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Characterization and comparison of phytase production by *Bacillus* and *Paenibacillus* strains from Thai soils

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ABSTRACT

Aims: The objective of this research was to isolate, screen and identify phytase-producing bacteria from soils and a potent isolate was selected for its phytase production.
Methodology and results: Eight spore-forming bacteria isolated from agricultural soils in Thailand were screened for their phytase production. They were identified as *Bacillus* and *Paenibacillus* strains based on their phenotypic characteristics and 16S rRNA gene sequence analyses. The phytase production by *Bacillus amyloliquefaciens* CH3-1 [Group I(a)] was 20.956 ± 0.099 U/mL, while *Bacillus subtilis* SR9-3 [Group I(b)] produced 20.588 ± 0.099 U/mL. Five isolates in Group I(c), identified as *Bacillus aryabhattai*, produced phytase at levels ranging from 2.436 ± 0.116 to 20.910 ± 0.000 U/mL, while *Paenibacillus cineris* CM5-3 (Group II) produced 1.261 ± 0.111 U/mL. A potent strain, CH3-1, produced the highest phytase when cultivated in Phytate Specific Medium (PSM) supplemented with 1% glucose, at pH 7.0 and incubated at 45 °C. Additionally, wheat bran and sorghum seed (0.5%) substrates were used to induce phytase production by replacing Na-phytate.
Conclusion, significance and impact of study: Phytase producing bacteria were isolated from soils in Thailand. Gram-positive spore forming thermotolerant *Bacillus* strains displayed higher phytase activity than a *Paenibacillus* strain. A potent strain, CH3-1, could utilize agricultural waste as a substrate, which may be useful for animal feed supplementation.

Keywords: Bacillus, *Paenibacillus,* phytase, phytate, thermotolerant bacteria

INTRODUCTION

Phytic acid [*myo*-inositol, 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate)] or phytates as mixed cation salts of phytic acid are a storage form of phosphate in nature (Jorquera *et al*., 2008). It is found in cereals, nuts, legumes and oil seeds used as food and feed. Phytic acid reduced the availability of various metal ions such as Fe, Zn, Mg and Ca, which affects digestion in humans and monogastric animals such as fish, poultry and swine (Kim *et al*., 1999; Singh *et al*., 2013). Phytases (*myo*-inositol hexakis phosphate phosphohydrolase) found in nature in plants, animal tissues, some microorganisms, are a group of enzymes that hydrolyse phytic acid or phytates releasing inorganic phosphate. At present, microbial phytases are used for biotechnological applications including environmental protection, aquaculture, agriculture, food and feed industries (Jorquera *et al*., 2008). Phytases are used as animal feed supplements to resolve the phosphorus bound in phytic acid in animal feeds (Fu *et al*., 2011). Therefore, enhancing phytate degradation by phytase enzyme in crops is useful to improve the absorption of nutrients. Furthermore, most industrial processes are done at high temperatures and the use of thermostable enzymes could possibly be advantageous. This research aimed to isolate, screen, identify and determine bacterial strains with high phytase production.

MATERIALS AND METHODS

Sources and isolation methods

Eight soil samples collected from an agriculture area in Thailand were used for bacterial isolation (Table 1). One gram of each soil samples was diluted in 99 mL of a 0.85% NaCl solution and 0.1 mL each of the appropriately diluted samples was transferred to Phytate Specific

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Medium (PSM) agar plates (per liter: 10.0 g D-Glucose, 2.0 g $CaCl_2$, 5.0 g NH_4NO_3, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 5.0 g Na-Phytate, 15.0 g agar; pH 6.5). Each plate was spread using a glass rod and incubated at 45 °C for 3 days (modified from Kerovuo and Tynkkynen, 2000). The phytate-degrading capacity of the isolates was measured by using a 2% (w/v) cobalt chloride solution and incubation at room temperature for 5 min. After this, a freshly prepared solution containing equal volumes of a 6.25% (w/v) ammonium molybdate solution and a 0.42% (w/v) ammonium vanadate solution was added, and a clear zone surrounding colonies was observed in which phytase was present (Yanke *et al*., 1998). The halo zone and colony diameters were measured as hydrolysis capacity, where (HC) = halo zone diameter/colony diameter (Dobre *et al*., 2015). The selected isolates were maintained on Luria Bertani (LB) agar slants and stored at 4 °C.

