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Full Length Research Paper

Analysis of phytase producing bacteria (*Pseudomonas* sp.) from poultry faeces and optimization of this enzyme production

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Phytase producing bacteria were isolated in media (phytase specific medium) with phytin and glucose as the only sources of phosphate carbon, from soil and poultry faeces. Among the five isolated strains, one identified that *Pseudomonas* sp. has high phytas activities. The result showed that isolated *Pseudomonas* produced 23 mm clear zone on solid media and 800 U/ml in phytase specific broth medium. The enzyme is produced extracellularly in the late stage of exponential growth. The maximum production of phytase by this isolated was obtained after 72 h incubation at 28°C and 180 rpm shaking. The best carbon and nitrogen sources for maximum phytase production were 1.5% glucose and 0.5% malt extract, respectively.

Key words: Phytase*, pseudomonas*, poultry, faeces, optimization.

INTRODUCTION

Phytic acid (*myo*-inositol hexakis phosphate, phytate) is the major storage form of phosphorus in cereal, oil and legume (Bae et al., 1999; Chang et al., 2004; Chunshan et al., 2001; Han et al., 1987). Phytase, a specific group of phosphatase hydrolizes phytic acid to myo-inositol and phosphoric acid (Batal and Abdelkarim, 2001; Bindu et al., 1998; Casey and Walsh, 2004; Cho et al., 2003; Chunshan et al., 2001; Han et al., 1987; Lei and Stahl, 2001; Maenz and Classen, 1998; Powar and Jagannathan, 1982). In terms of animal nutrition, monogastric animals such as swine, poultry and human are not capable of metabolizing phytate phosphorus owing to the lack of digestive enzymes hydrolyzing the substrate, and therefore inorganic phosphate is added to their diet to meet the phosphorus requirement, while undigested phytate phosphorus is excreted in manure and poses a serious phosphorus pollution problem, contributing to the eutrophication of surface water in areas of intensive livestock production. In addition, phytic acid also act as an antinutritional agents forming complexes with proteins and various metal ions, thereby decreasing the dietary bioavailability of these nutrients (Batal and Abdelkarim, 2001; Bindu et al., 1998).

Recently, phytase have been of interest for biotechnological application. Up till now phytase has been mainly used as a feed supplement in diets largely for pig and poultry (Maenz and Classen 1998; Zyla, 2001). Because of their great industrial significance there is an ongoing interest in isolation of new microbial strain producing phytase and optimization of this enzyme (Lan et al., 2002). The objectives of this study include the isolation of bacteria with phytase producing ability and optimization of this enzyme production.

MATERIALS AND METHOD

Isolation of phytate degrading bacteria

Bacterial strain were isolated from the soil and faeces of birds. Approximately 0.1 g of these samples was suspended in 5 ml of

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0.9% saline solution and aliquot (0.1 ml) of this suspension was streaked on to phytase specific medium [1.5%glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate (Sigma)]. Four microbial colonies capable of hydrolyzing sodium phytate which can be recognized by their surrounding clear halo were obtained by re-plating single colonies (Chunshan et al., 2001).

Screening for best phytate degrading strains

Bacterial strains tested by plate assay using phytase specific medium with 1.5% agars. The halo and colony diameters were measured after 14 days of incubation at 28°C (Rodrigues and Fraga, 1999)

Quantities estimation of phytate degrading bacteria

The strains were inoculated in 100 ml of phytase specific medium and were culture in a rotary shaker (180 rpm) at 28°C for 3 - 7 days. Cell collected from 1 ml of culture by centrifugation at 10000 g for 15 min then the collected cells were resuspended in acetate buffer (0.2 M, pH 5.5) and used for the phytase activity assay (Chunshan et al., 2001).

Measurement of enzymatic activity

The phytase activity assay was carried out using whole cell as well as the supernatant. Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consist of 0.8 ml acetate buffer (0.2 M, pH 5.5 containing 1 mM sodium phytate) 0.2 ml of cell suspension or supernatant. After incubation for 30 min 37°C, the reaction was stopped by adding 1 ml of 10% tricholoroacetic acid. A 1.0 ml aliquot was analyzed for inorganic phosphate librated by the method of Harland and Harland (Chunshan et al., 2001). One enzyme unit (U) is the amount of enzyme librating 1 ppm of inorganic phosphate in 1 min.

