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PHYTASE PRODUCTION BY *Enterobacter cloacae*

SULIASIH* AND SRI WIDAWATI

Research Center for Biology, Indonesian Institute of Sciences, Bogor 16911, Indonesia

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ABSTRACT

This study aims to isolate the phytase producing bacteria (PPB), a plant growth promoting rhizobacteria (PGPR), from *Vigna sinensis* rhizosphere and to optimize its physicochemical conditioning. Phytase is an enzyme that can hydrolyze the phosphoester bond in organic phosphorus (phytic acid) to form ester phosphate and inorganic phosphate, the available forms of phosphorus. To test its ability to hydrolyze organic phosphates (calcium phytate), the phytase was screened in solid and liquid phytase screening medium (PSM). After isolation, a total of 13 bacteria were positive for this enzyme's production as indicated by the clear zones of hydrolysis observed around the colony. *Enterobacter cloacae* strain B1 had the largest hydrolysis efficient (3.43) on solid medium. The phytase-production of the *Enterobacter cloacae* strain grown in liquid PSM, showed 0.92 U/mL after 48 hours of incubation. This strain produced optimum levels of phytase in the presence of lactose and monoammonium phosphate ($NH_4H_2PO_4$), as carbon and nitrogen sources, respectively, at 30 ^oC and pH 5.0. The PPB obtained in this study are recommended for further research as to their use as plant biological fertilizers.

Keywords: *Enterobacter cloacae*, phytase, phytase producing bacteria

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria commonly found in the rhizosphere, root surface or any area associated with plant roots. PGPR is able to boost plant growth and protect plants from diseases and abiotic stress through various mechanisms. Among the many important mechanisms of PGPR are: biological nitrogen fixation, ACC deaminase activity, production of siderophores, phytohormones and phosphate dissolution (Grover *et al.* 2011; de Souza *et al.* 2015). In the rhizosphere, microorganisms play very important roles in the transformation and mobilization of micro and macro nutrients in the soil, thus increasing plant growth (Jha *et al.* 2012). Soil phosphorus is an important source of nutrient for plant growth, as well as development of other macro nutrients. However, phosphorus has low natural availability due to the very slow process of phosphorus solubilization into the available form. In contrast, its transformation to the insoluble form is fairly rapid (Jorquera *et al.* 2011).

Nearly 30 - 65% of the total phosphorus (P) in the soil is in its organic P form and is not available for plant use. Phytate is one of these dominant soil organic P. Phytate possesses strong bonds with mono or divalent cations and is also able to form complexes with other nutrients, such as metal ions (Ca, Mg, Fe, Cu) (Cerino *et al.* 2012; Selle *et al.* 2012; Shim & Oh 2012). Some PGPRs are able to dissolve organic or inorganic phosphate, thereby making P available for plant growth. PPB are members of PGPR that have the ability to hydrolyze phytate by secreting phytase that provides inorganic P that are available for plants (Greiner *et al.* 2007; Shivange *et al.* 2010; Richardson & Simpson 2011). It is contained in plants, microorganisms and animal tissues. PPB are widely found in agricultural fields, grasslands and forests. Among the isolated phytase-producing bacteria from various rhizospheres are: Enterobacter, *Bukholderia, Pseudomonas* and *Pantoea* from the

*Corresponding author, e-mail: lishadari@yahoo.co.id
All authors contributed equally to this work

rhizosphere of legume white lupin (*Lupinus albus*) and other plant rhizosphere (Yoon *et al.* 1996; Unno *et al.* 2005; Jorquera *et al.* 2008).

Phytase production is greatly affected by the composition of the medium used for bacterial culture, particularly the physical and nutritional conditions that can significantly affect the enzymatic production. The objective of the experiment was to isolate PPB from leguminous plant rhizosphere and to optimize phytase production by *Enterobacter cloacae* under various physical conditions, such as incubation time, initial pH, temperature and when using different sources of carbon and nitrogen.