Cultivation and assay of phytase activity

Two percent (v/v) of the cultures (1.5×10^8 CFU/mL adjusted using McFarland No. 0.5) were inoculated into 20 mL of PSM and cultivated on a rotary shaker (200 rpm) at 45 °C for 2 days. The culture broth was collected and centrifuged at 4,000 rpm for 20 min at 4 °C and the supernatant was used for determination of phytase activity (Kumar *et al*., 2013). Phytase activity was determined according to the ammonium molybdate method described by Qiu *et al*. (2004). The reaction mixture (consisting of 0.5 mL of supernatant and 1 mL of 5 mM sodium-phytate prepared in a 2 mM sodium-acetate buffer at pH 6.5) was incubated at 45 °C for 30 min. The reaction was stopped by adding 1.5 mL of a colouring reagent (consisting of 50 mL, 5% ammonium molybdate; 25 mL, 0.25% ammonium metavanadate; 16 mL, 65% nitric acid and 9 mL deionized water). The liberated phosphate was measured at 615 nm (Kumar *et al*., 2013). One enzyme unit was defined as the amount of enzyme liberating 1 nmol/mL of inorganic phosphate in 1 min under the assay conditions.

Identification methods

Phenotypic characterization

Morphological and cultural characteristics of the isolates were investigated using the cells grown on an LB agar plate, incubated at 37 °C for 2 days. The Gram reaction, cell shape, spore formation, catalase, oxidase, MR-VP production, indole production, nitrate reduction, Simon's citrate utilization, H_2S production; hydrolysis of L-arginine, casein, gelatin, starch, L-tyrosine and Tween-80; acid production from carbohydrates, growth at different temperatures (30-60 °C), at different pH values (5.0-9.0) and different NaCl concentrations (w/v) were determined as previously described (Barrow and Feltham, 1993; Tanasupawat *et al*., 1998).

Genotypic characterization

The 16S rRNA gene was PCR amplified using primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') as well as the amplified 16S rRNA gene sequence and then analyzed by Macrogen®, Korea. The sequences of strain were aligned with selected sequences obtained from GenBank by using CLUSTAL_X Version 1.83 (Thompson *et al*., 1997). The alignment was edited manually to remove gaps and ambiguous nucleotides prior to construction of phylogenetic trees. A phylogenetic tree was constructed by the neighbor joining method (Saitou and Nei, 1987) with the MEGA 6 program (Tamura *et al*., 2013). The confidence values of individual branches in the phylogenetic tree were determined using bootstrap analysis (Felsenstein, 1985) based on 1,000 replications. The values for sequence similarity among the closest strains were determined using the EzTaxon server (Kim *et al*., 2012).

Effects of culture condition for phytase production

Potent selected strains were inoculated into 20 mL of PSM broth and incubated as described above. The effects of temperature were evaluated by incubating the culture at 30, 35, 40, 45 and 50 °C in PSM at pH 6.5. Different carbon sources including arabinose, fructose, galactose, glucose, glycerol, lactose, maltose, mannose and sucrose were used to evaluate their effects on phytase production. The initial pH of PSM culture was varied as 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Na-phytate, rice bran, wheat bran, peanut shell, sisal meal, peanut seed, sorghum seed, corn seed and sunflower seed were selected as substrates for phytase production. The selected strains were cultured in the PSM broth and various substrates were used to replace Na-phytate as described above.

RESULTS AND DISCUSSION

Isolation and screening

Eight isolates from soil samples in Thailand, Chiang Mai, Lampang, Nakhon Phanom, Chachoengsao, Phetchaburi, Nakhon Si Thammarat and Surat Thani were screened on PSM agar and exhibited clearing zones when incubated at 45 °C for 3 days. The halo zone of the isolates was calculated as a hydrolysis capacity that ranged from 1.36 to 2.06 (Table 1). The phytase activities of all isolates were assayed using the supernatant cultivated in PSM broth and incubated on a rotary shaker (200 rpm) at 45°C for 2 days. The isolates exhibited phytase activities ranging from 1.261 ± 0.111 to 20.956 ± 0.099 U/mL (Table 1). The isolate, CH3-1, had maximal phytase activity.

Identification

Eight isolates were Gram-positive endospore forming rods. They grew in 2% NaCl, at pH 7.0 and at 30-45 °C.

