

## Protease-Producing Bacteria from Soil in Nasinuan Community Forest, Mahasarakham Province, Thailand

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The current work aimed to screen for and identify protease-producing bacteria from the untapped resource Nasinuan forest, Kantarawichai District, Mahasarakham Province, Thailand. Nineteen bacterial isolates with protease-producing capacity on 1% skimmed milk agar were identified using 16S rRNA sequencing. Seventeen bacteria were gram-positive, rod shaped and identified as *Bacillus* spp. and only two bacteria were identified as *Enterobacter* sp. and *Staphylococcus cohnii*. Their closest relatives were found in India, Oman, Italy, Indonesia, Malaysia, China and USA. The top six highest halo : colony ratios from pure isolates were ranked in the following order: 1.2PT1 (2.43) > 1.2PT2 (2.23) > 2.2PT3(2.21) > 2.1PT3 (2.17) > 2.3PT3(2.16) > 2.4PT1 (2.16). *Bacillus thuringiensis* 2.3PT3 was found to exhibit the highest protease enzyme activity of  $3.72 \pm 0.08$  U/mg protein at the optimal conditions of 65°C and pH 8.0 after 30 min incubation with 1% casein in 0.05 M PBS buffer. This protease-producing bacterial strain might be of great potential for applications in food, agricultural and pharmaceutical industries in Thailand.

**Keywords :** Protease, Bacteria, Soil, Nasinuan Forest, Thailand.

Proteases, one of the most important industrial enzymes, account for a major share of 60% of total global enzyme market<sup>1</sup>. Proteases are the hydrolytic enzymes which break down peptide bonds between proteins with paramount applications in pharmaceutical and industrial sector. Proteases have a myriad of functions in food, textile industries and important biopharmaceutical applications such as infant formula preparation (American Academy of Pediatrics Committee on Nutrition, 1998), contact-lens enzyme cleaners

and enzymatic deriders<sup>2</sup>. The proteolytic enzymes can also be used in clinical/medical field offering a gentle and selective debridement, promoting the natural healing step in the successful local management of skin ulcerations by removing the necrotic material efficiently<sup>3</sup>.

To supply sufficient industrial proteases to meet the global increasing demand, we have to investigate the cost effective way of producing industrially important enzymes. Proteases derived from microbial sources are preferred over the

enzymes from plant or animal sources<sup>4</sup> due to microbial wide-range biochemical diversity, their rapid proliferation, the limited space required for cell cultivation and the convenience with which the enzymes can be genetically manipulated to generate new enzymes for various applications<sup>5</sup> and they exhibit almost all the desirable characteristics for their biotechnological applications. A plethora of *Bacillus* derived alkaline proteases have been purified and characterized because of their significant proteolytic activity, broad substrate specificity, stability, short period of fermentation, simple downstream purification and low cost<sup>6,7</sup>. The protease producing bacterial strains are as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus thuringiensis*<sup>8</sup>.

Thus far, a number of protease-producing bacteria has been reported, however, no study on protease-producing bacteria from soil in the Nasinuan Community Forest, Kantarawichai District, Mahasarakham Province, Thailand has been done. This forest seems to be rich in microbial biodiversity that can be applied for the production of industrial enzymes including protease. This is the first report to identify protease-producing bacteria isolated from Nasinuan Forest. These bacterial proteases have potential applications in food processing, animal feed, agriculture and pharmaceutical industry in Thailand.

