Isolation and Identification of Lipase-Producing Bacteria from Soil in Nasinuan Forest, Kantarawichai District, Mahasarakham Province

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Abstract:

Nasinuan Forest was located at Kantarawichai District, Mahasarakham Province Thailand. This area has been considered to be quite saline; however contains a variety of bacteria that may be able to produce industrial enzymes. Lipase is one of the industrial enzymes which play an important role in industry in Thailand such as detergent industry, food, fat and oil industries and cosmetics. This study aimed to identify lipase-producing bacteria from soil in Nasinuan Forest and determine the optimum conditions for lipase production and lipase activity. The results showed that 35 isolates were positive for lipase after being tested on tributyrin agar. The top five isolates with the highest halo : colony ratios included *Bacillus subtilis* 1.3LP2 > *Brevibacillus brevis* 2.1LP4 > *Micrococcus* sp. 3.2LP3 > *Bacillus thuringiensis* 1.1LP3 > *Staphylococcus* sp. 1.4LP4, respectively. The results of gram staining found that four isolates were gram-positive with rod-shaped and only gram-positive with cocci-shaped was found as *Staphylococcus* sp. 1.4LP4. These lipase-producing bacteria were closest to *Bacillus* spp., *Staphylococcus* sp., *Brevibacillus* sp. and *Micrococcus* sp. using 16S rRNA gene analysis whose from Italy, India, China, Egypt and Brazil. The optimum conditions for the highest lipase activity by *Bacillus subtilis* 1.3LP2 are pH 7.0 at 55 °C with the lipase activity of 160.92 ± 0.91 U/mL. This is the first report of lipase-producing bacteria isolated from soil in Nasinuan forest, Mahasarakham, Thailand. These bacterial enzymes can be applied in various industries in Thailand such as detergent industry, food, fat and oil industries and cosmetics.

Keywords: Bacteria, Soil, Lipase enzyme, Industry, 16S rRNA gene

Introduction

Lipase (EC 3.1.1.3) is an enzyme that can be found in nature, this enzyme acted by catalyzing hydrolysis of molecular ester bonds with triglycerol and long chain fatty acids which containing monoglycerides, dioxins and free fatty acids as the final product [1]. Lipase producing bacteria often have a variety of habitats such as waste, processing plants, vegetable oil, soil contaminated from oil mill area, etc. There have many factors of inducing lipase production such as temperature, pH, nitrogen and carbon source, inorganic salts, agitation and dissolved oxygen concentration [2]. The reliable way to detect microorganisms that can produce lipase is to use tributyrin as substrate and degrading zone around the colony was visualized [3]. Most of lipase- producing bacteria are gram-negative bacteria especially *Pseudomonas*, which have at least seven species that can produce lipase, namely *Pseudomonos aeruginosa*, *Pseudomonos alcaligenes*, *Pseudomonos fragi*, *Pseudomonos glumae*, *Pseudomonos cepacia*, *Pseudomonos fluorescens* and *Pseudomonos putida* [4].

Bacillus spp. can produce lipase as reported by Chaturvedi et al. [5] mentioned to lipase-producing bacteria isolated from contaminated soil areas, India. Solid State Fermentation (SSF) technique occurred led to discovery of *Bacillus subtilis* with lipase production. In addition, several reports mentioned that bacteria species *Staphylococcus* sp. [6] and *Brevibacillus* sp. [7] separated from soil and hot spring source can produce lipases as well as those were found in India. In general, soils are often the source of a variety of microorganisms due to richness in various nutrients and minerals. The advantages of using microorganisms

for enzyme production are the ease to cultivate and low production costs. Therefore, this work studied optimum condition for bacterial lipase production. There are many factors that are very important for enzyme production such as substrate concentration, the starter cultures, acidity and temperature. This research aimed to isolated lipase-producing bacteria obtained from the soil in the Nasinuan Forest, Kantarawichai District, Mahasarakham Province and optimization conditions for lipase activity were evaluated.

Materials and methods

Collection of soil sample

The soil samples were collected from Nasinuan Forest, Kantarawichai District, Mahasarakham Province, Thailand. They were collected from 60 rai randomly in zones 1-3 (Figure 1).

