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 thermostolerant 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, thermostolerant 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
Medium (PSM) agar plates (per liter: 10.0 g D-Glucose, 2.0 g CaCl₂, 5.0 g NH₄NO₃, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g MnSO₄·7H₂O, 5.0 g Na-Phytyate, 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 phytyate-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 phytyase 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 (1.5x10⁸ 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 phytyase activity (Kumar et al., 2013). Phytyase 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-phytyate 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, H₂S 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'-GGATTAGATACCTGGTA-3') and 907R (5'-CCGTCATTCTMTTTRGTTT-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, manitol, 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, sial meal, pea 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, H₂S
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 (weakly), 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. aryabhattai B8W22 with 100.0, 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 I(b) included only CM5-3. Colonies were 0.1-0.3 cm, cream-coloured, round/irregular, smooth/lobate, flat and opaque on LB agar. 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 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 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       | Lamphun      | 1.57                     | 11.584 ± 0.494          |
| NN3-2       | Nakhon Phanom| 2.06                     | 2.436 ± 0.116           |
| NNS-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          |

(U/mL) and LP3-2 (1.492 bps) were closely related to B. aryabhattai B8W22 with 100.0, 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, cream-coloured, round/irregular, smooth/lobate, flat and opaque on LB agar. 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 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).
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           | +           | +           | -           | +       | +        | +         | +         |          |
|                          | +           | +           | -           | +       | -        | +         | +         |          |
| Growth at pH 6           | +           | +           | -           | +       | -        | +         | -         |          |
| Growth at pH 8           | +           | +           | -           | +       | +        | -         | +         |          |
| 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       | +        | -         | -         | -         |
| 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. cinereus, which produced 1.261 ± 0.111 U/mL of phytase. B. 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. cinereus 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.
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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