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ISOLATION AND CHARACTERIZATION OF PHYTASE PRODUCING BACTERIAL ISOLATES FROM SOIL

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ABSTRACT

Phytase is an enzyme which breakdown the complex organic (unavailable) forms of phosphorous such as phytic acid into simpler inorganic (available) forms. Aim of the present study was to isolate, characterize and optimize physicochemical conditions of phytase producing bacteria. Phytase producing bacterial isolates were screened on phosphate solubilizing media. Isolates (PHY02, PHY03, PHY06, PHY07, PHY12, and PHY30) were identified by morphological, microscopic, and biochemical characteristics as *Lactobacillus casei*, *Enterobacter intermedius*, *Bacillus badius*, *Escherichia coli*, *Shigella spp.*, and *Klebsiella spp.***,** respectively. All isolates (PHY02, PHY03, PHY06, PHY07, PHY12, and PHY30) produced optimum level of phytase on 37°C (27, 9, 19, 40, 19, and 32 IU/ml, respectively), pH 6.5 (26, 15, 19, 41, 19, and 32 IU/ml, respectively) and 1% NaCl (27, 15, 17, 41, 18, and 32 IU/ml, respectively). PHY03, PHY06, PHY07 and PHY12 had significantly higher ($P<0.05$) enzyme production when glucose was used as sole source of corbon, while 0.3% lactose was preferred by PHY02 and PHY30. Peptone was preferred nitrogen sources for PHY02, PHY12, PHY07 and PHY30, while tryptone was preferred by PHY03 and PHY06, respectively. Phytase producing bacterial isolates may be further analyzed and developed as indigenous biofertilizers.

Key words: Rhizosphere, Phytase, *E. coli*, *Lactobacillus casei*, *Bacillus badius*

INTRODUCTION

Phosphorous is one of major nutrient and essential constituent of life (Maathuis and Frans, 2009). Phosphorous is present in both organic (generally as phytic acid) and inorganic forms in soil. Plants can uptake only inorganic form of phosphorous from soil, which generally is a growth limiting factor because of its low availability (Grotz and Guerinot, 2002). To cope with low availability of phosphorous, an enormous amount of phosphorous based fertilizers are used. Artificial fertilizers added to soil not only increase production cost but also cause environmental pollution (Sasirekha *et al.*, 2012).

Phytate is one of the major storage forms of phosphorous in plants. Although, phytate is present in soil as plant based organic matter, it remains unavailable for plants. (Lazali *et al.*, 2013; Mittal *et al.*, 2012; Shivange *et al.*, 2012). Phytate has strong binding capacity to mono or divalent cations in soil (Cerino *et al.*, 2012). Due to its unusual molecular structure phytate can also form complexes with other nutrients such as metallic ions (Ca, Mg, Fe, Cu etc.) rendering them unavailable to plants (Selle *et al.*, 2012; Shim and Oh, 2012). Phytase is an enzyme that can hydrolyze the complex organic forms of phosphorous such as phytate into simpler forms by sequential hydrolysis of phosphate ester bonds and release the usable form of inorganic phosphorous (Shin *et al.*, 2001; Shivange *et al.*, 2010). It is a natural enzyme often used for breaking down and increasing the nutritional quality of phytate containing compounds. Phytase is widely distributed among plants, microorganisms and animal tissues. Bioaugmentation of soil by phytase producing bacteria as biofertilizers can increase the bioavailability of phosphorous to plants, increase plant growth and decrease phosphorous pollution (Kumar *et al.*, 2013; Miao *et al.*, 2013). Moreover, uptake of other essential metal ions also increase by adding phytase producing microbes in soil (Pontoppidan *et al.*, 2012).

Because of its potential biotechnological applications, interest in the isolation of new bacterial isolates, producing novel and efficient phytases, is increasing (Shim and Oh, 2012). There are only few reports of phytase producing bacteria in Pakistan, therefore present study was designed to isolate, characterize and optimize the growth conditions of phytase producing bacteria from Pakistan which may further develop as an indigenous biofertilizers.