MATERIALS AND METHODS

Bacterial Isolation

Phytase producing bacteria were isolated from the soil samples taken from the legume plant rhizosphere in Cibinong, West Java. One gram of soil was dissolved in a 9 mL 0.8% sterile NaCl solution and was serially diluted. Around 0.2 mL of the final solution was placed in a sterile Petri dish and then poured with phytase screening medium (PSM) agar (1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.01% NaCl, 0.05% KCl, 0.001% $FeSO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 0.001% $MnSO_4$, pH 6.5 with 0.5% calcium phytate) (Kerovuo *et al.* 1998). The petri dish was incubated for 7 days at room temperature and monitored for colonies growing halo zones. Colonies with halo zones around them were further purified with repeated subcultures. The colony and halo zone diameters were measured after 1 - 7 days of incubation. The halo zone formed surrounding the colony revealed phytate hydrolization and was expressed as hydrolysis efficiency, as follows; Hydrolysis Efficiency (HE) = (Diameter of halo zone - diameter of colony)/Diameter of colony (Dobre *et al.* 2015).

Identification of the Selected Producing Phytase Bacteria

Thirteen phytase producing bacteria (PPB) isolates from the legume rhizosphere, namely PPB strain B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12 and B13, demonstrated the ability to hydrolyze Ca phytate (hydrolysis efficiency) in solid PSM, of which PBB strain B1 has the highest hydrolysis efficiency (3.43). The strain was further identified and analyzed following a method developed by Otsuka *et al.* (2008) based on 16S rRNA gene sequences with 16S-9F (5-GAGTTTGATCCTGGCCC-3) and 16S-15 10R (5-GGCTACCTTGTTACGA-3) primer.

Phytase Activity

The bacterial isolates were inoculated in a 50 mL of liquid phytase media, then incubated in a rotary shaker (200 rpm) at room temperature (30 °C) for 24, 48, 72 and 92 h. The culture was then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was extracted as extracellular source of phytase and calcium (Ca) phytate was used as the substrate in the phytase activity assessment. The enzymatic activity was determined by measuring the amount of inorganic phosphate produced. The reactant mixture comprising 0.5% calcium phytate was dissolved in sodium acetate buffer (0.1 M, pH 5.5), and 0.1 mL of the supernatant. After incubation for 30 min at 45 °C, the reaction was inhibited by adding 5% trichloroacetic acid. Around 160 µL of reagent dye, consisted of 10 N H_2SO_4, 10% ammonium molybdate and 5% $FeSO_4$, was then added. The isolate was allowed to stand for 30 min after incubating at 45 °C. The absorbance was measured by using a spectrophotometer set at 660 nm wavelength. One enzyme unit (IU) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate in 1 min (Kumar *et al.* 2013).

Physico-chemical Optimization on Phytase Production (Sreedevi & Reddy 2012)

Effect of Incubation Time

To investigate the optimum time for growth and production, 10% of 10^9 cfu/mL *Enterobacter cloacae* inoculum was inoculated into 100 mL of liquid PSM using a 250 mL Erlenmeyer flask and incubated in a rotary shaker at 120 rpm for 4 days at room temperature. The culture was harvested at an interval of 24, 48, 72 and 96 h.

Effect of pH

To obtain the optimum pH, *E. cloacae* was grown in various initial pH i.e., 3.0, 4.0, 5.0, 6.0,

7.0 and 8.0. The liquid PSM was adjusted using 1 N HCl and 1 N NaOH.

Effect of Temperatures

To evaluate the effect of temperature on the production levels, *E. cloacae* was incubated for 48 h in liquid PSM at different temperatures (30, 40 and 50 °C) at pH 6.5.

Effect of Nitrogen (N) Sources

To determine the effect of N sources on production, the organic nitrogen (tryptone and beef extract) and inorganic nitrogen {$(NH_4)H_2PO_4$ and $NH_4(NO_3)$} were used to replace $(NH_4)_2SO_4$ in the liquid PSM at pH 6.5 at 30 °C.

Effect of Carbon (C) Sources

To determine the effect of carbon on production, the bacteria were inoculated with $(NH_4)_2SO_4$ as the N source, in the liquid PSM at pH 6.5 and 30 °C, and supplemented with different C sources i.e., glucose, dextrose, lactose and maltose.