Figure 1 Studied area. Zone 1, 2 and 3 of 9.6 hectare where soil samples were collected

Bacterial isolation and screening for lipase activity

Soil sample (10 g.) was suspended in 90 mL of sterile 0.85% NaCl solution and serial dilution was performed. The suspension of 100 µL was spread on tributyrin agar (g/ 100 mL) consists of 0.5% peptone, 0.3% yeast extract, 1% (v/v) tributyrin and 1.5% agar (pH 7.0) and incubated at 37 °C for 3 days. The degrading zone was visualized and halo : colony ratios were calculated following the method of Haq et al [8]. The highest halo : colony ratios from 5 isolates were collected and subculture to obtain pure isolates using streaking technique on general bacterial medium (g/L) consists of 1.0% yeast extract, 0.5% tryptone and 0.5% NaCl (pH 7.0) then incubated at 37 °C overnight. The morphological properties of each bacterial isolate were identified including color, shape, surface and edge of colony and Gram staining results were recorded.

Screening of lipase production

Five of pure isolates of lipase-producing bacteria were cultured and adjusted at 10^8 CFU/mL equivalent to 0.5 McFarland [9]. These isolates were inoculated into 30 mL lipase liquid medium (g/100 mL) consists of 0.2% peptone, 0.1% $NH_4H_2PO_4$, 0.25% NaCl, 0.04% $MgSO_4.7H_2O$, 0.04% $CaCl_2.2H_2O$, 1.0% olive oil and 2% (v/v) tween 80 in flask (50 mL) incubated at 37 °C and shaking at 150 rpm for 24, 48 and 72 h. The extracellular enzyme (crude enzyme extract) in supernatants were obtained from centrifugation at 10,000*g* and kept at 4 °C for lipase activity analysis following a method of Winkler and Stuckman [10]. The crude enzyme extract with highest lipase activity was determined.

Optimization of lipase activity

pH

The crude extract of the highest lipase activity from above analysis was used to determine its optimum pH for activity using various pHs in suitable buffers in a range of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 incubated at 37 °C for 30 min and lipase activity was recorded.

Temperature

The crude extract of the highest lipase activity with optimum pH was used to determine its

optimum temperature for activity using various temperatures at 4, 25, 35, 45, 55, 65, 75, 85 and 95 °C for 30 min and lipase activity was recorded.

Protein quantitative analysis

Protein content was determined following a method of Lowry [11] using BSA as standard agent.

Statistical analysis

Measurements were obtained in triplicates as means ± standard deviation (SD). Statistical analysis was performed using One-way analysis of variance and Duncan Multiple's Range Test by the software SPSS package version 17.0 at $P < 0.05$.

16S rRNA gene sequencing and phylogenetic analysis

The pure isolates were inoculated from 20% glycerol stock to general bacterial medium for 18 h and identified using genomic DNAs obtained from the above method and using universal primers : 27F 5'-GAGAGTTTGATYCTGGCTCAG-3' and reverse primer 1492R 5'-AAGGAGGTGATCCARCCGCA-3'. In 25 µL PCR mixture, it was composed of genomic DNA 0.5 ng, 2X Master Mix (One PCR) of 100 mM Tris-HCl (pH 9.1), 0.1% TritonTMX-100, 200 mM dNTP, 1.5 mM $MgCl_2$, 0.005 U Taq DNA Polymerase and 0.2 µM forward and reverse primers with volume adjustment with nuclease-free water sterile. PCR thermocycler (Thermo Scientific Hybaid Px2) was programed as follows : (1) initial denaturation for 2 min at 94 °C for 1 cycle ; (2) denaturation at 94 °C for 45 s ; annealing at 54 °C for 45 s, and extension at 72 °C for 1 min for 32 cycles ; (3) final extension at 72 °C for 7 min. Samples were held at 4 °C till further analysis. The PCR products of 16S rRNAs (1,500 bp) were detected on 0.8% agarose gel, purified using the PCR product purification kit (Vivantis, Malaysia), sent to First Base Co. Ltd. (Malaysia) for DNA sequencing. The 16S rRNA gene sequences were then compared with others available in GenBank using BLASTN program (Basic Local Alignment Search Tools) [12]. The phylogenetic tree was constructed using Muscle method for sequence alignment and neighbor-joining method using MEGA7 (www.megasoftware.net) with 1,000 replicates of bootstrap values [13].