## MATERIALS AND METHODS

### Soil samples

Soil samples were randomly collected below the soil surface 15 cm and kept in polystyrene bags from Nasinuan Community Forest, Kantarawichai District, Mahasarakham Province, Thailand (area of 9.6 hectare; coordinate of 16.340941, 103.210799). during July 2009

### Isolation of protease-producing bacteria

Soil sample (10 g) was suspended in 90 mL of sterile 0.85% NaCl solution. The suspensions (100  $\mu$ L) of serial dilutions were spread on skimmed milk agar (g/L): 10.0 Tryptone, 5.0 Peptone, 3.0 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 K<sub>2</sub>HPO<sub>4</sub>, 0.2 MgSO<sub>4</sub>, 1.0 Casein, 15.0 agar pH 7.0 and incubated at 37 °C for 3 days. Any colonies with formation of clear zone around the colonies were subcultured in liquid broth and streaked at least five times to obtain pure isolates as confirmed by Gram staining

and 1000X light microscopic observation. The pure isolates were point inoculated on skimmed milk agar and incubated at 37 °C for 7 days. The diameters of the clear zones over the diameters of the colonies were measured using a ruler as the halo : colony ratio.

### 16S rRNA gene sequencing and phylogenetic analysis

Pure bacterial isolates were identified using genomic DNAs obtained from the above method and universal primers: forward primer 27F 5'-GAGAGTTTGGATYCTGGCTCAG-3' and reverse primer 1492R 5'-AAGGAGGTGATCCARCCGCA-3'. In 25  $\mu$ L 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% Triton TMX-100, 200 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 0.005 U Taq DNA Polymerase and 0.2  $\mu$ M forward and reverse primer with volume adjustment with nuclease-free water. PCR thermocycler (Thermo Scientific Hybaid Px2) was programmed 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)<sup>9</sup>. The Phylogenetic tree was constructed using Muscle method for sequence alignment and maximum likelihood method using MEGA X with 1,000 replicates of bootstrap values<sup>10</sup>. All 16S rRNA partial sequences of our protease-producing isolates were deposited on NCBI database.

### Protease enzyme activity

The method followed the previous report<sup>11</sup>. Each isolate was subcultured in casein induction liquid broth (g/L): 10.0 Tryptone, 5.0 Peptone, 3.0 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 K<sub>2</sub>HPO<sub>4</sub>, 0.2 MgSO<sub>4</sub>, 1.0 Casein and incubated at 37 °C, 150 rpm for 3 days. The clear supernatant (crude extracellular protease enzyme) was obtained after centrifugation at 10,000g for 15 min at 4 °C. The crude extract was concentrated using MWCO

10 kDa ultracentrifuge protein concentrator (Vivaspin, Sartorius, UK). Crude enzyme (0.5 mL) was mixed with 1 mL of 1% casein solution in 1 mL 0.05 M Potassium phosphate buffer pH 7.5. The samples were incubated at 37°C for 30 min. After incubation, 3 mL of 110 mM trichloroacetic acid (TCA) was added to each sample to stop the reaction and then centrifuged at 10,000 rpm for 15 min. The clear supernatant (1 mL) was mixed with 2 mL of 0.5 M sodium carbonate solution and 0.5 mL Folin's reagent. The mixture was recorded for  $A_{280\text{nm}}$  using a spectrophotometer. The reading was compared to a prepared blank solution (without crude enzyme). The  $A_{280\text{nm}}$  values of samples at  $T_{30\text{min}}$  were subtracted from those of samples at  $T_{0\text{min}}$  since casein still remained in the  $T_{0\text{min}}$  samples after enzyme induction process at 3 days. The process was carried out in triplicates. The concentration of tyrosine produced for each solution was obtained from the tyrosine standard curve. The activity of protease was calculated. One unit of protease activity is defined as the amount of protease required to catalyze the formation of 1  $\mu\text{mol}$  tyrosine per min under assay conditions<sup>11</sup>. The crude enzyme of the bacterial isolate having the highest activity was chosen for further work. In order to determine the specific enzyme activity of the selected isolates, the Folin-Lowry method for total protein estimation was used. The specific enzyme activity was measured using the following formula.

