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Optimization of Cultural Conditions for Maximum Phytase Production from *Enterobacter* sp Isolated from Poultry Litter

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A bacterial strain capable of producing phytase enzyme was isolated from dried poultry litter and identified as a gram negative bacterium that belongs to Enterobacter species. The result showed that isolated organism produced around 27 mm clear halo zone on solid phytase screening media with an activity of 590 U/ml. The enzyme is produced extracellularly in the late exponential growth phase. Important physical and nutritional parameters such as different temperature, pH, carbon, nitrogen and phosphorus sources required for optimal enzyme production was analysed. Maximum production of phytase enzyme was obtained when the medium was supplemented with 1% sucrose, 0.5% yeast extract at 35°C of pH 5.5 in the culture media. Phosphate supplementation had negative effect on phytase production.

Key words: Enterabacteriaceae, Enterobacter, Extracellular phytase, Poultry litter.

During ripening, cereal and legume seeds accumulate a substantial amount of phytic acid[1]. Although phytate serves as the major source of energy and phosphorus for seed germination, the bound phosphorus is poorly available to simple stomached animals. Thus, inorganic phosphorus, a non-renewable and expensive mineral, is supplemented in diets for swine, poultry, and fish to meet their nutrient requirement for phosphorus. Meanwhile, the unutilized phytate phosphorus from plant feeds is excreted, becoming an environmental pollutant in areas of intensive animal agriculture. Excessive phosphorus in soil runs off to lakes and the sea, causing eutrophication and stimulating growth of aquatic organisms that may produce neurotoxins injurious to humans[2].

Furthermore, Phytic acid is considered as an anti nutritional factor as they reduce the availability of cations[3] carbohydrates, aminoacids[4] enzymes[5] and also lowers fat digestibility[6]. As a whole, challenges in the above areas of animal nutrition, and environmental protection have prompted the fast emerging of phytase science and biotechnology[2].

Phytases catalyze phosphate monoester hydrolysis of phytic acid that results in the liberation of inorganic phosphate[7]. Phytases have a wide distribution in plants, microorganisms and in some animal tissues. Although phytases from

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several species of bacteria, yeast and fungi have been characterized[8], commercial production currently focuses on the soil fungus *Aspergillus*. However, due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases are a real alternative to the fungal enzymes[9].

Production of phytases is in the range of €150 million worldwide which is still raising further[10]. The increasing use of phytases as additives in many biotechnological applications, including animal feed, reinforces the interest of isolating new and efficient phytase-producing microorganisms, obtaining effective phytases capable of releasing as many of the food phosphates as possible into the digestive tract and selecting phytases that remain stable during food processing and storage, with the lowest production costs[11]. The objective of the present study was to isolate phytase producing bacteria from poultry litter and optimize culture conditions for maximum enzyme production.

MATERIAL AND METHODS

Isolation of phytate degrading organism

Litter samples were collected from poultry houses already stocked with broiler chickens in Erode district. 0.5g of the samples were resuspended in 25 ml of 0.9% saline and kept in a shaker for 15 mins. Then it was kept undisturbed for few minutes. The clear samples were serially diluted and plated in solid phytase screening media (Glucose 1.5%, Na phytate 0.25%, NH_4NO_3 0.2%, KCl 0.05%, $MgSO_4$ 0.05%, $MnSO_4$ 0.05%, $FeSO_4$ 0.03%). The plates were incubated at 35°C for 2-5 days.

Screening for best phytate degrading bacteria

Isolates that produced clearance zone were then plated in phytase screening medium containing calcium phytate as substrate. The clearing zone and colony diameter were measured. Strain that produced maximum clearance zone was then inoculated into production media (Glucose 3%, yeast extract 0.5%, $MgSO_4$ 0.05%, $CaCl_2$ 0.01%, $FeSO_4$ 0.02%, $ZnSO_4$ 0.001%, $MnSO_4$ 0.001%, $CuSO_4$ 0.0002%, pH 5.5 ± 0.2) and was cultured in a rotary shaker (180rpm) at 35°C for 84 hrs. Growth of the organism was measured at 600 nm at regular time intervals. Supernatant and cells at different time points were collected from 1 ml of culture by centrifugation at 10000 g for 15 mins. Collected cells were resuspended in acetate buffer (0.2 M, pH 5.5) and used for the phytase activity assay.

