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ORIGINAL ARTICLE

A novel phytase characterized by thermostability and high pH tolerance from rice phyllosphere isolated *Bacillus subtilis* B.S.46

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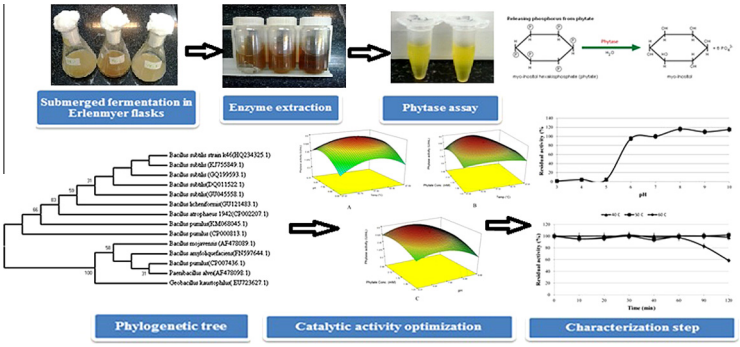
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, an extracellular alkali-thermostable phytase producing bacteria, *Bacillus subtilis* B.S.46, were isolated and molecularly identified using 16S rRNA sequencing. Response surface methodology was applied to study the interaction effects of assay conditions to obtain optimum value for maximizing phytase activity. The optimization resulted in 137% (4.627 U/mL) increase in phytase activity under optimum condition (56.5 °C, pH 7.30 and 2.05 mM sodium phytate). The enzyme also showed 60–73% of maximum activity at wide ranges of temperature (47–68 °C), pH (6.3–8.0) and phytate concentration (1.40–2.50 mM). The partially purified phytase demonstrated high stability over a wide range of pH (6.0–10.0) after 24 h, retaining 85% of its initial activity at pH 6 and even interestingly, the phytase activity enhanced at pH 8.0–10.0. It also exhibited thermostability, retaining about 60% of its original activity after 2 h at 60 °C. Cations such as Ca^{2+} and Li^{+} enhanced the phytase activity by 10–46% at 1 mM concentration. The phytase activity was completely inhibited by Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+} and the inhibition was in a dose dependent manner. *B. subtilis* B.S.46 phytase had interesting characteristics to be considered as animal feed additive, dephytinization of food ingredients, and bioremediation of phosphorous pollution in the environment.

Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) or its salt, phytate is the major storage form of phosphorus in plants and represents 1–1.5% of weight and 60–80% of total phosphorus in cereals, legumes, and oil seeds [1]. Phytate is considered an anti-nutritional factor because of its high negatively charged structure and strong ability to chelate and bind minerals such as calcium, magnesium, zinc and iron [2]. It is also known to form complexes with proteins under both acidic and alkaline pH conditions affecting the proteins' structure, thus decreasing the enzymatic activity, protein solubility and digestibility [3]. Phytate phosphorus is poorly utilized by non-ruminant animals such as pigs, poultry, human, and fish because of insufficient or lack of natural phytase activity in their gastrointestinal tract [4]. Animal feedstuffs are mainly of plant origin and therefore have a lot of phytate, but phytate phosphorous is not available for them and consequently, its excretion causes several environmental problems such as water pollution and eutrophication especially in areas of intensive livestock production [5,6].

Phytases (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate phosphohydrolases: EC 3.1.3.8 and EC 3.1.3.26) are a group of enzymes, which catalyze the stepwise removal of phosphates from phytic acid to less phosphorylated *myo*-inositol intermediates and inorganic phosphate. The presence of phytases has been reported in plants, animal tissues, and microorganisms [7]. Numerous researchers have shown that microbial phytases are more promising for the commercial production of phytase [7–9]. Although several strains of bacteria [10], yeasts [11], and fungi [9] have been isolated and studied for phytase production, currently commercial scale feed phytases are mainly derived from *Aspergillus niger* (3-phytase), *Peniophora lycii* and *Escherichia coli* (6-phytase) [7,12]. However, according to strict substrate specificity, higher heat stability, wide pH profile, and resistant to proteolysis, *Bacillus* phytases are potential alternatives to fungal ones [8,13,14]. Several *Bacillus* phytases isolated from different sources have been characterized [15–17]. There is no single phytase as an ideal phytase and therefore, there has been a continuous effort to isolate new bacterial strains producing novel and efficient phytases. Phytases are also of great interest for other applications including processing and reduction of phytate in food industry, production of individual *myo*-inositol phosphate derivatives for human health and medicine, environmental protection, soil nutrient enhancement and aquaculture [18–20].

To our knowledge, no study has been published on the application of response surface methodology (RSM) for optimizing the catalytic activity of phytase. In the present study, phytase activity of *Bacillus subtilis* B.S.46, isolated from the phyllosphere of rice plant, was optimized by RSM. Furthermore, characterization of partially purified phytase was also investigated.

Material and methods

Chemicals

All of the chemicals and reagents used in this study were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA).

Bacterial strain, inoculum preparation and phytase production

Submerged fermentation was used to evaluate the phytase activity of 70 microbial isolates obtained from the rhizosphere and phyllosphere of different fields and orchards in Iran (Agricultural Biotechnology Research Institute of Iran, Karaj, Iran). The isolates were first cultured on agar plates (g/L: nutrient broth (NB) 8, yeast extract 1, K_2HPO_4 1, KH_2PO_4 0.25, glucose 0.4, $MgSO_4$ 0.12, and agar 18) and incubated at 30 °C for 24 h. Inoculum was prepared by transferring a loop of fresh culture from the agar plate into a 50-mL tube containing 10 mL of sterile NB and incubated in a shaker incubator at 170 rpm and 30 °C for 18 h [21]. Next, each of the isolates was inoculated at the concentration of 2% into a 100-mL Erlenmeyer flask containing 25 mL of phytase production medium (g/L: sodium phytate 10, dextrin 12, yeast extract 4, meat extract 3, $MgSO_4$ 0.3).