The effect of phosphate on phytase production

To study the effect of phosphate on phytase production, the activity of this enzyme in nutrient broth, medium with tricalcium phosphate and medium with sodium phytate was assayed. The three medium was incubated under same condition as above. The phytase activity was measured after 7 days.

Optimization of culture condition

To determine the optimum condition for growth and phytase production, the best strain was inoculated into 100 ml of liquid medium in a 250 ml erlenmyer flask and incubated on shaker at 28°C for 7 days .The parameters tested were: carbon source such as 1% glucose, maltose, sucrose, fructose and manitol, and 0.5% glucose + 0.5% sucrose. Nitrogen sources were 0.1% glycine, malt extract, yeast extract and amoniom sulfate (Lan et al., 2002).

Estimation of growth

Growth was estimated by the absorbance at 600 nm. Sample from culture grown in medium with insoluble forms of phosphate were previously diluted 1:1 (v/v) using 1 N HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated.

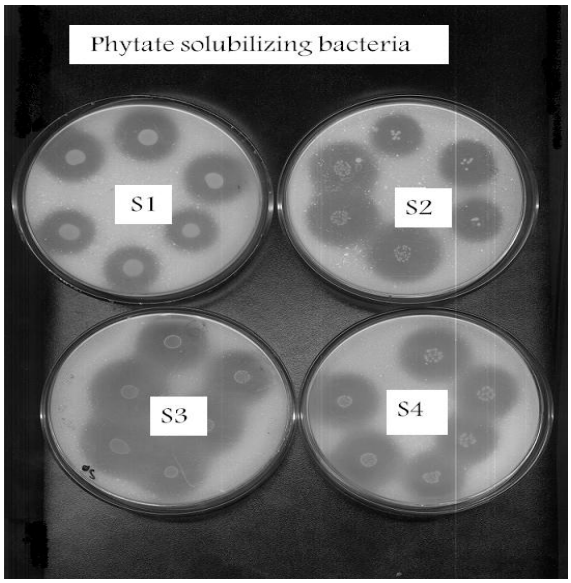

Figure 1. Four isolated *Pseudomonas* sp. strains forming peripheral halo zone on phytase specific agar medium. By this method S1 identified was the best stain.

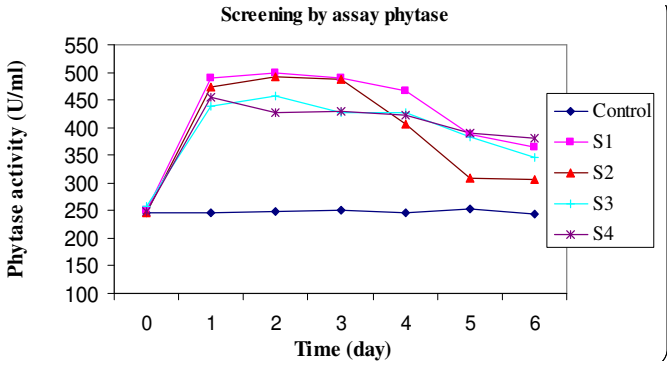

Figure 2. Quantitative estimation of phytate-degrading isolated *Pseudomonas* sp. by assaying phytase enzyme. S1 was found to be the best strain for degrading sodium phytate and produces 598 U/ml phytase enzyme.

RESULTS AND DISCUSSION

Isolation of phytate degrading bacteria

Four strains that can solubilize sodium phytate and form peripheral halo zone on phytase specific agar medium around colonies were isolated form poultry faeces and designed as S1, S2, S3 and S4. By plate screening, S1 was found to be the best strain and could produce approximately 4.2 cm clear halo around colonies (Figure 1). Quantitative screening methods done by phytase assay (Figure 2) also showed S1 to be the best strain. S1 produced maximum amount of phytase on the 2nd to 3rd day (598 U/ml). Identification of this strain, based on the result of morphological and biochemical tests, showed that it is *Psedumonus* sp. according to Bergi taxomical

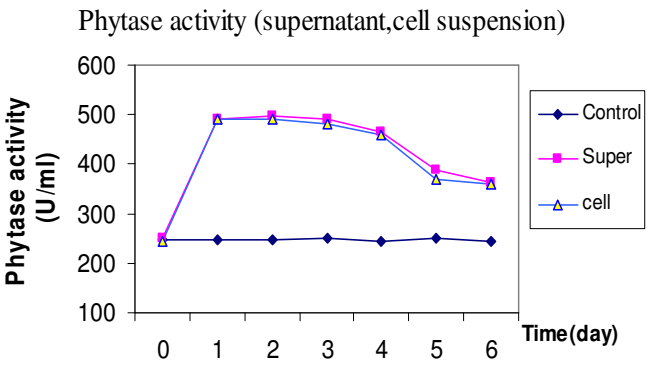

Figure 3. The amount of phytase enzyme of the isolated *Pseudomonas* sp. in supernatant and sample with whole cell were similar. This indicates that production of this enzyme was extracellular (590 - 612 U/ml).