Results and discussion

Soil sample information

Lipase can be found in several sources such as plants, animals and microorganisms. At present, microorganisms are popular to use in enzymes production. Especially, bacteria have advantages over the production of enzymes derived from plants and animals as bacteria can increase the number of cells quickly within a short period of time in a cheap culture medium. In general, the selection of bacteria with the ability to produce lipase is commonly used to observe degrading zone around colonies indicate the ability to synthesize lipase [14]. There are several reports mentioned the use of olive oil and palm oil as substrates for lipase production [15].

Table 1 Information on soil samples from Nasinuan Forest

Location of soil collection	Surrounding area	Electroconductivity (µS/cm)
1.1 (N 16 20 17.945, E 103 12 14.731)	*Azadirachta indica* and *Senna siamea* were grown around in this area	3.62 Low saline (0.15% NaCl)
1.3 (N 16 20 6.619, E 103 11 0.985)	Unknown trees	2.23 Low saline (0.1% NaCl)
1.4 (N 16 20 23.547, E 103 12 35.244)	Found termite in this area	2.09 Low saline (0.1% NaCl)

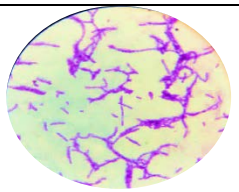
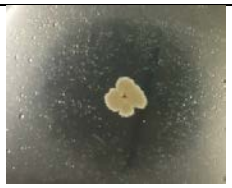
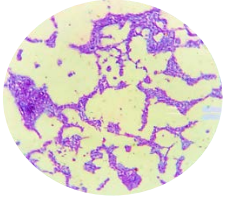
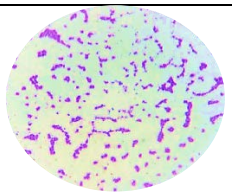
2.1 (N 16 20 36.882, E 103 12 32.315)	Found *Ageratum conyzoides* in this area	2.88 Low saline (0.15% NaCl)
3.2 (N 16 20 35.406, E 103 12 35.157)	*Catunaregam tomentosa* was grown in this area	2.24 Low saline (0.1% NaCl)

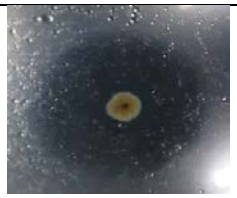
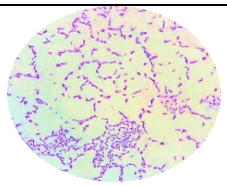
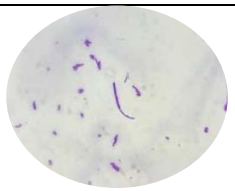
In this study, the pH and electrical conductivity of soils were recorded. These factors are related to salinity of soil samples collected from Nasinuan Forest, Kantarawichai District, Mahasarakham Province. The top 5 bacterial isolates were collected and isolated with highest lipase activity based on highest halo : colony ratios from the soil at locations 1.1, 1.3, 1.4, 2.1 and 3.2 with a pH range between 6.0 and 8.0 (Table 1). Normally, electrical conductivity values at 2-3 indicates that salinity or NaCl exists approximately about 0.1-0.15% in soil [16]. The soil showed slight salinity when the electrical conductivity of the soil is more than 2 µS/cm, it is considered as having some effect on the plants that grow around. Thus, in this case, low salinity in soil has little effect on plant growth in Nasinuan Forest.

Bacterial lipase isolation

In this study, 35 lipase-positive isolates were isolated and identified. The top 5 lipase-producing bacterial isolates (1.1LP3, 1.3LP2, 2.1LP4 and 3.2LP3) showed the highest degrading zones on 1% tributyrin agar with different halo : colony ratios. Four bacterial representative strains showed similar colony morphologies and appeared to be gram-positive and rod-shaped; however another strain appeared to be gram-positive and cocci-shaped (Table 2). The results showed halo : colony ratios ranged from 1.2 to 2.4 (Table 2). Our finding was similar to Ertugrul et al. [3] and these 35 isolates were submitted to 16S rRNA gene analysis in order to identify bacterial isolates.