RESULTS AND DISCUSSION

Bacterial Isolation

Phytase producing bacteria (PPB) are plant growth promoting rhizobacteria (PGPR) that have the ability to hydrolyze phytate by secreting phytase to produce phosphate esters and inorganic phosphorus, allowing P to become available for plant use (Greiner *et al.* 2007; Shivange *et al.* 2010; Richardson & Simpson 2011). The activities of all bacteria, assayed using PSM agar, were indicated by the formation of clear zones around the colony.

Thirteen bacterial isolates from the legume rhizosphere demonstrated the ability to hydrolyze Ca phytate in PSM solid (Fig.1). The hydrolysis efficiency, based on halo zone and colony diameter, ranged from 0.56 to 3.43 (Fig. 2). In another study, the hydrolysis efficiency from 54 PPB that were isolated from rhizosphere soil, (cattle shed soil and poultry farm soil) ranged from 4 to 200% (Sreedevi & Reddy 2012). Some PPB were isolated from various rhizospheres around legume *Lulinus albus* (L.) (Unno *et al.* 2005; Acuna *et al.* 2011). Similarly, PPB bacteria isolated from various plant rhizospheres grown on volcanic soils, such as wheat (*Triticum aestivum*), oats (*Avena sativa*), lupin (*Lupineus luteus*), *Lolium perenne* and *Trifolium repens*, also showed similar ability to use sodium (Na) phytate and Ca phosphate on agar media (Jorquera *et al.* (2008).

Bacterial Identification

The isolate B1 that produced the highest hydrolysis efficiency (3.43) was identified as *Enterobacter cloacae*. Partial sequences of 16S rRNA genes were compared to the NCBI GeneBank database by using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed using the neighbor-joining methods of the MEGA 7 program. Sequences from all species of *Enterobacter* genus were referred to the NCBI database. The corresponding GeneBank accession numbers were labeled after the name of the species and strains. Associated taxa were clustered in the 1,000 replicates from the boostrap test and the substitution model used Junkes-Cantor model with gamma (1). *Aquifex pyrophilus* Kol5a was used as the outgroup taxon to determine the root of the tree. The boostrap value of 77% for the *Enterobacter cloacae* subsp. and the B1 (B184a) sequence being found in the *Enterobacter cloacae* group, suggested that it had members within the *Enterobacter* genus and was similar with *Enterobacter cloacae* (Fig. 3).

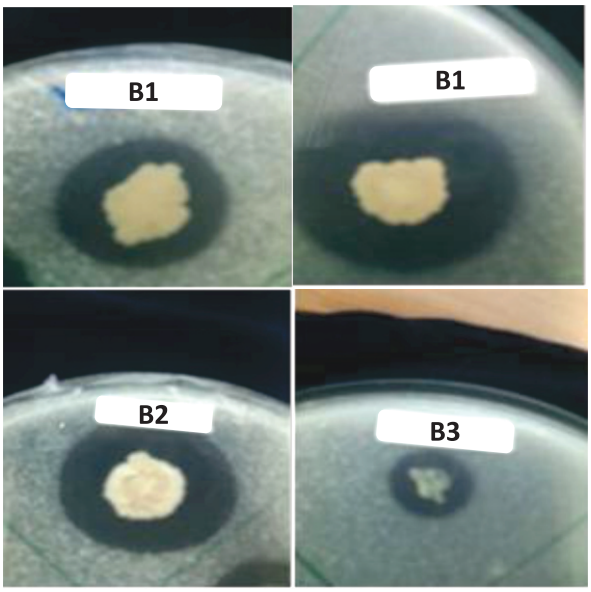

Figure 1 Halo zone around Phytase Producing Bacterial (PPB) colony on solid PSM
Notes: B1 = *Enterobacter cloacae*; B2 = unidentified isolate; B3 = unidentified isolate
Photo of colonies were taken from one Petri dish which was divided into 4 sections.
Each photo was taken from different Petri dishes.