All isolates did not grow at 55-60 °C. They showed a negative Voges-Proskauer reaction, nitrate reduction, H_2S

Table 1: Isolation number, location, hydrolysis capacity (HC) and phytase activity (U/mL) of isolates.

Isolate no.	Province	Hydrolysis capacity (HC)	Phytase activity (U/mL)
CM5-3	Chiang Mai	1.44	1.261 ± 0.111
LP3-2	Lampang	1.57	11.584 ± 0.494
NN3-2	Nakhon Phanom	2.06	2.436 ± 0.116
NN5-3	Nakhon Phanom	1.50	6.695 ± 0.344
CH3-1	Chachoengsao	1.42	20.956 ± 0.099
P6-2	Phetchaburi	1.90	7.404 ± 0.122
N8-2	Nakhon Si Thammarat	1.36	20.910 ± 0.000
SR9-3	Surat Thani	1.81	20.588 ± 0.099

production and gelatin hydrolysis. Acid was produced from D-fructose, D-glucose and sucrose. The microorganisms were identified as *Bacillus* (7 isolates) and *Paenibacillus* (1 isolate) based on their morphological, cultural, physiological, biochemical characteristics and 16S rRNA gene sequence analyses (Figures 1 and 2, Table 2).

Group I(a) consisted of 1 isolate, CH3-1. Colonies were 0.1-0.4 cm, cream-coloured, irregular, lobate, crateriform or flat and opaque on LB agar. CH3-1 grew in 2-5% NaCl at pH 4.0-9.0 and at 30-45 °C. It was positive for catalase, oxidase, citrate utilization, hydrolysis of urea and casein as well as assimilation of D-fructose, glycerol, D-maltose, D-mannitol, D-mannose, salicin and D-trehalose. On the basis of its 16S rRNA gene sequence and phylogenetic tree analysis (Figure 1), isolate CH3-1 (1,487 bps) was closely related to *B. amyloliquefaciens* ATCC 23350^T with 99.0% sequence similarity. Therefore, this isolate was identified as *B. amyloliquefaciens* (Priest *et al*., 1987; Borriss *et al*., 2011).

Group I(b) also had 1 isolate, SR9-3. Colonies were 0.1-0.4 cm, cream-coloured, irregular, lobate, raised and opaque on LB agar. SR9-3 grew in 2-5% NaCl at pH 5.0-8.0 and at 30-45 °C. It was positive for catalase, hydrolysis of urea, hydrolysis of L-arginine and starch. Acid was produced from L-arabinose, D-galactose, glycerol (weakly), D-maltose, D-melibiose and D-trehalose. On the basis of its 16S rRNA gene sequence and phylogenetic tree analysis (Figure 1), isolate SR9-3 (1,486 bps) was closely related to *B. subtilis* DSM 10^T with 99.2% sequence similarity. Therefore, this isolate was identified as *B. subtilis* (Nakamura *et al*., 1999; Rooney *et al*., 2009).

Group I(c) consisted of 5 isolates, LP3-2, N8-2, P6-2, NN3-2 and NN5-3. Colonies were 0.05-0.35 cm, creamy-white-coloured, irregular, lobate, crateriform/ umbonate/flat and opaque on LB agar. All isolates grew in 2-5% NaCl at pH 7.0 and at 30-45 °C. It was positive for hydrolysis of urea and L-arginine. Acid was produced from L-arabinose, glycerol, lactose, D-mannitol and D-xylose. On the basis of its 16S rRNA gene sequence and phylogenetic tree analysis (Figure 1), isolates NN5-3 (1,486 bps), NN3-2 (1,484 bps), P6-2 (1,482 bps), N8-2 (1,492 bps) and LP3-2 (1,492 bps) were closely related to *B. aryabhattai* B8W22^T with 100.0, 99.9, 99.9, 99.7 and 99.5% sequence similarity, respectively. This result showed high similarity of their 16S rRNA gene sequence when compared with the type strain. Therefore, based on these results, all isolates were identified as *B. aryabhattai* (Shivaji *et al*., 2009).