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Extracellular protein concentration (mg/ml)}}$$

### Optimal pH and temperature for protease enzyme activity

Casein (1%) in different pH solutions starting from 3 to 10 were tested. One mL of different 1% casein solution was added along with 1 mL of the respective buffers; 0.05 M citrate buffer (pH 3 to 5), 0.05 M sodium phosphate buffer (pH 6 and 7), 0.05 M Tris-HCl (pH 8 and 9) and 0.05 M glycine NaOH (pH 10). One mL of crude protease enzyme was added to these buffers as well. The samples were incubated at 37°C for 30 min. The specific protease activity was calculated. The pH at which the highest activity was observed was noted. Likewise, different substrate solutions were made by dissolving 1% casein in pH 7.0 solutions. One

mL of 1% casein starch was added along with 1 mL of 0.05 M sodium phosphate buffer (pH 7). One mL of crude enzyme was added to the buffers as well. The samples were incubated at 4, 25, 35, 45, 55, 65, 75, 85, and 95°C for 30 min. The specific protease activity was calculated. The temperature at which the highest activity was observed was noted. Both optimal pH and temperature were used to determine the final specific protease enzyme activity.

### Statistical analysis

One-Way Analysis Of Variance (One-way ANOVA) was used with Duncan Multiple Range's Test on SPSS Statistics Ver. 17.0. Results were expressed as means  $\pm$  SD with statistical difference when  $p < 0.05$ .

## RESULTS AND DISCUSSIONS

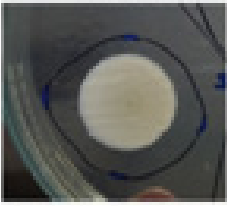
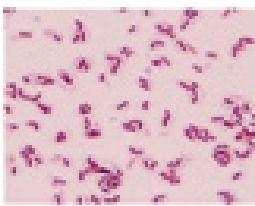
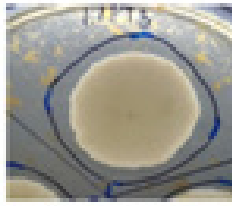


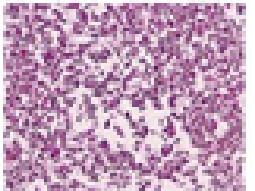

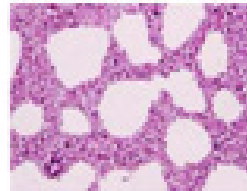
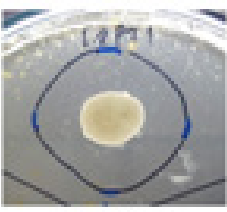


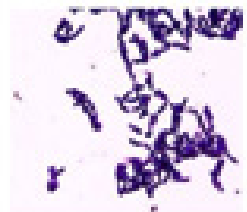
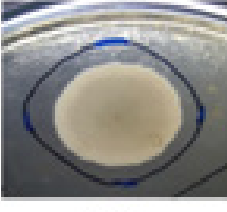
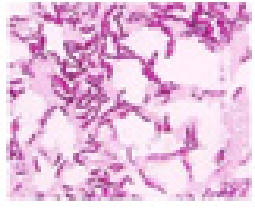

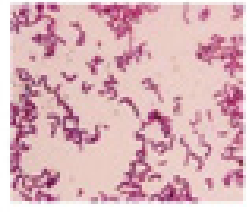
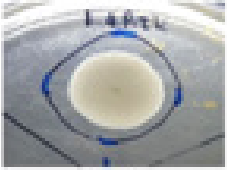
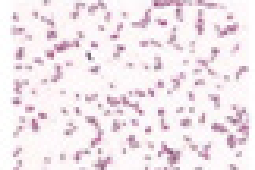