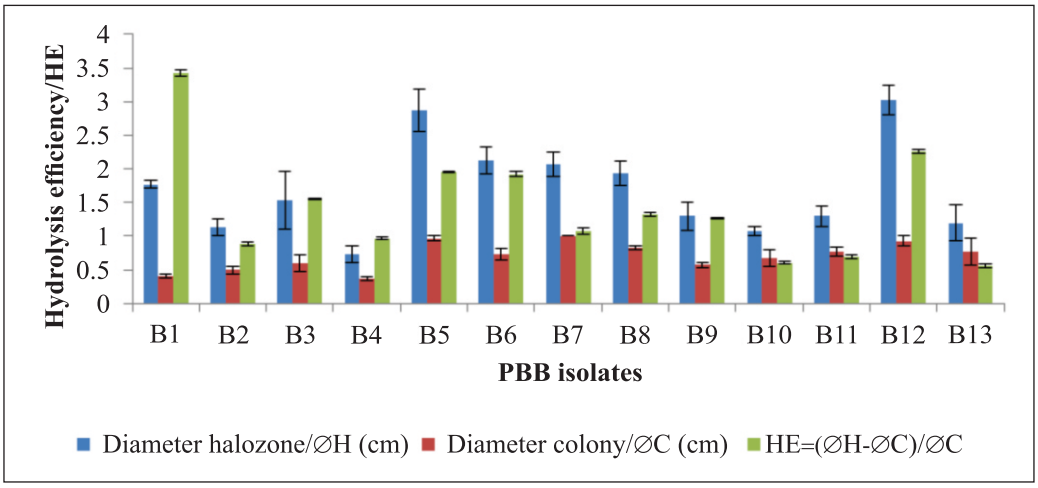

Figure 2 Hydrolysis efficiency of the isolates

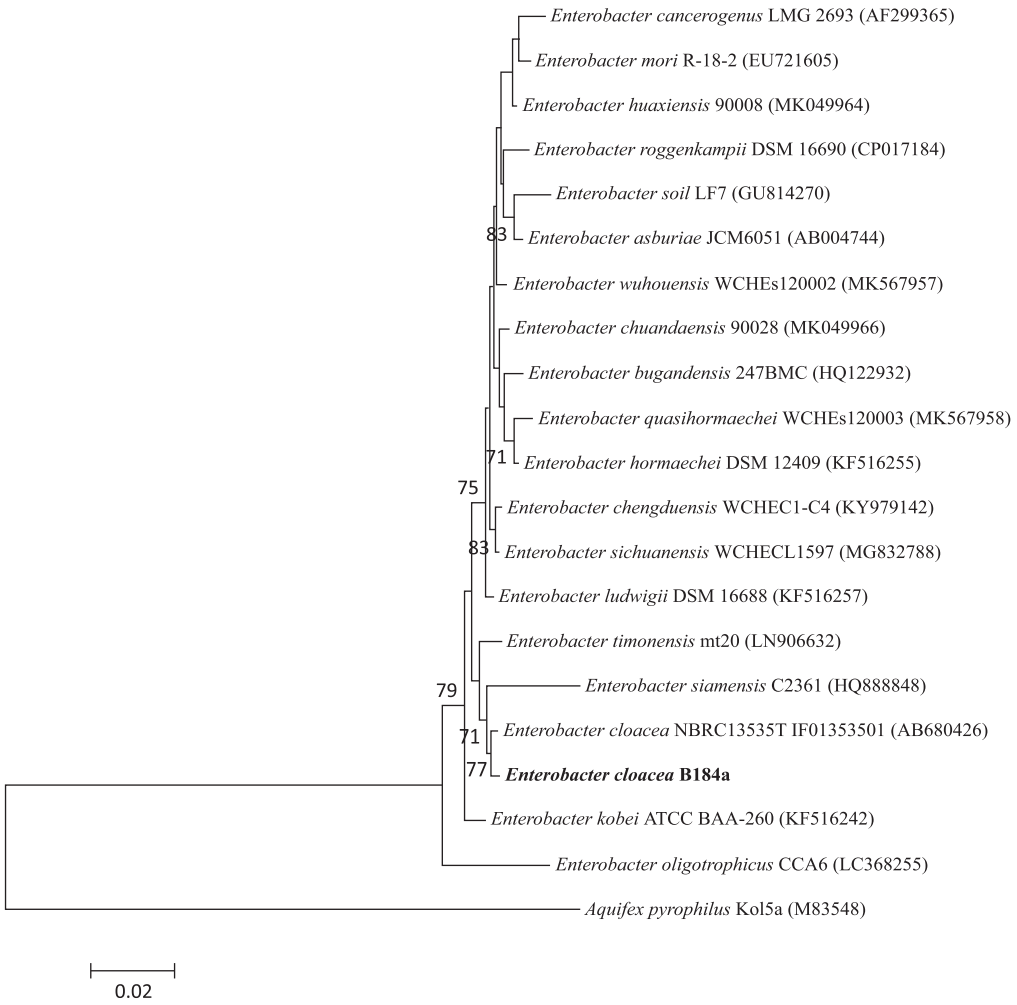

Figure 3 The phylogenetic tree of B1 isolates

Physico-chemical Optimization on Phytase Production

Effect of Incubation Time

Quantitative assesment on liquid PSM showed that *Enterobacter cloacae* bacteria was able to produce phytase of 0.92 U/mL at 48 h of incubation (Fig. 4). Similarly, *Bacillus laevolacticus* isolated from legume rhizosphere was able to produce phytase (Gulati 2007). *Bacillus licheniformis* grown in shakers under optimum conditions also resulted in high activity (0.276 U/mL) (Fu *et al.* 2011). *P. aeruginosa* isolated from rhizosphere soil samples showed an activity of 22.165 U/mL (Sasirekha *et al.* 2012). Comparably, the presence of activity produced by bacteria grown on PSM media containing Na phytate, both qualitatively and quantitatively, was about of 2.24 - 2.58 U/mL and 12.85 U/mL, respectively (Li *et al.* 2013; Tungala *et al.* 2013).

Incubation time plays an important role in maximum enzyme production. Phytase activity was observed after a 24-hour incubation period and a significantly high level of enzyme activity (0.92 U/mL) was obtained during 48 h of incubation, which then decreased at 72 and 