Table 2 Characteristics of 5 lipase-producing bacterial representative strains

Isolate	Colony morphology	Halo : colony ratios	Gram staining
1.1LP3	Irregular shape Flat structure Curled margin Cloudy white color	 1.35	 G+, rod
1.3LP2	Irregular shape Flat structure Curled margin Cloudy white color	 2.40	 G+, rod
1.4LP4	Circular shape Flat structure Undulate margin Cloudy white color	 1.25	 G+, cocci

2.1LP4	Punctiform shape Flat structure Entire margin Cloudy white color	 2.35	 G+, rod
3.2LP3	Filamentous shape Flat structure Filamentous margin White color	 1.60	 G+, rod

Identification of lipase-producing bacteria

In this study, five lipase-positive bacteria (1.1LP3, 1.3LP2, 2.1LP4 and 3.2LP3) were identified using 16S rRNA gene product analysis (1,500 bp) on agarose gel electrophoresis (Figure 2). A 16S rRNA gene analysis is the most popular technique on bacterial identification [18]. The 16S rRNA gene, approximately 1,500 bp can determine the difference between bacterial species because it has certain functions. There is a large size and there is very little change in each generation of evolution, so it is easy to classify bacteria species [19]. It is also correlate with the analysis of the phylogenetic tree [20] and more accurate than API kit as previously reported [21].This study found that 5 strains 1.1LP3, 1.3LP2, 1.4LP4, 2. 1LP4 and 3. 2LP3 were closest to *Bacillus thuringiensis*, *Bacillus subtilis*, *Staphylococcus* sp., *Brevibacillus brevis* and *Micrococcus* sp., respectively. These bacteria were isolated from the Nasinuan forest and showed 88-99 % sequence identity to those bacteria found in Italy, India, China, Egypt and Brazil (Table 3). Our results were similar to previous reports, *Bacillus subtilis* I-4 isolated from oil contaminated effluents of various industries from Pakistan, this strain was produced lipase with an optimum conditions pH 7.0 and temperature 50 °C [22]. Furthermore, our results were similar to previous reports were included *Staphylococcus* sp. Lp12 isolated from an oil contaminated soil in India [23], *Bacillus thuringiensis* TS11BP isolated from soil in Bhimavaram , India [24] and *Brevibacillus* spp. isolated from hot springs located in Gazan area in Saudi Arabia [25]. However, this current work discovered for the first time that *Micrococcus* sp. isolated from Nasinuan forest, Kantarawichai District, Mahasarakham Province can be a lipase producer.

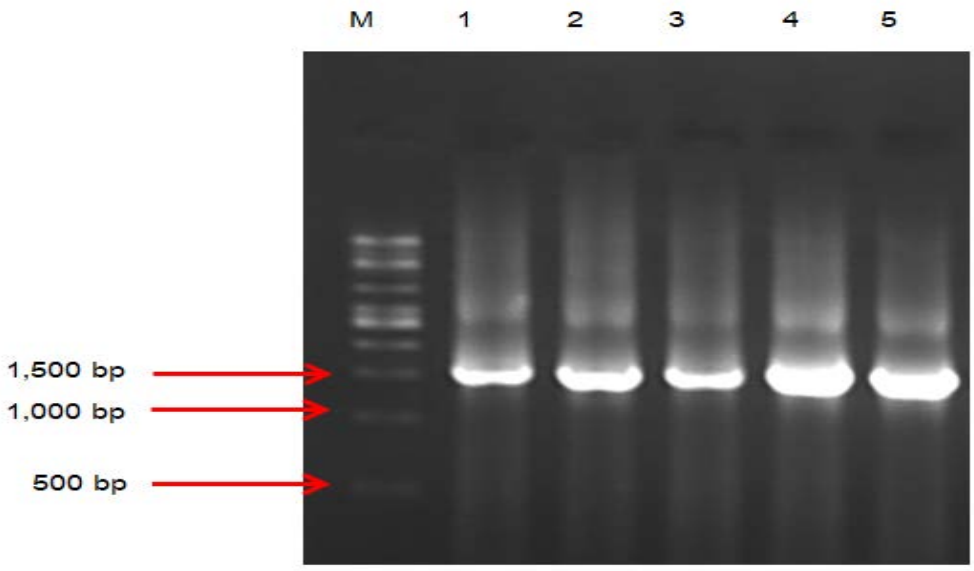


Figure 2 DNA bands of 16S rRNA gene from PCR-based 16S rRNA analysis on agarose gel (Approximately 1,500 bp)

Table 3 The 5 lipase-producing bacterial strains identified by 16S rRNA analysis.