MATERIALS AND METHODS

Isolation and screening of phytate hydrolyzing bacteria: Soil samples (n=15) were collected from different areas of Punjab from a depth of 2-5 cm and transported to bacteriology laboratory, UVAS at low

temperature. Tenfold serial dilutions of each sample were plated on Phytase Screening Media (PSM) and incubated at 37ºC as described previously (Kumar *et al.*, 2011). PSM was prepared by dissolving 3g glucose; 1g tryptone; 1g sodium phytate; 0.3g $CaCl_2$; 0.5g $MgSO_4$; 0.04g $MnCl_2$; 0.0025g $FeSO_4$; and 15g agar in 1 liter of distilled water. Following incubation, colonies showing zone of phytate hydrolysis (>6 mm diameter) were selected, purified and stored at -20ºC in 15% glycerol till further analysis.

Identification of bacterial isolates: Selected bacterial isolates were identified by colony morphology, microscopic examination (Gram staining, spore staining, capsular staining) and biochemical characteristics (Catalse test, Oxidase test, Urease test, Coagulase test and IMViC tests)) following Bergey's manual of Determinative Bacteriology, 9th edition (Holt *et al.*, 1994).

Physico-chemical optimization of enzyme production: Physical (temperature, pH, and osmotic pressure) and chemical conditions (carbon and nitrogen source) for optimum enzyme production were determined by using PSM broth as described previously (Gunashree and Venkateswaran, 2008). To optimize physical factors, bacterial cultures were grown in in PSM broth with different pH (5.5, 6.5, and 7.5), osmotic pressure (1%, 2%, 4%) and incubated at different temperatures (28°C, 37°C, and 42°C). For chemical optimization, bacterial cultures were also grown in PSM broth modified with different carbon sources (glucose, lactose and maltose) at different concentrations (0.1%, 0.3%. 0.5% each), and different nitrogen sources (peptone, tryptone and urea) at different concentrations (0.07%, 0.1%, and 0.3% each). After 48 hours incubation, enzyme activity of cell free supernatant of each isolate was determined.

Phytase activity assay: Ferrous sulphate molybdenum blue method was used for quantification of enzyme activity as described previously (Selvamohan *et al.*, 2012). Exponentially growing isolates in PSM broth were centrifuged to obtain cell free supernatant (CFS). The CFS (150µl) of each isolate was mixed with 600µl of substrate solution containing 0.1 M tris-HCl, 2 mM sodium phytate and 2mM $CaCl_2$. The mixture was incubated at 37°C for 30 minutes. Reaction was stopped by the addition of 5% trichloroacetic acid (TCA) followed by the addition of 150 µl of coloring reagent. Coloring reagent was freshly prepared by mixing 4 volumes of 1.5% (w/v) ammonium molybdate with 5.5% (v/v) sulfuric acid and 1 volume of 2.7% (w/v) ferrous sulfate solution. After the addition of coloring reagent, absorbance was taken at 700 nm. To determine the amount of inorganic phosphorous released the absorbance was compared to the standard curve, prepared by using different concentrations of K_2HPO_4 as a source of inorganic phosphorous. Enzyme activity was measured in terms of enzyme units from the amount of inorganic phosphorous released. The enzyme activity (IU/ml of CFS) was defined as the micromoles of inorganic phosphate liberated in one minute.

Statistical Analysis: Enzyme activity data (IU/ml) was presented as Mean ± S.D and analyzed one-way ANOVA followed by Tukey's multiple comparison tests by graphpad prism statistical software at $P < 0.05$.