Measurement of phytase activity

Phytate-degrading activity was determined at 35°C in 250 µl of 200 mM sodium acetate buffer, pH 5.5 containing 15 mM sodium phytate. The enzymatic activity was done by adding 500 µl of enzyme solution to the assay mixture. After incubating for 40 min at 35°C, the reaction was stopped by adding 1 ml of 2% TCA. The liberated phosphate was measured according to ammonium molybdate method[12] with some modifications. Added to the assay mixture was 1.5 ml of a freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molyb-date (2:1:1 v/v) and 100 µl citric acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was preparedover the range of 30-360 µmol phosphate. Activity (units) was expressed as 1 nmol phosphate liberated per min. Blanks were run by addition the ammonium molybdate solution prior to adding the enzyme to the assay mixture.

Determination of optimum temperature and pH for phytase production

To determine the optimum temperature and pH for maximum phytase production, the bacterial strain was cultivated at temperatures ranging from 20- 50 °C at an interval of 5 °C and pH ranging from 3.0-8.0 with an interval of 0.5. The crude enzyme was checked for its inorganic phosphate release by the standard method described above.

Optimization of culture conditions

To determine the optimum conditions for growth and phytase production, the strain was inoculated into 50 ml of liquid medium in 250 ml Erlenmeyer flask and incubated on shaker at 35°C for 36 hrs. The parameters tested were: carbon sources such as glucose, fructose, lactose, maltose, mannose, xylitol, arabinose, galactose, sucrose, lactose and maltose at 1% level; nitrogen sources such as yeast extract, peptone, ammonium sulphate, ammonium nitrate, potassium nitrate and urea at 0.5% level; various phosphates such as potassium dihydrogen phosphate, p-nitrophenyl phosphate, ammonium phosphate, phosphoric acid at 0.025% concentration were used.

RESULTS AND DISCUSSION

Isolation and screening of phytate degrading bacteria

Based on the clearance zone in phytase screening medium, twelve colonies that can degrade sodium phytate were isolated from poultry litter. Among them one colony was found to have higher phytate degrading capacity and produced approximately 27 mm clear halo in phytase screening media containing calcium phytate as substrate (Fig 1). According to Bergi taxonomical manual, strain identification based on morphology and biochemical tests showed that the organism belongs to Enterobacter species.

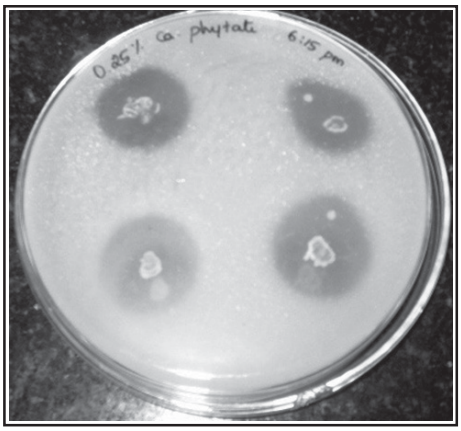

Fig. 1. Colonies showing clearance zone in solid phytase screening medium

Phytase activity assay was carried out using whole cell as well as supernatant at different time duration. The amount of phytase in the supernatant was found to be more compared to whole cell (Fig 2). This indicates that the enzyme is produced extracellularly and is not cell associated. Bacterial phytases are mostly cell associated, with the exception of *B. subtilis, Lactobacillus amylovo-ru*s and *Enterobacter* sp.4[8]. Recently Shobirini *et al*.[13] have also reported extracellular phytase producing bacteria that belonged to Enterobacter species from Malaysian maize plantation.

After 36 hrs of incubation, the enzyme activity in the supernatant reached a maximum value of 590 U/ml. From the growth curve (Fig 3) it was found that the strain reached its stationary growth phase after cultivation for 24 hrs. During its exponential growth phase the organism produced only small amount of phytase. Rate of phytase production was more in the late log phase. Because the synthesis of the enzyme started as soon as the growth rate began to fall, Konietzny and Greiner[9] suggested that either a nutrient or an energy limitation known to occur in stationary phase could be at the origin of its induction.