Isolate	Closest relative*	Accession No.*	%Identity*	Origin*
1.1LP3	*Bacillus thuringiensis* BD17-E12	HF584771.1	99	Grapevine root system, Italy
1.3LP2	*Bacillus subtilis* APBSWPTB156	MG733629.1	99	Bio aerosols, India
1.4LP4	*Staphylococcus* sp. CLC-F24	MH518206.1	98	China
2.1LP4	*Brevibacillus brevis* ABS9	KU973528.1	88	Soil, Egypt
3.2LP3	*Micrococcus* sp. HEXBA06	JQ658425.1	98	Todos os Santos Bay oil contaminated Mangrove, Brazil

*Based on results from BLAST search (www.blast.ncbi.nlm.nih.gov/Blast.cgi)

Phylogenetic tree analysis

Phylogenetic tree of five lipase-producing bacteria strains showed a relationship between the 16S rRNA genes of five strains *Bacillus* spp., *Staphylococcus* sp., *Micrococcus* sp. and *Brevibacillus* sp. The evolution of five lipase bacterial strains was constructed by MEGA 7.0 (www.megasoftware.net). In our study finding, *B. thuringiensis* 1.1LP3 was evolutionarily similar to *B. thuringiensis* IA RI-IIWP-38 (NCBI accession no. KF054891.1) isolated from the soil of the rhizosphere grown in wheat, India. *Staphylococcus* sp. 1.4LP4 was similar to *Staphylococcus cohnii* RCB1038 (NCBI accession no. KT261250.1) isolated from soil sources in the cave ceiling, India. *B. subtilis* 1.3LP2 was evolved similarly to *B. subtilis* JM1C6 (NCBI accession no. EU221334.1) isolated from rhizosphere grown in wheat, India. *Micrococcus* sp. 3.2LP3 was similar to *Micrococcus* sp. SK13 (NCBI accession no. LC068961.1) isolated from commercial gasoline sources, Korea and *Brevibacillus brevis* 2.1LP4 was evolved similarly to *Brevibacillus brevis* YQH20 (NCBI accession no. HQ202569.1) isolated from soil sources, China (Figure 3).