RESULTS

Six bacterial isolates (PHY02, PHY03, PHY06, PHY07, PHY12, and PHY30) were selected as phytase producers on the basis of their distinguished ability to hydrolyze phytate > 6mm) on PSM. Isolates were identified as *Lactobacillus casei* PHY02, *Enterobactor intermedius* PHY03, *Bacillus badius* PHY06, *Escherichia coli* PHY07, *Shigella sonnei* PHY12, and *Klebsiella pneumonia* PHY30 following the keys given in Bergey's manual of Determinative Bacteriology.

Effect of physical parameters (temperatures, pH and osmotic pressure) on enzyme production by selected isolates is given in Figure 1. Optimum production of phytae by *Lactobacillus casei* PHY02, *Enterobactor intermedius* PHY03, *Bacillus badius* PHY06, *Escherichia coli* PHY07, *Shigella sonnei* PHY12, and *Klebsiella pneumonia* PHY30 (27, 9, 19, 40, 32, and 19 IU/ml, respectively) was at 37°C (Fig 1A). *Escherichia coli* PHY07 showed highest enzyme production, followed by *Klebsiella pneumonia* PHY30 and *Lactobacillus casei* PHY02. *Bacillus badius* PHY06 showed similar enzyme activity at 37°C and 42°C but it was significantly reduced at low temperature ($P<0.05$). Effect of pH on phytase production by selected isolates is represented in figure 1B. All isolates produced maximum amount of phytase at pH 6.5. At pH 6.5, enzyme activities of CFSs of PHY02, PHY03, PHY06, PHY07, PHY12, and PHY30, were 26, 15, 19, 41, 19, and 32 IU/ml, respectively. Increase in osmotic pressure had negative effect on enzyme production. Highest enzyme activity was observed at 1 % NaCl (Figure 1C).

Effect of carbon source on phytase production of selected isolates is shown in figure 2. PHY03, PHY06, PHY07, PHY12 had significantly higher ($P<0.05$) enzyme production in glucose, while PHY02 and PHY30 showed higher ($P<0.05$) enzyme activity at 0.3% lactose. Effect of nitrogen source on phytase production by different isolates is presented in figure 3. PHY02 and PHY12 had better enzyme production at 0.1% peptone, while PHY07 and PHY30 had significantly higher phytase activity in media modified with 0.3% peptone. Addition of tryptone in growth medium significantly enhanced the enzyme production by PHY03, and PHY06.