Group II included only CM5-3. Colonies were 0.1-0.3 cm, creamy-coloured, round/irregular, smooth/lobate, flat and opaque on LB agar. Isolate CM5-3 grew in 2% NaCl at pH 7.0-9.0 and at 30-45 °C. It was positive for catalase, oxidase and hydrolysis of starch. Acid was produced from L-amygdalin, L-arabinose, D-galactose, lactose, D-maltose, D-mannitol, D-melibiose, D-melezitose (weakly), salicin, D-trehalose and D-xylose. On the basis of its 16S rRNA gene sequence and phylogenetic tree analysis (Figure 2), isolate CM5-3 (1,482 bps) was closely related to *P. cineris* LMG 18439^T with 99.9% sequence similarity. Isolate CM5-3 showed high similarity of its 16S rRNA gene sequence when compared with the type strain. Therefore, it was identified as *P. cineris* (Logan *et al*., 2004).

Effects of culture condition for phytase production

The maximal phytase activity of isolate CH3-1 was 20.956 ± 0.099 U/mL. Thus, it was selected for examination of the effects of cultivation parameters on its phytase production. Optimization of isolate CH3-1 was done in PSM medium. Firstly, cultivation of isolate CH3-1 at 30, 35, 40, 45 and 50 °C was done. Subsequently, the effects of various carbon sources and initial pH on its phytase production were studied. The phytase activity of CH3-1 was 9.683 ± 0.000, 12.972 ± 0.114, 17.516 ± 0.105, 20.591 ± 0.000 and 15.097 ± 0.102 U/mL when incubated at 30, 35, 40, 45 and 50 °C, respectively (Figure 3). Thus, the results showed that 45 °C was the optimal temperature for supporting growth of CH3-1 for phytase production. Arabinose, fructose, galactose, glucose, glycerol, lactose, maltose, mannose and sucrose were used as carbon sources in PSM medium. Results obtained showed that the best carbon source for phytase

Table 2: Differential phenotypic characteristics of the isolates.

Characteristics	Group I (a)	Group I (b)	Group I (c)					Group II
	CH3-1	SR9-3	LP3-2	N8-2	P6-2	NN3-2	NN5-3	CM5-3
Growth in 3-5% NaCl	+	+	+	+	+	+	+	-
Growth at pH 5	+	+	-	-	+	-	+	-
6	+	+	-	-	+	-	+	-
8	+	+	-	+	+	+	+	+
9	+	-	-	+	+	+	+	+
Growth at 50 °C	-	-	-	+	-	-	-	-
Catalase test	+	+	-	-	+	+	+	+
Oxidase test	+	-	+	-	-	+	+	+
Methyl red	-	-	-	-	+	+	-	-
Indole production	-	-	-	+	-	+	-	-
Citrate utilization	+	-	-	+	-	+	-	-
Urease	+	+	+	+	+	+	+	-
Motility test	-	-	+	+	-	+	-	+
Hydrolysis of:								
L-Arginine	-	+	+	+	+	+	+	-
Casein	+	-	-	+	-	-	-	-
Starch	-	+	+	+	+	+	-	+
L-Tyrosine	-	-	-	-	-	+	-	-
Tween 80	-	-	-	-	+	-	-	-
Acid from :								
L - Amygdalin	-	-	-	w	-	+	-	+
L - Arabinose	-	+	+	+	w	+	+	+
D - Galactose	-	+	-	-	+	+	+	+
Glycerol	+	w	W	+	+	+	w	-
Lactose	-	-	W	w	+	+	+	+
D - Maltose	+	+	-	-	+	+	+	+
D - Mannitol	+	-	+	+	+	+	+	+
D - Mannose	+	-	W	w	w	+	-	-
D - Melibiose	-	+	-	-	+	+	w	+
D - Melezitose	-	-	-	-	+	-	+	w
Salicin	+	-	-	-	w	+	w	+
D - Trehalose	+	+	-	w	+	+	+	+
D - Xylose	-	-	+	+	w	w	+	+

+, positive or strong acid production; w, weakly positive or moderate acid production; -, negative or no reaction.

