesosphere and neutral pH conditions. This was similar to the amylolytic activity of other *Bacillus* species, which were active and stable under the mesosphere and neutral pH conditions (Abdel-Fattah et al. 2013; Ashraf et al. 2018; Ashwini et al. 2011; Luang-In et al. 2019; Saxena and Singh 2011). Therefore, the amylases from the *B. paralicheniformis* strain DNP0507 were preferable for applications with mild conditions, such as baking and food industries. In this study, the amylolytic activity was found to be enhanced by some metal ions, such as $Cu^{2+}$, $Co^{2+}$, and $Pb^{2+}$ whereas slightly inhibited by $Mg^{2+}$. A previous report similarly showed that $Cu^{2+}$ and $Co^{2+}$ could activate, and the $Mg^{2+}$ could inhibit the amylolytic activity of amylases from *B. licheniformis* (Afrisham et al. 2016). Unfortunately, these three metal ions might be not as promising as the activator of amylases due to their toxicity. The amylolytic activity of amylases from the *B. paralicheniformis* strain DNP0507 was strongly inhibited by the cheating properties

of EDTA. This result agreed with the study of Božić et al. (2011). It was evident that amylases require the metal ions for their catalytic mechanisms. Interestingly, the presence of $Ca^{2+}$ had no enhancing effect on the amylolytic activity of amylases from the *B. paralicheniformis* strain DNP0507. These amylases were possibly designated as a $Ca^{2+}$ independent enzyme. The $Ca^{2+}$ independent amylases were considered as an advantageous feature for the fructose syrup production by the stepwise processes of amylase-glucose isomerase enzymes (Wu et al. 2018).

Every year, massive amounts of starchy waste are generated and cause serious environmental problems. In this study, the hydrolysis of the starchy residues from the fried chicken processes was performed by the partially purified amylases from the *B. paralicheniformis* strain DNP0507. The hydrolysis result showed no significant difference among the amylases from the commercial and *B. paralicheniformis* strain DNP0507 enzymes. Therefore, the amylases from the *B. paralicheniformis* strain DNP0507 were preferred for the starchy waste valorization under mild conditions.

In conclusion, mangrove forests are certainly considered as a potential source for the isolation of amylase-producing bacteria. There were five genera of amylase-producing bacteria isolated from the mangrove forest soils in Thailand and genetically belonging to species of *Bacillus*, *Desulfurella*, *Peribacillus*, *Priestia*, and *Pseudomonas*. Notably, the *B. paralicheniformis* strain DNP0507 was the most active amylolytic bacterium. Its amylases could be considered as potential candidates for baking and food applications under mesosphere and neutral pH conditions. Moreover, they could be used in the hydrolysis of starchy residues and starchy waste valorization. Therefore, further studies would be recommended for the complete purification of the enzymes, the determination of the enzyme molecular weight, and the optimization of enzyme production.

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