92 h (Fig. 4). The production period was different from one bacteria to another. The production started 24 hours after incubation and increased to optimum levels thereafter. This result conforms with Kasli *et al.* (2016) that the maximum phytase production of *Enterobacter cloacae* strain PSB 45 was found at 48 hours of incubation. Similarly, the optimum production of *E. aerogenes* was at 48 h (Muslim *et al.* 2018). In another study, a stationary growth phase occurred around 48 h (109 U/mL) and phytase production occurred after 36 hours of cultivation (Shamna *et al.* 2012). Moreover, the maximum activity of *Pseudomonas aeruginosa* and *Aspergillus niger* were found at 24 h and 48 h of incubation, respectively (Ogbonna *et al.* 2017).

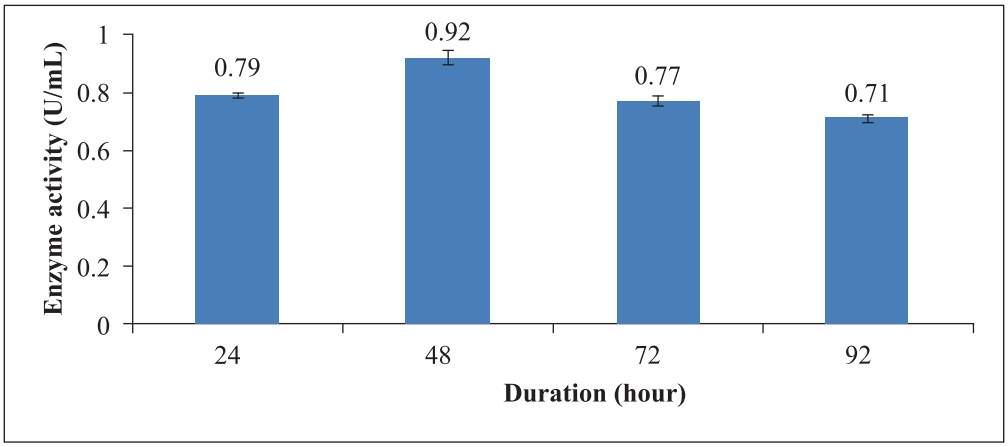

Figure 4 Effect of incubation duration at pH 6.5 and 30 °C on phytase production by *Enterobacter cloacae*

Other researchers observed that optimum production level of *Enterobacter* sp4 occurred after 44 h of incubation (Trivedi *et al.* 2017), while another after 72 h of incubation (Yoon *et al.* 1996). Time variations depended on nutrient availability in the medium and bacterial culture conditions. The surrounding environmental condition also affected the bacterial cultivation time.