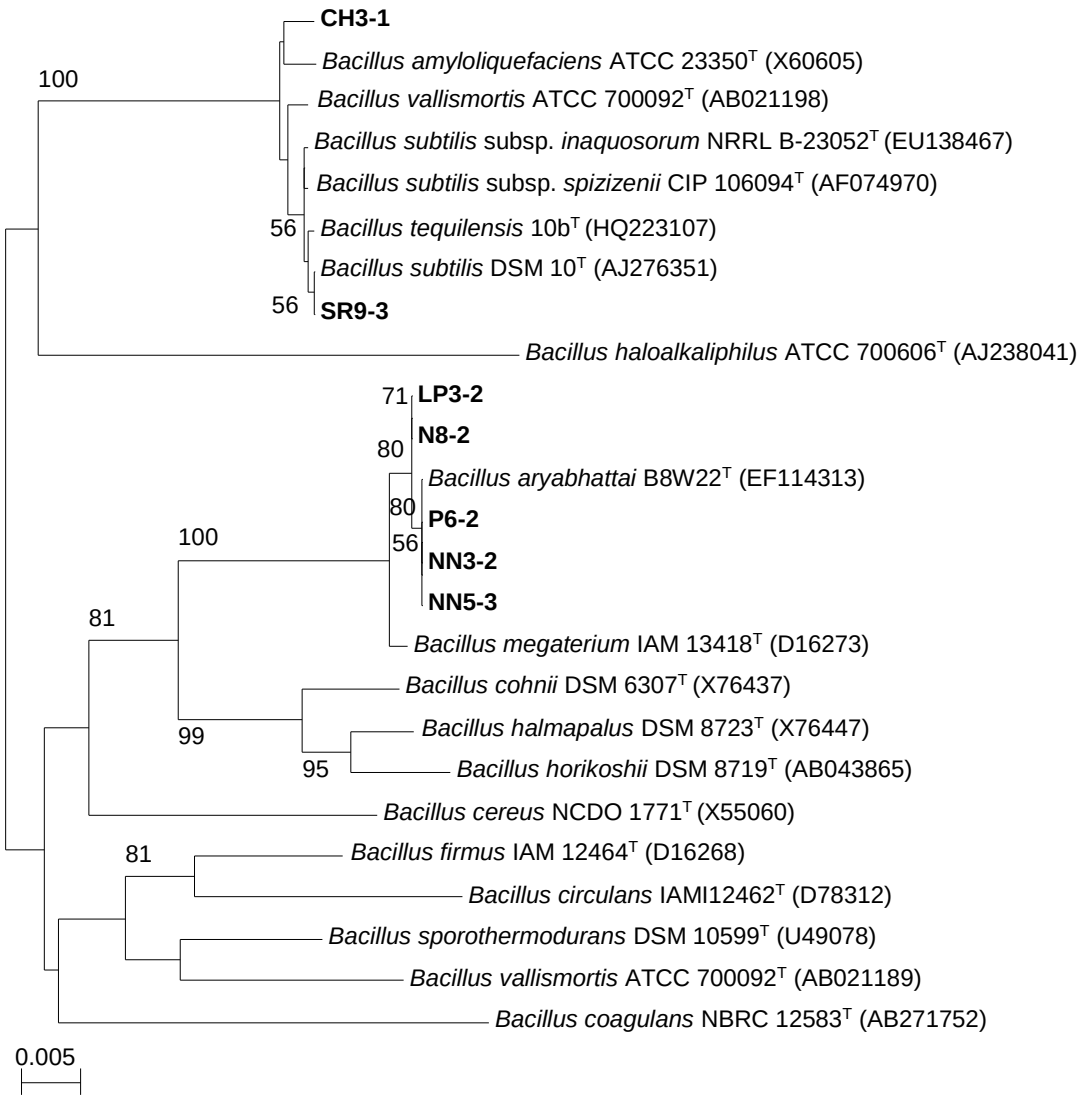

Figure 1: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between representative isolates in Group I and *Bacillus* species. Based on 1,000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.005 substitutions per nucleotide position.