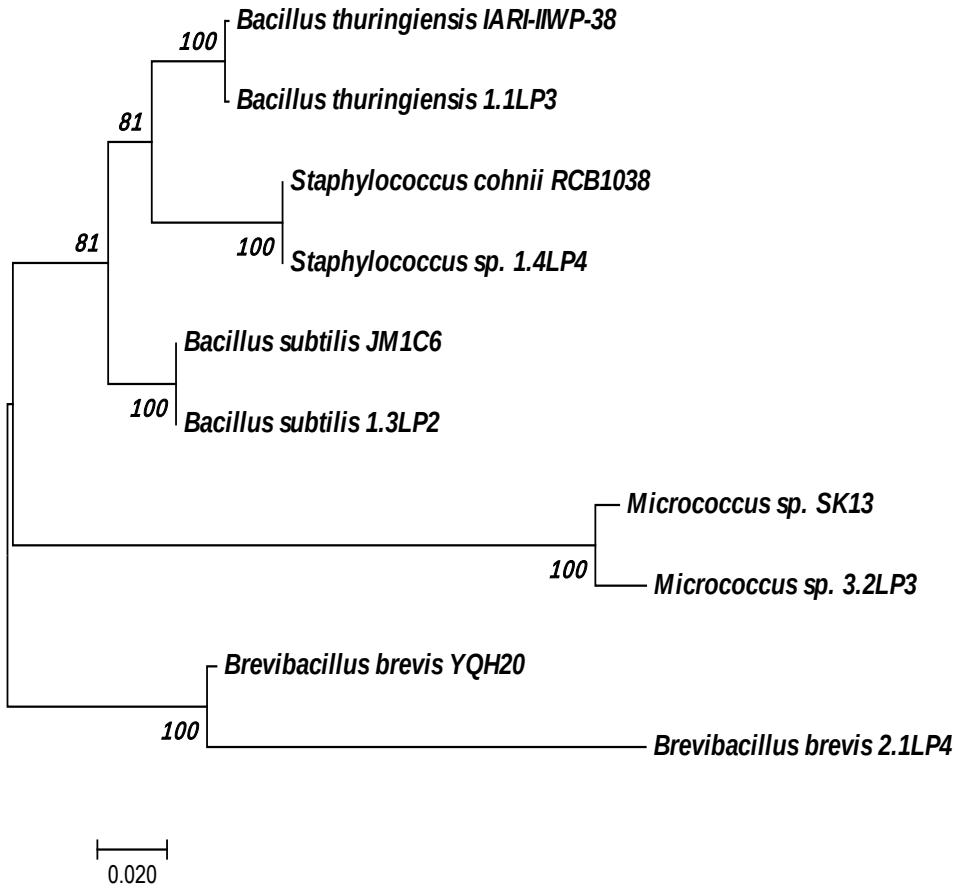


Figure 3 phylogenetic tree of 5 lipase bacterial strains from this study and other 5 reference strains from previous reports

Optimum conditions for lipase production

Mazhar et al. [26], isolated lipase-producing bacteria from soil sources in Pakistan and found that the optimum conditions for lipase production and lipase activity of *B. subtilis* PCSIRNL-39 were temperature at 45 °C and pH 7.0. In addition, Femi-Ola et al. [27] mentioned that optimum conditions for lipase activity of *B. subtilis* was incubated at 60 °C (pH 8.0). In general, production and function of lipase is found in the pH range of 6.0-10.0 [28].

In this study, the optimum conditions for lipase production were evaluated. Our study found that *B. subtilis* 1.3LP2 gave the highest lipase activity at 97.78 ± 2.41 U/mL at 37 °C for 72 h compared to the other 2 bacterial species including *B. thuringiensis* 1.1LP3 and *Micrococcus* sp. 3.2LP3 (Figure 4A). Therefore, crude extract of *B. subtilis* 1.3LP2 at 72 h induction time was used to determine optimum conditions of lipase activity. The results showed that crude extract of *B. subtilis* 1.3LP2 gave the highest enzyme activity of 105.12 ± 12.04 U/mL at pH 7.0 and 37 °C after 30 min (Figure 4B) and highest enzyme activity with 160.92 ± 0.91 U/mL after incubation with 1% olive oil induction at 55 °C, pH 7.0 after curing for 30 min (Figure 4C), respectively. Olive oil used as substrate for screening of lipase producing bacterial has been referred as one of the best inductors and substrate for lipase production [29].

Our results show that *B. subtilis* 1.3LP2 has optimum temperature at 55 °C (pH 7.0). This results were similar with previous report that investigated the optimum conditions of the *Bacillus* sp. PD-12 on lipase production, incubation at 55 °C (pH 7.0) using olive oil as substrate and incubated for 1 h [30]. This is very interesting result because they are in agreement with our finding, but crude extract in this study was incubated only for 30 min and gave lipase activity. This is first report of lipase from *B. subtilis* 1.3LP2 isolated from Nasinuan Forest that offers possible advantage for food enhancement, biofuel production, removal of fat-containing stain and cosmetics ingredients.