### Isolation of protease-producing bacteria








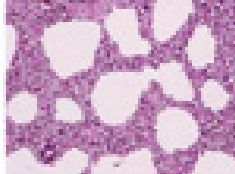





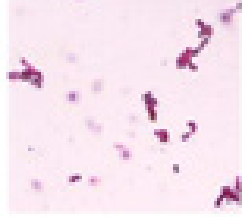


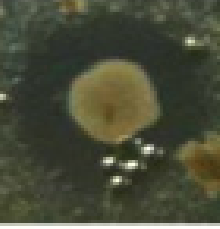

In this study, 19 protease-positive isolates showed clear zones on skimmed milk agar with different halo : colony ratios. The colonies showing clear zones were taken as positive protein-degrading bacterial colonies. Seventeen bacterial isolates showed similar colony morphologies and appeared to be Gram-positive and rod-shaped (Table 1) while 3.5PT11 isolate was gram-negative and rod-shaped and 3.5PT7 isolate was gram-positive and coccus-shaped. The top six highest halo : colony ratios from pure isolates were ranked in the following order: 1.2PT1 (2.43) > 1.2PT2 (2.23) > 2.2PT3 (2.21) > 2.1PT3 (2.17) > 2.3PT3 (2.16) > 2.4PT1 (2.16) (Table 1). These six bacterial isolates were then used for secondary screening for protease activity in 3-day induction liquid broth containing 1% casein. It appeared that 2.3PT3 isolate had the highest protease activity (data not shown) among all isolates and thus used for further experiment.

### Strain identification of protease-producing bacteria

All 19 protease-positive bacterial strains were subjected to 16S rRNA gene sequencing for strain identification. The BLAST results displayed that all protease-positive isolates belong to the genus *Bacillus*, except for two isolates belonging to *Enterobacter* and *Staphylococcus* (Table 2). Their closest relatives were found in India, Oman, Italy, Indonesia, Malaysia, China and USA with a range of 95-99% sequence identity. Our results are similar

Table 1. Characteristics and halo : colony ratios of 19 protease-producing bacterial strains

Halo:colony ratio	Gram staining	Halo:colony ratio	Gram staining
1.1PT2  1.54	Bacilli, G+ 	1.1PT3  1.23	Bacilli, G+ 
1.1PT8  1.73	Bacilli, G+ 	1.1PT9  1.41	Bacilli, G+ 
1.2PT1  2.43	Bacilli, G+ 	1.2PT2  2.23	Bacilli, G+ 
1.2PT3  1.42	Bacilli, G+ 	1.4PT2  1.22	Bacilli, G+ 
1.4PT6  1.32	Bacilli, G+ 	2.1PT3  2.17	Bacilli, G+ 

Halo:colony ratio	Gram staining	Halo:colony ratio	Gram staining
<p><b>2.2PT1</b></p>  <p><b>1.90</b></p>	<p><b>Bacilli, G+</b></p> 	<p><b>2.2PT3</b></p>  <p><b>2.21</b></p>	<p><b>Bacilli, G+</b></p> 
<p><b>2.3PT3</b></p>  <p><b>2.16</b></p>	<p><b>Bacilli, G+</b></p> 	<p><b>2.4PT1</b></p>  <p><b>2.16</b></p>	<p><b>Bacilli, G+</b></p> 
<p><b>2.4PT2</b></p>  <p><b>1.88</b></p>	<p><b>Bacilli, G+</b></p> 	<p><b>3.1PT3</b></p>  <p><b>1.55</b></p>	<p><b>Bacilli, G+</b></p> 
<p><b>3.5PT7</b></p>  <p><b>1.22</b></p>	<p><b>Cocci, G+</b></p> 	<p><b>3.5PT11</b></p>  <p><b>1.19</b></p>	<p><b>Bacilli, G-</b></p> 
<p><b>3.6PT7</b></p>  <p><b>1.88</b></p>	<p><b>Bacilli, G+</b></p> 		

to previous findings. *Bacillus* sp. APP-07 isolated from Laundromat soil of Solapur, Maharashtra, India produced alkaline protease with an optimum pH 10.5 and temperature 55°C<sup>12</sup>. Similarly, *Bacillus licheniformis* TKU004, an isolated bacterial strain from Taiwanese soil, was found to produce protease<sup>13</sup>. In addition, *Enterobacter agglomerans* and *Enterobacter aerogenes* have been previously discovered as protease producers with the highest proteolytic activities at pH 9.0<sup>14</sup>. However, no report has identified *Staphylococcus cohnii* as a protease producer before.