Effect of pH

From a pH range of 3.0 - 8.0, the optimum phytase production (0.92 U/mL) for *Enterobacter cloacae* was at pH 5 (Fig. 5). The pH of cultivation media plays a significant role in the bacterial production of phytase, as pH directly impacts extracellular enzyme activity and the metabolism of microorganisms (Moreira *et al.* 2014; Farouk 2015). The highest production by lactic acid-producing bacteria was obtained at pH 5.0 (Tang *et al.* 2010). Similarly, the optimum activity of bacterial strain BAFA faifi 103, BAFA faifi 11 and BAFA faifi 117 occurred at the pH of 5.0 (Farouk *et al.* 2015). *Pseudomonas* sp. also generated the highest activity at pH 5.00 (Selvamohan *et al.* 2012). The optimum pH to produce phytase for two isolates (9B and 15C) was also pH 5.0 (Jorquera *et al.* 2017). However, *Enterobacter* sp. 4, *E. intermedius* PHY03, *E. cloacae* PSB45 and *E. aerogenes* produced the maximum phytase at pH 5.5, 6.5, 7.0 and 5.5, respectively (Yoon *et al.* 1996; Aziz *et al.* 2015; Kasli *et al.* 2016; Muslim *et al.* 2018).

Effect of Temperature

Another essential factor for detecting activity is the temperature. The highest phytase production (0.89 U/mL) of *Enterobacter cloacae* was observed at the incubation temperature of 30 °C (Fig. 6). When temperature was increased, there was a noticeable decrease in enzyme production. The optimum production temperature for *Citrobacter farmer* strain phas32 was 30 °C (Ebrahimian *et al.* 2018). The optimum enzyme activity of *L. plantarum* also occurred at a temperature of 30 °C (Saribuga *et al.* 2014). The highest production of *Rhizopus oligosporus* (Gautam *et al.* 2002), *Aspergillus ficuum* TUB F-1165 (Gunashree & Venkateswaran 2008) and *Aspergillus niger* (Sandhya *et al.* 2015) also occurred at 30 °C. However, *Enterobacter* spp. isolated from legume plant rhizosphere and *Peudomonas* sp. from soil around cattle shed had the highest activity at 37 °C (Yoon *et al.* 1996; Kim *et al.* 2002). The maximum production from *Pseudomonas* sp. was at 37 °C (Sasirekha *et al.* 2012a; 2012b). Production from both *P. aeruginosa* and *A. niger* were at 37 °C (Ogbonna *et al.* 2017). Accordingly, the optimum production temperature of most microorganisms ranged from 25 °C to 37 °C (Vohra & Satyanarayana 2003). On the contrary, the optimum phytase production from *Enterobacter cloacae* and *E. aerogenes* obtained at 70 °C and 50 °C, respectively (Kasli *et al.* 2016; Muslim *et al.* 2018), were much higher than in this study.