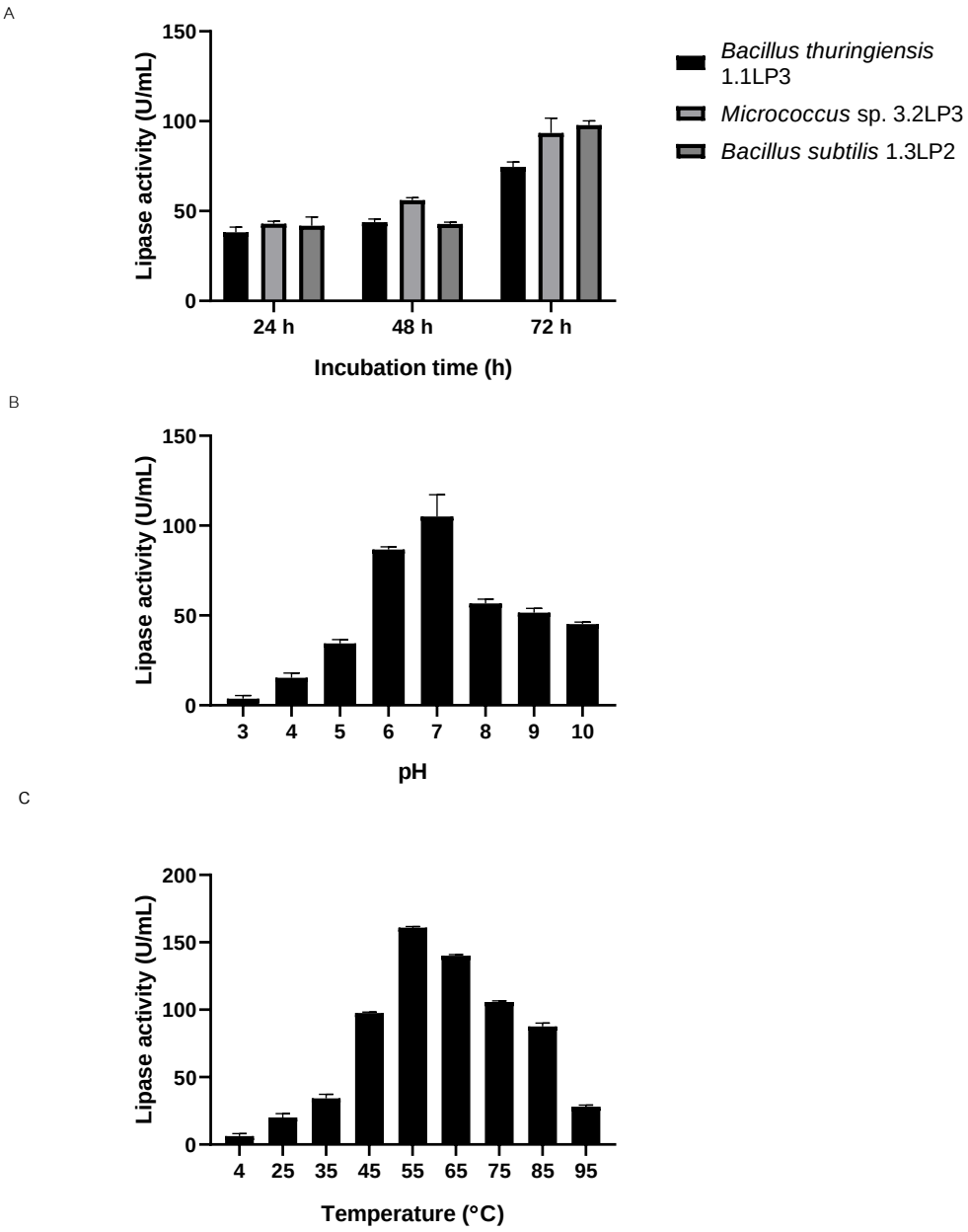


Figure 4 Lipase activity from lipase-producing bacteria (A) Lipase activity from *B. thuringiensis* 1.1LP3, *B. subtilis* 1.3LP2 and *Micrococcus* sp. 3.2LP3 (B) Optimal pH for lipase from *B. subtilis* 1.3LP2 and (C) Optimal temperature for lipase from *B. subtilis* 1.3LP2.

Conclusion

This is the first report of identification of lipase-producing bacteria isolated from soil samples in Nasinuan Forest, Nasinuan Sub-district, Kantarawichai District, Mahasarakham Province, Thailand. The 5 strains of lipase-positive bacteria were identified as *Bacillus* spp., *Staphylococcus* sp., *Brevibacillus* sp. and *Micrococcus* sp., respectively. The *Bacillus subtilis* 1.3.LP2 has the highest lipase activity after screening through liquid medium containing 1% olive oil as substrate. Optimum conditions for lipase activity were pH

7.0 and 55 °C. This lipase might be applied in a various industry in Thailand such as detergent industry, food (shelf life enhancement), fat and oil industries and cosmetics.

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