**Phylogenetic analysis**

The phylogenetic tree of 19 protease-positive bacterial strains and 5 reference strains with putative protease enzymes showed that *Enterobacter* sp. 3.5PT11 was evolutionarily similar to *Enterobacter* sp. 638 (EU340965.1). These two bacteria evolved differently from the other bacteria. Likewise, *B. subtilis* 1.1PT8, 1.1PT9, and *B. subtilis* subsp. *stercoris* 1.2PT3 were evolutionarily similar to *B. subtilis* subsp. *subtilis* 168 (MH283878.1). The rest of *Bacillus*

**Table 2.** Nineteen protease-positive bacterial strains identified by 16S rRNA analysis

Isolate	Accession no. <sup>a</sup>	Closest relative <sup>b</sup>	Accession no. <sup>c</sup>	% Identity <sup>d</sup>	Origin <sup>e</sup>
1.1PT2	MK648326.1	<i>Bacillus anthracis</i> ANA1	MG593550.1	99	Marine sediments, India
1.1PT3	MK648327.1	<i>Bacillus cereus</i> IARI-ME-36	KJ752763.1	99	Acidic soil, India
1.1PT8	MK648328.1	<i>Bacillus subtilis</i> TP5	KX822704.1	99	Plant growth promoting endophytes Oman
1.1PT9	MK648329.1	<i>Bacillus subtilis</i> BCB-19	MG832888.1	99	Herbal vermicompost, India
1.2PT1	MK648330.1	<i>Bacillus thuringiensis</i> BD17-E12	HF584771.1	99	Grapevine root, Italy
1.2PT2	MK648331.1	<i>Bacillus cereus</i> F4a	MK088302.1	99	Tea rhizosphere soil, India
1.2PT3	MK648332.1	<i>Bacillus subtilis</i> subsp. <i>stercoris</i> HY5	MK332369.1	99	Fermented food, India
1.4PT2	MK648333.1	<i>Bacillus toyonensis</i> DFT-2	KY750686.1	97	Seawater of industrial area, Indonesia
1.4PT6	MK648334.1	<i>Bacillus cereus</i> DFT-4	KY750688.1	99	Seawater of industrial area, Indonesia
2.1PT3	MK648335.1	<i>Bacillus cereus</i> F4a	MK088302.1	99	Tea Rhizosphere soil, India
2.2PT1	MK648336.1	<i>Bacillus cereus</i> RE01-BS05	KJ742939.1	98	Fermented shrimp paste, Malaysia
2.2PT3	MK648337.1	<i>Bacillus cereus</i> DFT-6	KY750690.1	99	Seawater of industrial area, Indonesia
2.3PT3	MK648338.1	<i>Bacillus thuringiensis</i> BD17-E12	HF584771.1	98	Grapevine root, Italy
2.4PT1	MK648339.1	<i>Bacillus cereus</i> CP1	JX544748.1	98	Unknown source, China
2.4PT2	MK648340.1	<i>Bacillus cereus</i> 2Y-2	FJ493043.1	99	Wheat, China
3.1PT3	MK648341.1	<i>Bacillus thuringiensis</i> IARI-IIWP-38	KF054891.1	99	Wheat rhizosphere, India
3.5PT7	MK648342.1	<i>Staphylococcus cohnii</i>	AY395015.1	99	Midgut of gypsy moth larva, USA
3.5PT11	MK648343.1	<i>Enterobacter</i> sp. T311	KM406403.1	96	Irrigation water, Pakistan
3.6PT7	MK648344.1	<i>Bacillus cereus</i> DFT-1	KY750685.1	95	Seawater of industrial area, Indonesia

<sup>a</sup> GenBank accession no. of our strains deposited on NCBI website (<http://www.ncbi.nlm.nih.gov/pubmed>)

<sup>b</sup> Closest species with highest % identity and highest Max score on BLAST search

<sup>c</sup> GenBank accession no. of closest relative strains on NCBI website

<sup>d</sup> Based on BLAST search results, identity (%) of strains compared to the closest relatives.

<sup>e</sup> Based on BLAST search results, origin of the closest relatives.

spp. evolved similarly. However, *S. cohnii* 3.5PT7 seemed to be evolutionarily different from all bacteria (Fig. 1).