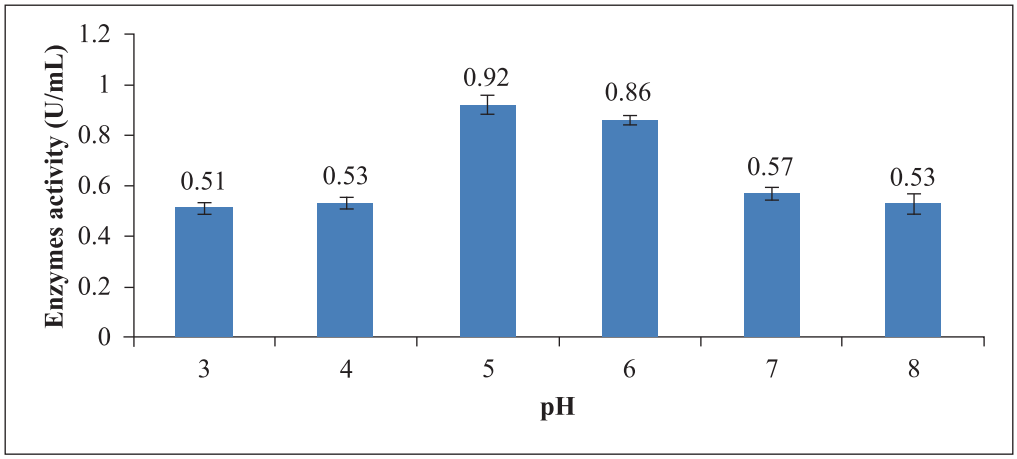

Figure 5 Effect of pH on phytase production by *Enterobacter cloacae*

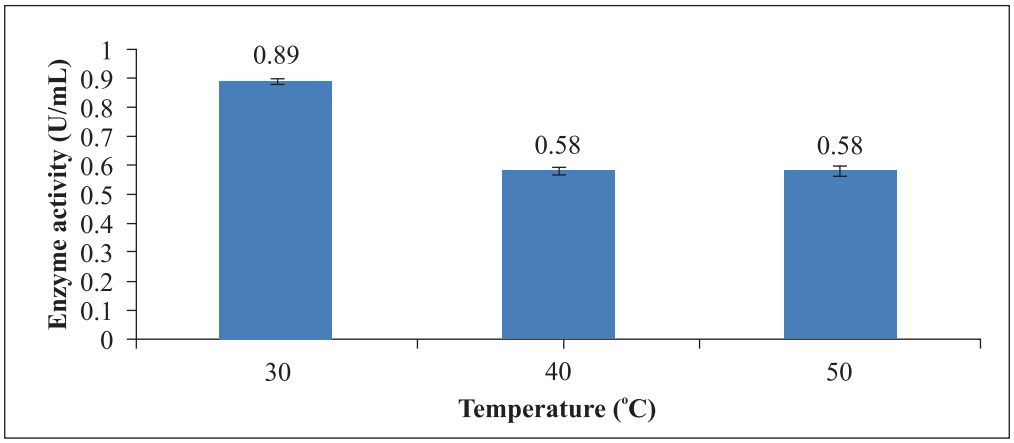

Figure 6 Effect of temperature on phytase production by *Enterobacter cloacae*

Effect of Carbon (C) Sources

Among the different sources, the highest carbon yield was obtained from lactose (0.91 U/mL), followed by glucose 0.83 U/mL, dextrose 0.81 U/mL and maltose 0.73 U/mL (Fig. 7).

The appropriate type and amount of nutrient sources are important factors that will help increase production. Utilization of the best C source in improving productivity is well-known. In this study, bacteria *Enterobacter cloacae* showed the highest production when incubated on a media with lactose as C source. In a similar study, *Lactobacillus casei* PHY02 and *Klebsiella pneumonia* PHY30 produced higher productivity when grown on media with lactose, while *Enterobacter intermedius* PHY03 preferred glucose (Aziz *et al.* 2015). The use of C from lactose also produced the highest activity from both *P. aeruginosa* and *A. niger* (Ogbonna *et al.* 2017). Furthermore, the maximum phytase activity was observed when lactose and wheat bran were used as C sources (Demirkan *et al.* 2014).

Effect of Nitrogen (N) Sources

Aside from C sources, the inorganic nitrogen {$(NH_4)H_2PO_4$, NH_4NO_3} and organic nitrogen (tryptone and beef extract) sources also affected the production of phytase after 48 h of incubation (Fig. 8). Production was higher in media enriched with inorganic N ($NH_4H_2PO_4$) as the nitrogen source. In other studies, the maximum production for *Mycelophoythora thermophile* (Vohra & Satyanarayana 2003), and *Klebsiella* sp (Mittal *et al.* 2012) occurred when $NH_4H_2PO_4$ was used as the nitrogen source. Inorganic N sources, such as $NH_4H_2PO_4$ (Gulati *et al.* 2007) and NH_4NO_3 (Fu *et al.* 2011), provided higher phytase production as compared to organic N. Similarly, the highest production was obtained from NH_4NO_3 enriched media (Tahir *et al.* 2010; Sreedevi & Reddy 2012).