DETERMINATION OF OPTIMUM TEMPERATURE AND pH FOR PHYTASE PRODUCTION

An optimum temperature exists for every organism that may enhance the growth and facilitate the production of metabolites. In this study, the organism was incubated at various temperatures ranging from 20°C to 50°C. As the incubation temperature increased, phytase production tends to increase as shown in fig 4. At 35°C the organism showed maximum phytase activity of 590 U/ml. Lan *et al*[14] has also observed that during early fermentation period, enzyme production increased with increasing temperatures for *M. jalaludinii.*

The pH of medium is the other most important factor for a fermentation process, which influences the microbial growth and activity. Optimum pH required for maximal phytase production was evaluated using various initial pH levels (3 to 8.5). Results presented in fig 5 showed that maximal enzyme production was recorded with the pH of 5.5 (590 U/ml). Any further increase in pH of the medium decreased the enzyme production. A high pH generally leads to poor growth and results in variations in enzyme yield. If the pH of the medium was adjusted initially to 2.5-3.0, only less amount of phytase production was observed. Purva *et al*[15] has observed a significant increase in phytase yield with parallel decline in pH suggesting the strong correlation between decline in pH and phytase production.

Optimization of culture conditions

To initiate growth and metabolism, bacteria requires carbon sources in easily available form. Hence the effect of addition of different carbon sources at 1% w/v level to the medium on

enzyme production was evaluated. Fig.6 indicated that sucrose was utilized favorably for phytase production with the maximum yield of 618 U/ml. Some carbon sources like galactose and xylitol were poor inducers of phytase enzyme. Hosseinkhani *et al*[16] has reported 0.5% glucose + 0.5% sucrose has resulted in maximum phytase production by *Pseudomonas* species.

Impact of supplementation of nitrogen sources to the fermentation medium on enzyme production was evaluated by incorporating various nitrogen sources. It was evident from the fig 7 that yeast extract added at 0.5% level produced high levels of phytase enzyme with an activity of 590 U/ml. It was found that all the other nitrogen sources except peptone did not support phytase.enzyme

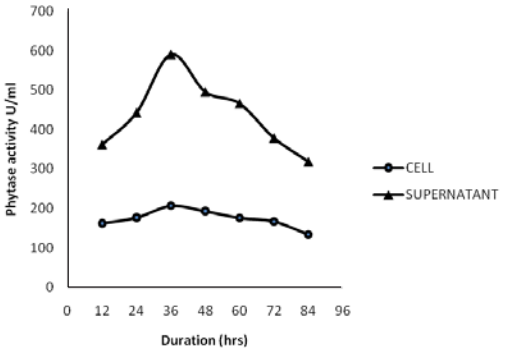

Fig. 2. Comparison of phytase enzyme production in supernatant and cell suspension

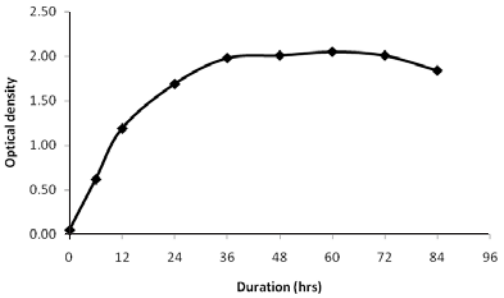

Fig. 3: Growth of *Enterobacter* sp in phytase production media

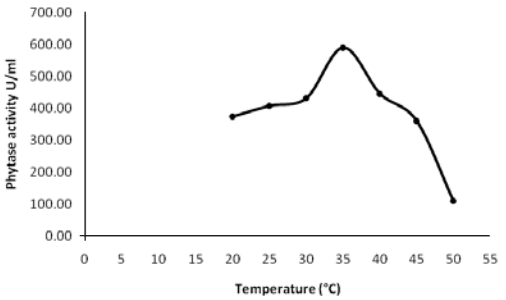

Fig. 4. Effect of different temperature on phytase production by *Enterobacter sp*