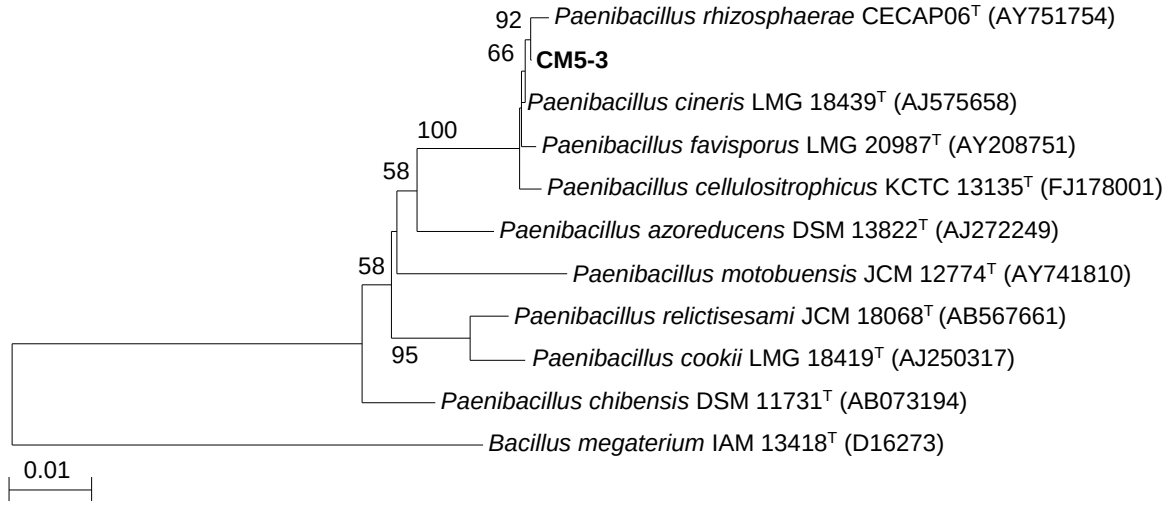

Figure 2: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between representative isolate in Group II and *Paenibacillus* species. Based on 1,000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position.

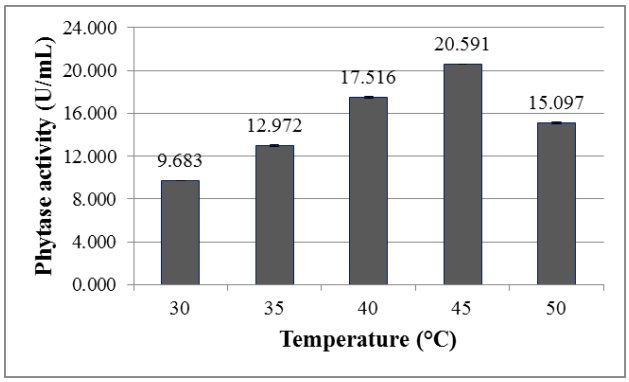

Figure 3: Effect of different temperatures on phytase production of isolate CH3-1.

production was glucose (22.776 ± 0.000 U/mL), at 45 °C when incubated for 2 days. Lactose and mannose were poor carbon sources for enzyme production since they did not induce phytase activity, as shown in Figure 4.

Cultivation of isolate CH3-1 at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0, showed phytase activities of 1.233 ± 0.650, 4.342 ± 0.186, 5.414 ± 0.186, 8.469 ± 0.279, 8.737 ± 0.246, 27.952 ± 0.093, 28.783 ± 0.186, 15.115 ± 0.093, 8.522 ± 0.093, 6.593 ± 0.093 and 3.162 ± 0.650 U/mL, respectively (Figure 5). The best pH for phytase activity was 7.0 followed closely by pH 6.5.

Na-phytate was the best substrate for inducing phytase activity, but it is expensive. Rice bran, wheat bran, peanut shell, sisal meal, peanut seed, sorghum seed, corn seed and sunflower seed are alternative substrates that can be used as low cost animal feed ingredients. Phytase activities on Na-phytate, rice bran, wheat bran, peanut shell, sorghum seed, corn seed and sunflower seed were 20.397 ± 0.478, 18.256 ± 0.000, 21.833 ± 0.837, 7.747 ± 0.359, 21.185 ± 0.000, 1.465 ± 0.239 and 5.156 ± 0.120 U/mL, respectively, while sisal meal and peanut seed were no activity. The results showed that using wheat bran and sorghum seed resulted in better enzyme activity better than Na-phytate. Furthermore, use of rice bran as a substrate showed similar phytase production to that of Na-phytase (Figure 6).

Eight isolates were Gram-positive spore forming bacteria that could grow at 45 °C. The Group I isolates were identified as *B. amyloliquefaciens*, *B. subtilis* and *B. aryabhattai*, which produced phytase at levels ranging from 2.436 ± 0.116 to 20.956 ± 0.099 U/mL. Group II isolate was identified as *P. cineri*s, which produced 1.261 ± 0.111 U/mL of phytase. *Bacillus amyloliquefaciens* (Olajuyigbe, 2016), *B. subtilis* (El-Toukhy *et al*., 2013; Alhadi *et al*., 2015) and *B. aryabhattai* (Sajidan *et al*., 2015) strains have been reported as phytase producers, while ours is the first report of *P. cineris* as a phytase producer.