**Optimal pH and temperature of protease enzyme activity**

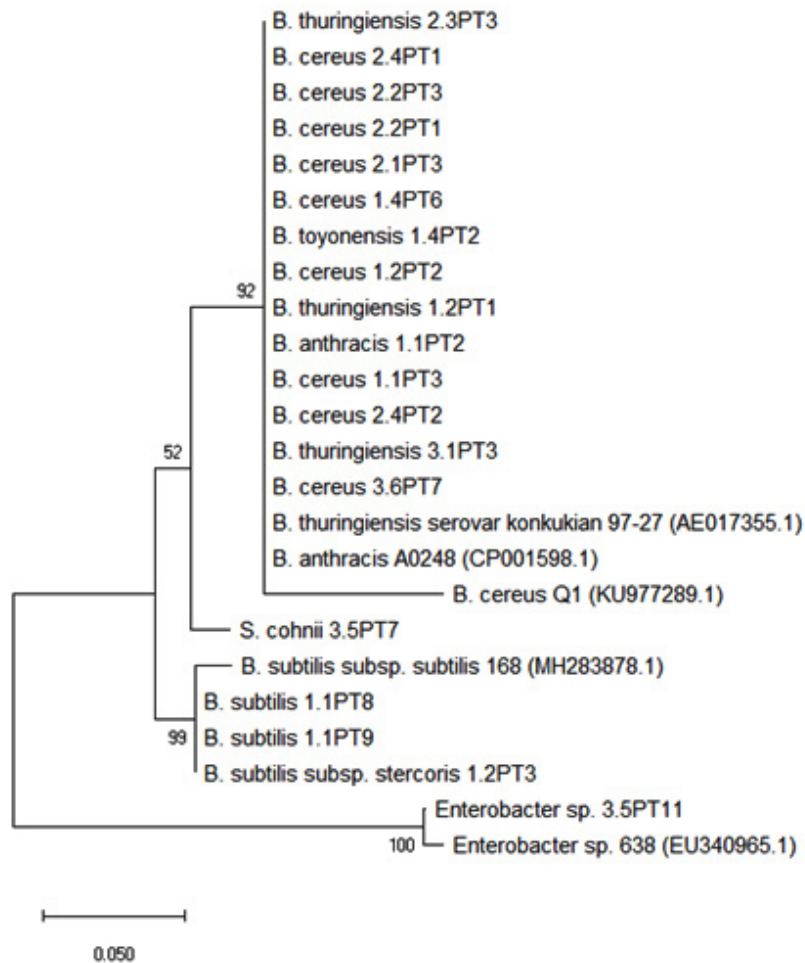
*B. thuringiensis* 2.3PT3 showed the highest specific activity at pH 8.0 when 37°C was fixed and at 65°C when pH 7.0 was fixed (Fig. 2). Thus, both optimal conditions (pH 8.0 and 65°C) were used to determine specific protease activity

and  $3.72 \pm 0.08$  U/mg (Table 3) was obtained.