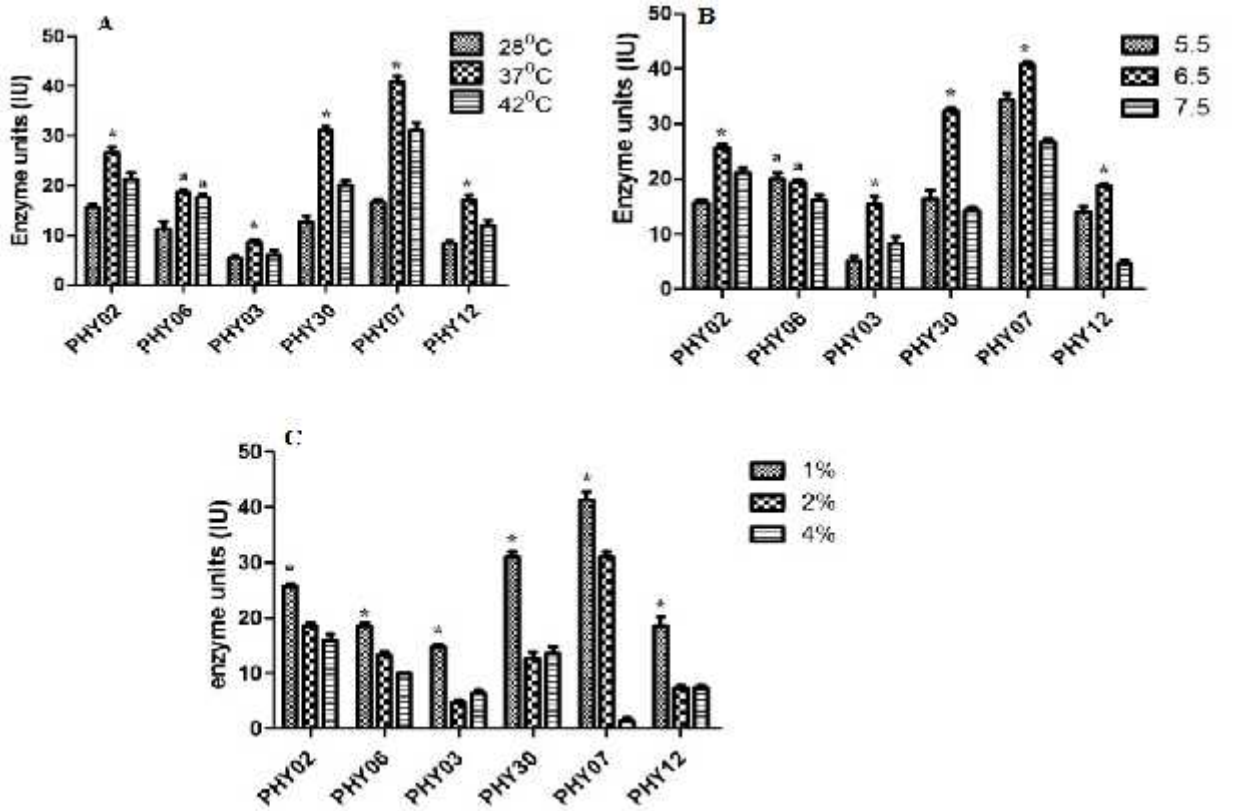

Fig 1: Effect of Physical parameters on enzyme production by selected isolates. A: Temperature, B: pH, C: Osmotic pressure

[*] Indicate statistically significant difference (P< 0.05) from the other two treatments in the same group

[a] Indicates no significant difference with each other but statistically significant difference (P< 0.05) third treatment in the same group