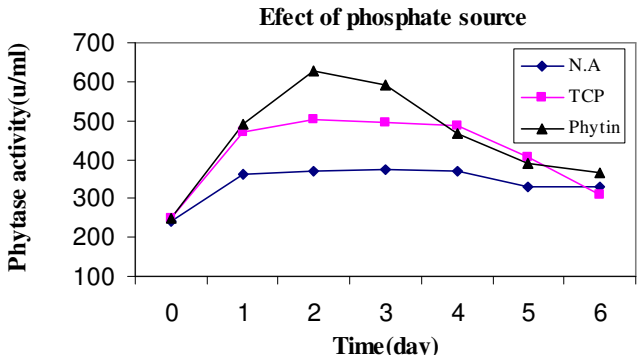

Figure 4. Effect of phosphate on phytase production using the isolated *Pseudomonas* sp. **N.A**, nutrient broth; **TCP**, medium with tricalcium phosphate; and **phytine**, medium with sodium phytate as phosphorus source. Sodium phytate may be inducing phytase production.

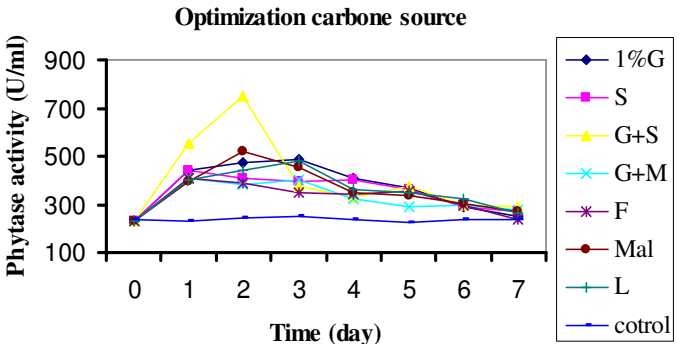

Figure 5. Optimization of culture condition (carbon source) of the isolated *Pseudomonas* sp. G, glucose; S, sucrose, G+S glucose + sucrose; M, manitol, F, fructose; Mal, maltose, L, lactose; and con, control. Result showed the best carbon source was G+S which produced 749.2 U/ml phytase.

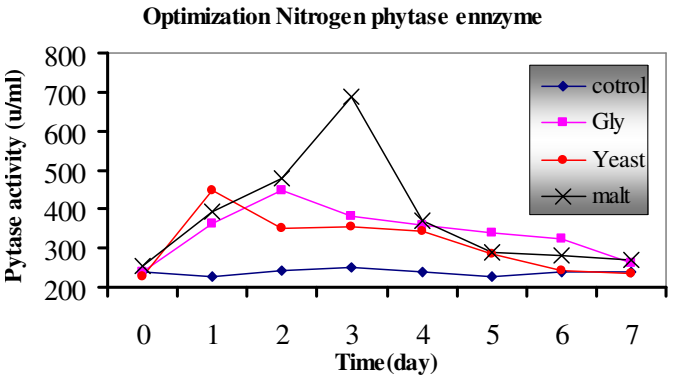

Figure 6. Optimization of culture condition (nitrogen source) of the isolated *Pseudomonas* sp. Malt, malt extract; Yeast, yeast extract; and Gly, glycine. Result showed that in medium with malt extract as nitrogen source, phytase production increased to 714U/ml.

method.

The phytase activity assay was carried out using whole cell as well as supernatant. The amount of phytase in the two samples was similar (Figure 3) indicating that phytase production in the isolated *Pseudomonas* sp. is extracelluler.

The effect of phosphate on phytase production

Assay activity of this enzyme carried out in three medium with different phosphorus sources; nutrient broth, medium with tricalcium phosphate and medium with sodium phytate. Result showed phytase activity in medium with sodium phytate increased after 3 days (598 - 650 U/ml) indicating that sodium phytate induces phytase production (Figure 4).