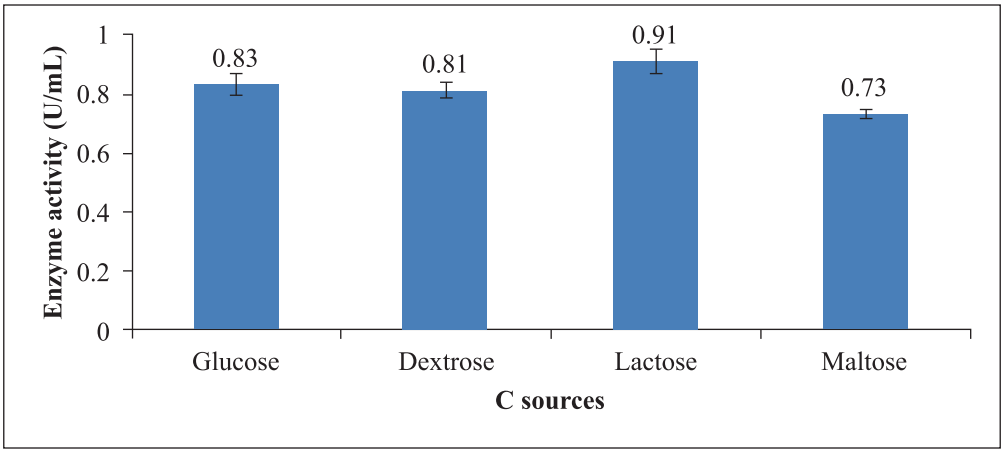

Figure 7 Effect of C sources on phytase production by *Enterobacter cloacae*

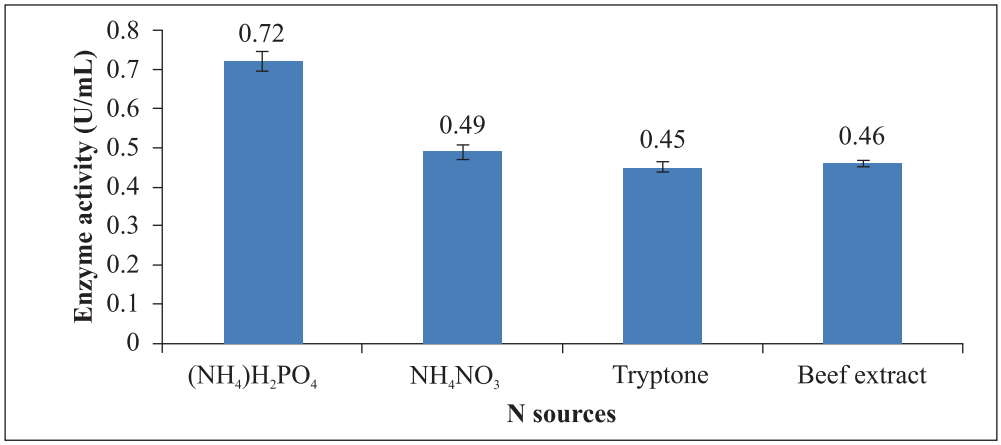

Figure 8 Effect of N sources on phytase production by *Enterobacter cloacae*

CONCLUSION

Thirteen phytase producing bacteria (PPB) were isolated from the legume rhizosphere. The *Enterobacter cloacae* strain B1 grown in solid PSM had the highest hydrolysis efficiency (3.43). The strain produced optimum levels when lactose and $NH_4H_2PO_4$ were utilized as the carbon and nitrogen sources, respectively, at 30 °C, pH 5.0 and at 48 hours of incubation This study recommends for the further investigation on the use of *Enterobacter cloacae* strain B1 as plant biological fertilizers.

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