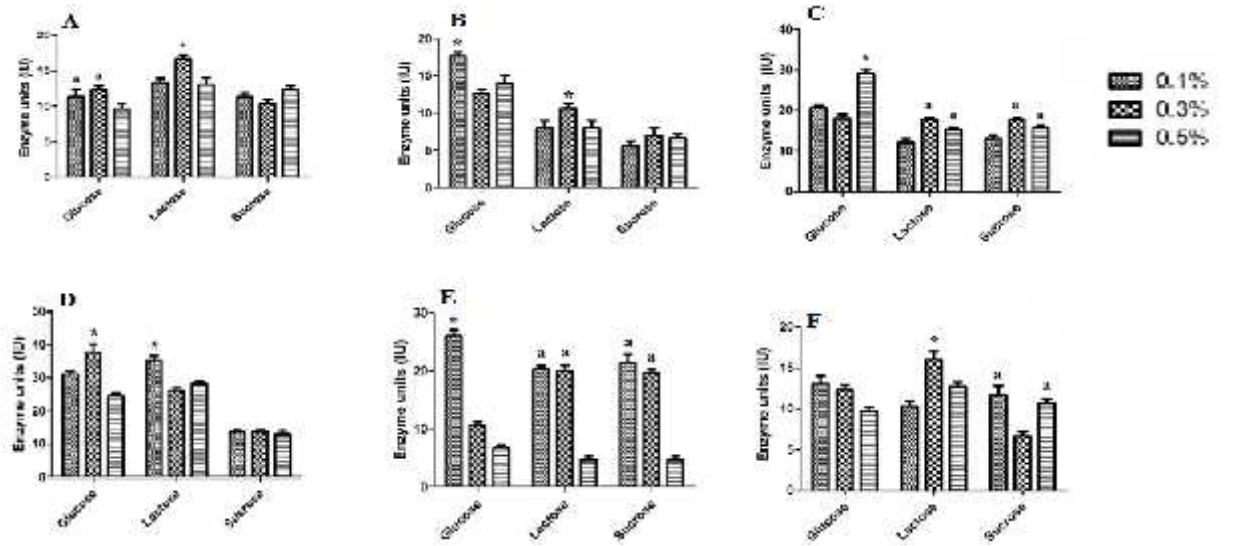

Fig 2: Effect of Carbon source on enzyme production by selected bacterial isolates. A: *Lactobacillus casei* PHY02, B: *Enterobactor intermedius* PHY03, C: *Bacillus badius* PHY06, D: *Escherichia coli* PHY07, E: *Shigella sonnei* PHY12, and F: *Klebsiella pneumonia* PHY30

[*] Indicate statistically significant difference (P< 0.05) from the other two treatments in the same group

[a] Indicates no significant difference with each other but statistically significant difference (P< 0.05) third treatment in the same group

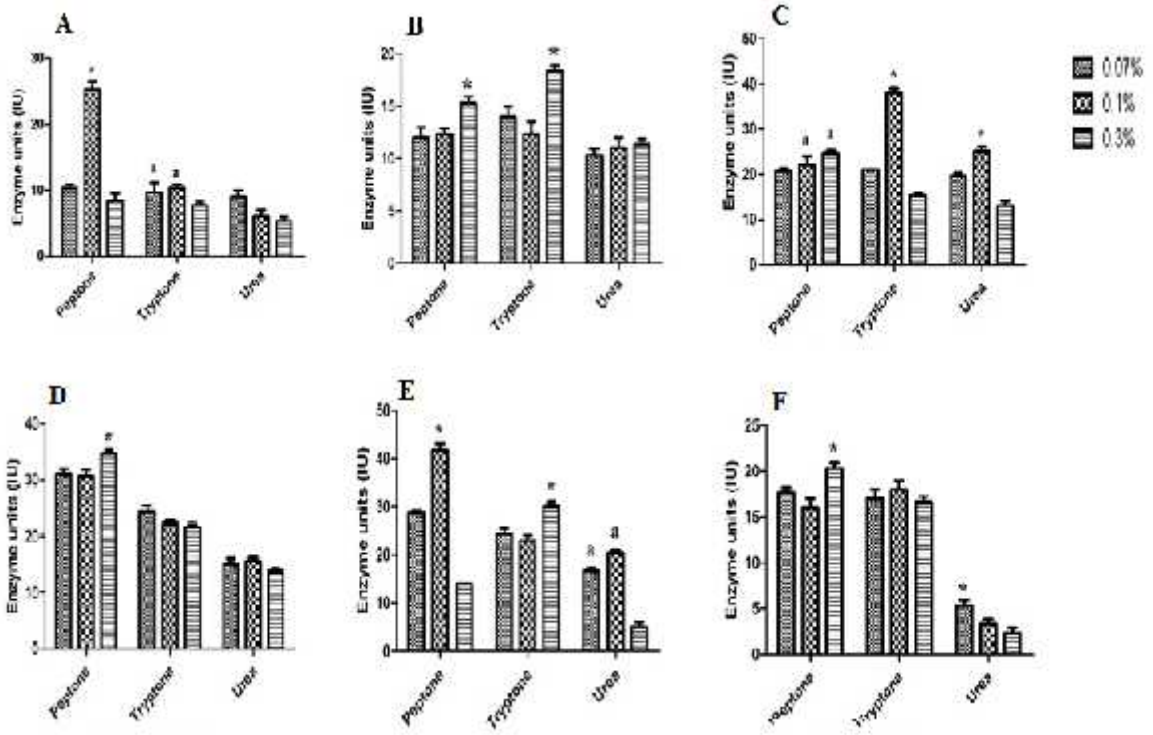

Fig 3: Effect of Nitrogen source on enzyme production by selected bacterial isolates. A: *Lactobacillus casei* PHY02, B: *Enterobactor intermedius* PHY03, C: *Bacillus badius* PHY06, D: *Escherichia coli* PHY07, E: *Shigella sonnei* PHY12, and F: *Klebsiella pneumonia* PHY30