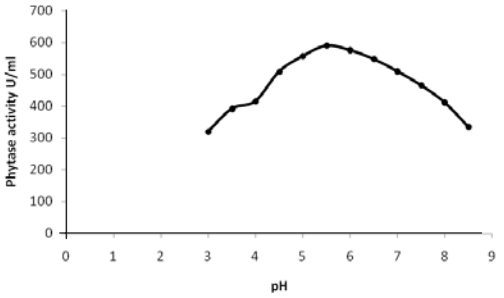

Fig. 5. Effect of different pH on phytase production by *Enterobacter sp*

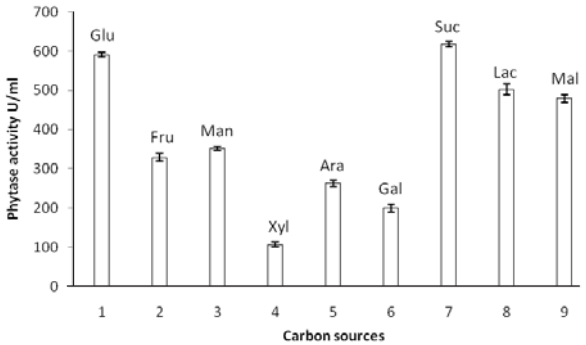

Fig. 6. Effect of different carbon source on phytase production. Glu-Glucose, Fru-Fructose, Man-Mannose, Xyl-Xylitol, Ara-Arabinose, Gal- Galactose, Suc-Sucrose, lac- Lactose, Mal- Maltose

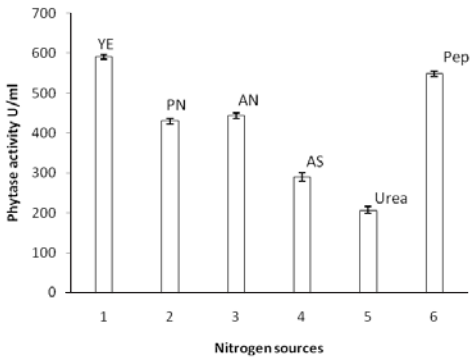

Fig. 7. Effect of different nitrogen source on phytase production. YE-yeast extract, PN-Potassium Nitrate, AN-Ammonium Nitrate, AS-Ammonium Sulphate, Pep-Peptone

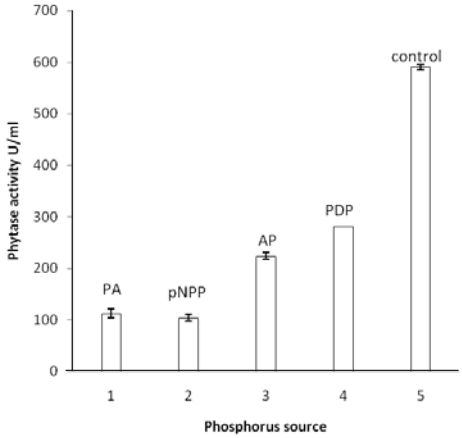

Fig. 8. Effect of different phosphorus source on phytase production. PA-potassium nitrate, pNPP-para nitrophenyl phosphate, AP-Ammonium phosphate, PDP-potassium dihydrogen phosphate, control – without any phosphate source

production. For *Candida* sp, Polypeptone at 0.7% concentration was found to be a good nitrogen source[17].

Inhibition of phytase synthesis by phosphate present in different ingredients used for the enzyme production by several species of yeasts and moulds has been reported by Nayini and Markakis[18]. Therefore different phosphorus sources were used in the medium. The results indicated that certain inorganic phosphate sources at 0.025% had a negative impact on phytase production as shown in fig 8. Phosphoric acid addition showed minimum enzyme production and p-Nitrophenol did not support the growth of the organism and enzyme production. Sreeramalu et al[19] have reported that the optimal phosphate level for phytase production in Lact. amylovorus was 240 mg/l. Similarly, Singh and Sathyanarayana[20] and Vats and Banerjee[21] have shown negative effect of phosphate on phytase production.

CONCLUSION

The ability of soil organisms to solubilize various forms of precipitated phosphorus is well documented[22]. Faeces of poultry have lots of phytate phosphorus and are degraded in the environment by microorganisms. In this study, phytate degrading bacterial strain which belonged to Enterobacter species was isolated from dried poultry litter. The organism produced phytase enzyme extracellularly. In order to design a suitable bioprocess for maximal phytase production an effort was made to optimize the important physical and nutritional parameters that influence the production of phytase by the isolated strain. The above results showed that the optimum conditions for phytase production by the strain were as follows: sucrose 1%, yeast extract 0.5%, $MgSO_4$ 0.05%, $CaCl_2$ 0.01%, $FeSO_4$ 0.02%, $ZnSO_4$ 0.001%, $MnSO_4$ 0.001%, $CuSO_4$ 0.0002% and pH 5.5 at 35°C for 36 hrs. Ongoing studies are being conducted to optimize the phytase supplementation to poultry diet and check to for its efficiency in poultry feed.

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