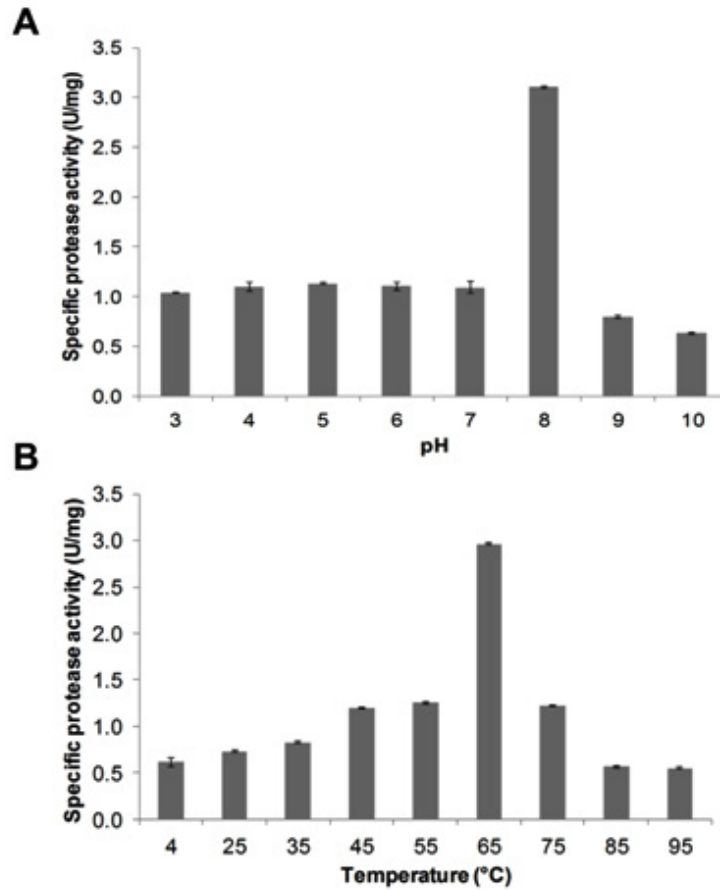
It has been reported that *Bacillus infantis* SKS1 isolated from garden soil of north India showed specific protease activity at 7.93 U/mg<sup>15</sup>. *Bacillus* sp. SKS1 was active at pH 10 and wide range of temperatures (40°C to 70°C) suggesting its application in industry demanding moderate heat and alkaline conditions. Alkaline serine protease produced by *Bacillus cereus* strain S8 (MTCC NO 11901). The optimum activity of

**Table 3.** Specific protease enzyme activity at optimal pH and temperature

Strain	Activity (U/mg)	Optimal Temp. (°C)	Optimal pH
<i>B. thuringiensis</i> 2.3PT3	$3.72 \pm 0.08$	65	8.0



**Fig. 1.** Phylogenetic tree of 19 protease-positive bacterial strains and 5 reference strains



**Fig. 2.** Optimal pH (A) and temperature (B) of protease enzyme activity from *B. thuringiensis* 2.3PT3

the protease was observed at pH 10.0 and 70°C<sup>16</sup> (Lakshmi *et al.* 2019). In addition, the protease produced by *Bacillus pumilis* MK6-5<sup>17</sup> was active at 50°C-55°C (pH 11) whereas *Bacillus licheniformis*<sup>18</sup> and *Bacillus firmus* 7728<sup>19</sup> produced proteases which are active at 37°C (pH 8.5) and 40°C (pH 9) respectively.

Our results show that protease of *B. thuringiensis* 2.3PT3 showed its maximum activity at 65°C (pH 8) as it was thermostable under alkaline conditions. Similarly, the previous report of *Bacillus subtilis* RJAS 19 has shown the optimum conditions for protease at pH 9.5 and 65°C<sup>20</sup>. This suggests that our protease from *B. thuringiensis* 2.3PT3 has a potential to be used in industries with alkalinity and high temperature.

Through enzyme characterization, further study on the thermostability, pH stability, effect of different metal ions and different substrates

is necessary. It is hoped that the production of commercial protease in Thailand enzyme industries may be more increased and in turn will be benefiting the country's economy due to self-reliance on its own resources to produce protease enzyme.

### CONCLUSION

This is the first report of identifying 19 protease-producing bacterial isolates from soil in Nasinuan Community Forest, Maha Sarakham. Most bacteria were identified as *Bacillus* spp. and two from *Enterobacter* and *Staphylococcus* genus. By far, *Bacillus*-derived proteases are the most industrially exploited. The results in this work are in accordance with the previous reports, as several *Bacillus* species are known to be protease producers. These bacteria can be used for protease



production and applied locally and nationally in food processing, agriculture, and pharmaceutical industries in Thailand. Thus, this will help lower the cost of industrial protease import from other countries, offer sustainability of local protease production and enhance the economy of the nation.

#### ACKNOWLEDGMENTS

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