[*] Indicate statistically significant difference (P< 0.05) from the other two treatments in the same group

[a] Indicates no significant difference with each other but statistically significant difference (P< 0.05) third treatment in the same group

DISCUSSION

Phytase, an enzyme responsible for the breakdown of organic phosphorous containing compounds, is widely distributed in plants, microorganisms and in some animal tissues (Konietzny and Greiner, 2002; Vohra and Satyanarayana, 2003). Microbial phytase are considered to have enormous biotechnological potential (Vucenik and Shamsuddin, 2003). Although, many plants produce phytase enzyme to some extent, the presence of phytase producing bacteria in soil has been attributed with increased available phosphorous and higher plant growths (Ranjan *et al.*, 2013). Different researchers have screened different habitats for phytase producing bacteria including poultry feaces (Selvamohan *et al.*, 2012), boggy water (Shamna *et al.*, 2012), soil (Acuña *et al.*, 2011), soils of cattle and poultry sheds, fields of cereals and pulses, rotten fruits and vegetables, and rhizospheric soil (Mittal *et al.*, 2012). Present study describes the phytase producing bacteria from wheat rhizosphere, which are believed to be rich source of phytase producing bacteria.

All isolates had phytase production over a wide range of physical and chemical conditions. For all isolates (PHY02, PHY03, PHY06, PHY07, PHY12, and PHY30), optimum physical conditions for enzyme production were 37°C, 6.5 pH, and 1% NaCl. Although, cell free supernatants of all isolate showed enzyme activity on 28°C and 42°C, pH 5.5 and 7.5, and 2% and 4% NaCl, highest activities were at 37°C, 6.5 pH, and 1% NaCl ($P < 0.05$). Yoon *et al.*, (1996) also isolated *Enterobactor spp.* from rhizospheric soil of leguminous plant and observed maximum production of phytase at 37°C and pH ranging from 6.0 to 8.0. Depending on origin of bacterial isolates, optimum conditions for enzyme production may vary. In another study, lactic acid producing bacteria had highest phytase activity at pH 5.0 (Tang *et al.*, 2010). Escobin-Mopera *et al.*, (2012) reported highest phytase production at pH 4.0 and 50°C by *Klebsiella pneumonia* 9-3B

Favorable nutrient source, in appropriate amount, is vital for enhanced production of phytase. PHY03, PHY06, PHY07, PHY12 had significantly higher ($P<0.05$) growth and enzyme production when incubated in media modified with glucose. Glucose has been revealed as favorable carbon source in previous studies as well (Shamna *et al.*, 2012). PHY02 and PHY30 had highest enzyme activity in media modified with 0.3% lactose sugar. Addition of nitrogen source in growing media also had significant effect on enzyme production.

PHY02, PHY03, and PHY12 had better enzyme production at 0.1% peptone while PHY07 and PHY30 had significantly higher production in media modified with 0.3% peptone. Different nitrogen sources such as yeast extract was found better source of nitrogen for phytase production from *B. subtilis* by Shamna *et al*., (2012). *B. badius* PHY06 had significantly higher enzyme production in media supplemented with tryptone. Results indicate that optimum conditions and preferred nitrogen and carbon sources are specie and strain specific.

Overall, study presents different phytase producing bacteria and their physicochemical optimization. *Escherichia coli* PHY07 produced highest amount (42 IU/ml) of enzyme followed by *Klebsiella pneumonia* PHY30, *Shigella sonnei* PHY12, *Lactobacillus casei* PHY02, *Bacillus badius* PHY06 and *Enterobactor intermedius* PHY03. Encouraging results from the present study may insinuate for further development of these strains as an indigenous biofertilizers.

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