Optimization of culture condition

The parameters tested were carbon source such as 1 % glucose, maltose, sucrose, fructose or manitol and 0.5% glucose + 0.5% sucrose (Figure 5). Result of the experiment show the best carbons source for this strainwas 0.5% glucose + 0.5% sucrose with phytase activity of 749.2 U/ml. Nitrogen sources tested were 0.1% glycine, malt extract and yeast extract. Malt extract was found to be with phyase production of 714 U/ml (Figure 6).

Change of pH

Result of estimated daily change of pH in the four strains showed that all could produce organic acid that leads to decrease in the pH of medium. The pH of media at first was 7 and gradually decreased to 3.5 (Figure 7).

Estimation of growth of *Pseudomuna* sp.

Sample from culture grown in insoluble forms of phosphate were diluted 1:1 (v/v) using 1 N HCl, dissolving the

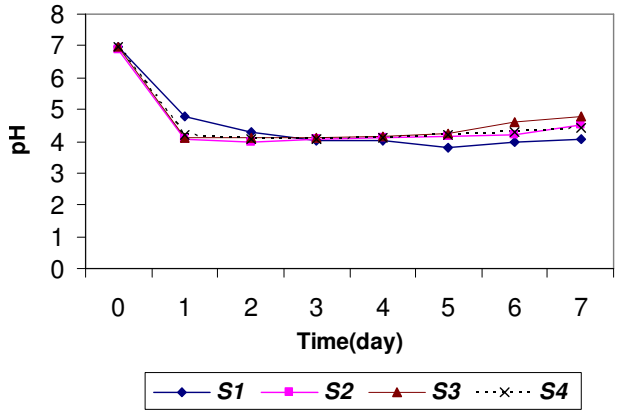

Figure 7. Estimated daily change of pH of the isolated *Pseudomonas* sp. strains (S1, S2, S3 and S4). pH of mediums at first were 7 and gradually decreased until 3.5, probably due to production organic acid which increases phytase solubilization.

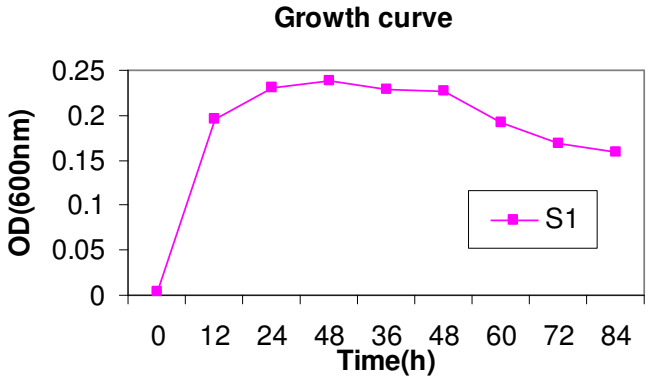

Figure 8. Growth curve of *Pseudomonas* sp. in phytase-specific medium and after 72 h this bacterium reached in stationary phase. The period between 48 - 72 h is maximum for phytase production

residual insoluble phosphate and measured at 600 nm. Figure 8 showed growth curves of *Psedomonus* sp. in phytase specific medium and reaching stationary phase after 72 h.

Conclusion

The ability of soil microorganism to solubilize various forms of precipitated phosphorus is well documented (Rodrigues and Fraga, 1999). Despite the quantitative importance of organic phosphorus compounds (such as phytin) in the soil, knowledge on the extent and mechanisms of the use by plants is still limited (Chunshan et al., 2001; Lan et al., 2002; Rodrigues and Fraga, 1999). Several types of phosphatase, such as phytase, are able to increase the rate of the dephosphorylation (hydrolysis) of organic compound. These enzymes are normally present in soils, where they originate from microorganisms. In this study, we isolated four strains with phytate degrading ability. Faeces of birds have a lot of phosphorus and it is degraded in the environment by microorganisms, which could be phosphate solubilizing bacteria. This enzyme was extra cellular because the result of cell suspension and supernatant was similar (Yadav and Tarafdar, 2003). The best carbon source is glucose + sucrose, while the best nitrogen source is malt extract (Han et al., 1987). Therefore, the organisms isolated and identified may influence phosphate nutrition of plants and could be applied for diet of poultry and pig (Casey and Walsh, 2004).

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