The use of glucose as a carbon source for CH3-1 in PSM at pH 7.0 and incubated at 45 °C were the optimal conditions for phytase production. However, there are reports on the optimal conditions of *B. amyloliquefaciens* PFB-02 cultivated at pH 5.0 and 40 °C (Olajuyigbe, 2016). The CH3-1 strain produced phytase at higher temperatures. Greiner and Konietzny (2006) reported that *B. amyloliquefaciens* produced 20 U/mg of phytase at pH 7.0-8.0 and 70 °C, while Kim (1997) reported that *a B. amyloliquefaciens* strain was not induce by glucose, fructose, maltose or sucrose. However, glucose was found to be the best carbon source for phytase production of *B. subtilis* BPTK4 (Demirkan *et al*., 2014). Thus, the factors that affect phytase production depend on the bacterial species and strain used. In current study, wheat bran, sorghum seed and rice bran were used as substrates to replace the more costly Na-phytate. Thus,

agriculture wastes as substrates could induce phytase and are also useful for animal feed supplements.

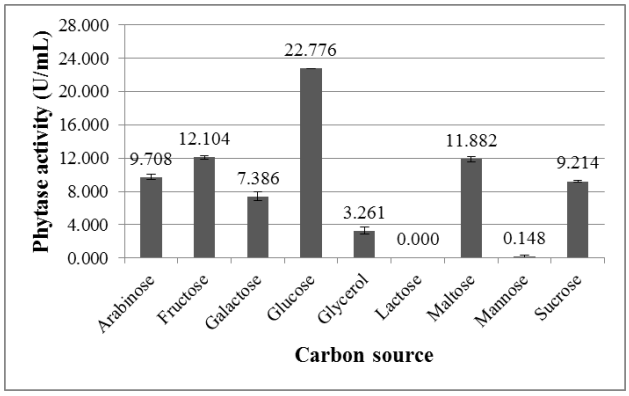

Figure 4: Effect of various carbon sources on phytase production of isolate CH3-1.

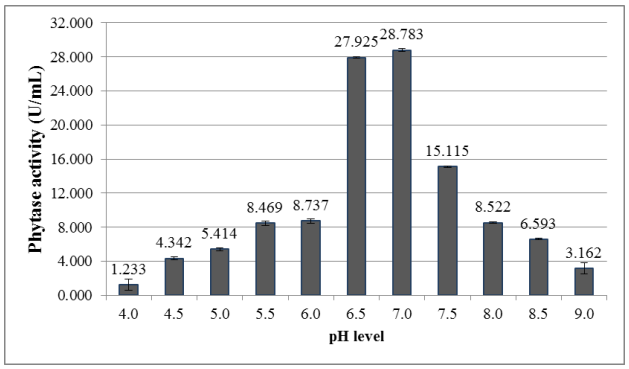

Figure 5: Effect of different pH values on phytase production of isolate CH3-1.

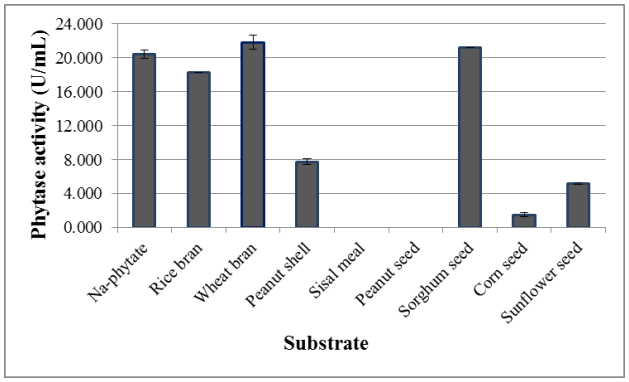

Figure 6: Effect of substrates on phytase production of isolate CH3-1.

CONCLUSION

In this investigation, phytase producing thermotolerant bacteria were isolated from soils in Thailand. The isolates were identified as *B. amyloliquefaciens*. *Bacillus amyloliquefaciens*, *B. subtilis* and *B. aryabhattai* and *P. cineris* exhibited phytase activity ranging from 1.261 ± 0.111 to 20.956 ± 0.099 U/mL. Isolate CH3-1 displayed a maximal phytase production. It produced phytase a higher level than the *P. cineris* strain.

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