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Screening for efficient phytase producing bacterial strains from different soils

S. Sreedevi[1*], B.N. Reddy[2]

[1]*Department of Microbiology, St. Pious X Degree & P.G. College, Hyderabad-500076, A.P. India*

[2]*Department of Botany, OU College for Women, Hyderabad- 500095, A.P. India*

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Abstract

Phytic acid (*myo*-inositol hexakisphosphate) is the main form of phosphate constituting about 80% of the total phosphorus in cereals, legumes and oilseeds used as animal feeds. Phytases are the enzymes which hydrolyze phytic acid to less phosphorylated *myo*-inositol derivatives releasing inorganic phosphate. The supplementation of animal feed with phytases reduces the cost of diets by removing or reducing the need for supplemental inorganic phosphate and increases the bioavailability of phosphorous in monogastric animals. Rhizosphere soil, Cattle shed soil and poultry farm soil collected from various regions of Hyderabad, Andhra Pradesh, India were used as source material for isolation and screening of phytase producing bacteria. A total of 162 colonies were obtained on wheat extract agar media plates. Fifty four colonies showed clear zones of hydrolysis around them which were replated and hydrolysis efficiency calculated .Twenty one isolates showed more than 50% efficiency which were investigated for phytase production in submerged fermentation. C43 isolate was found to produce significantly high phytase activity than other isolates.

* **Corresponding Author:** S. Sreedevi ✉ sreedevi163@yahoo.com

Introduction

Poultry and pig diets are currently based primarily on cereals, legumes and oil seed products which are grown over 90% of the world's harvested area. About two-thirds of phosphorus present in these feedstuffs occurs as phytates, the salts of phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6- hexakisphosphate) (Harland and Morris, 1995; Baruah *et al.,* 2004; Kim *et al.*, 1998; Wang *et al.*, 1980). Soluble inorganic and cellular phosphorus (phosphorus bound in nucleic acids, phosphorylated proteins, phospholipids, phosphor-sugars) represents the remaining phosphorus. Considerable amounts of phytate can be found in plant-based food products such as rice bran, oat meal, barley flour, wheat bran, beans, sesame bran, sunflower meal, soybean, cowpea, and sorghum (Roopesh *et al.*, 2006; Dost and Tokul, 2006; Lestienne *et al.*, 2005; Kaur and Satyanarayana, 2005; Ebune *et al.*, 1995). Phytates are regarded as antinutritional factors that decrease feed quality, because they chelate proteins and essential minerals such as calcium, iron, zinc, magnesium, manganese, copper and molybdenum and prevent their absorption (Graf, 1983; Thomson and Yoon, 1984; Lee *et al.,* 1988; Lei *et al.,* 1993; Pallauf and Rimbach, 1996). In addition, undigested phytates cause significant environmental pollution (Graf, 1983; Nayini and Markakis, 1983; Common, 1989; Nasi, 1990; Wodzinski and Ullah, 1996; Adeola, 1999). Monogastric animals such as swine and poultry are incapable of digesting phytate phosphorus due to the lack of, or low levels of, phytase activity in their digestive systems (Lantzsch *et al.,* 1995). Phytases are the enzymes (*myo*-inositol hexakisphosphate phosphohydrolases) which hydrolyze phytic acid to less phosphorylated *myo*-inositol derivatives (in some cases to free *myo*-inositol), releasing inorganic phosphate (Gibson and Ullah, 1990). The supplementation of animal feed with phytases reduces the cost of diets by removing or reducing the need for supplemental inorganic phosphate and increases the bioavailability of phosphorous in monogastric animals. Apart from contributing to improving nutritive value, these feed enzymes can also have a positive impact on the environment by allowing better use of natural resources and reducing pollution.

These Phytases are widespread in nature and can be derived from a host of sources including plants, animals and microorganisms. Microbial sources are more promising for the production of phytases on a commercial scale (Reddy *et al.,* 1982; Pandey *et al.,* 2001; Nam-Soon Oh and Man-Jin In, 2009). Several fungal, bacterial and yeast strains have been reported as the source of phytase. Some of the phytase producing microorganisms include bacteria such as *Bacillus* (Powar and Jagannathan, 1982; Shimizu, 1992; Kim *et al.,* 1998), *Escherichia coli* (Greiner *et al.,* 1993; Choi *et al.,* 2002), *Enterobacter* (Yoon *et al.,* 1996), *Lactobacillus* (Angelis *et al.*, 2003), *Klebsiella* (Greiner *et al.,* 1997), *Pseudomonas* (In *et al.,* 2004), *Citrobacter* (Kim *et al.,* 2003), Fungi such as *Aspergillus* (Shieh *et al.,* 1969; Shimizu, 1993; Ebune *et al.,* 1995; Mullaney *et al.,* 2000; Casey and Walsh, 2003), *Penicillium* sp. (Tseng *et al.,* 2000) and *Rhizopus* (Sutardi and Buckle, 1988) and Yeasts such as *Saccharomyces cerevisiae* (Nayini and Markakis,1984), *Schwannoiomyces castellii* (Lambrechts *et al.,* 1992). Due to several biological characteristics, bacterial phytases have considerable potential in commercial and environmental applications.

Bacteria are though ubiquitous in their occurrence, the most common sources for their isolation are soils, lakes and river mud. Most phytase producing microorganisms from nature were isolated from soils (Shieh and Ware, 1968; Howson and Davis, 1983; Tseng *et al.*, 2000). Soil bacteria are a potential source of a phytase that could be developed commercially (Anis Shobirin *et al.*, 2009). The phytases have been isolated from various sources such as maize plantation (Anis Shobirin *et al.*, 2009), from the rhizosphere soil of leguminous plant methi (*Medicago falacata*) (Gulati *et al.*, 2007), from *Kimchi,* a milk fermentation product (Nam-Soon Oh and Man-Jin In, 2009) marine bacteria (Bushra Uzair and Nuzhat Ahmed, 2007), rumen of cows

(Raun *et al*., 1956; Yanke *et al*., 1998), poultry faeces (Baharak Hosseinkhani *et al*., 2009) traditional waste water of rice fermentation – *kali* and liquid cattle feeds – *kudithi* and soil sample of poultry waste dumps (Mukesh *et al.,* 2004). Corn, Citric Pulp, wheat bran and rice bran were used as a source material to isolate phytase producing fungi (Spier *et al*., 2008).

The increasing potential of phytase application prompts screening for newer phytase producing microorganisms, which can meet the conditions favourable to the industrial production. Although several phytases have been isolated, cloned and characterized, an optimal phytase for industrial applications is still lacking. Therefore, there is a constant need for new phytase candidates. Despite the recognized importance in biotechnology, information on bacterial phytases and phytase-producing bacteria is clearly limited and major efforts are required to improve the knowledge of phytases present in bacteria and their utilization. The present investigations have been undertaken to isolate a phytase producing bacterial strain and subsequent development of an efficient and economically suitable fermentation process for high production of phytase.In our present study we have selected rhizosphere soil of Legumes, cattle shed soil and poultry farm soil samples for isolation of potent phytase producing bacteria.

Materials and methods

Sample collection

A total of 14 soil samples – four rhizosphere soil samples of Legumes (RS1,RS2,RS3,RS4 and RS5), five cattle shed soil samples (CS1,CS2,CS3,CS4 and CS5) and four poultry farm soil samples (PF1,PF2,PF3 and PF4) were collected from various localities in Hyderabad, Andhra Pradesh.

Isolation of phytase producing bacteria

One gram of each sample was suspended in 10 ml of sterile distilled water and was serially diluted and 10^{-3} and 10^{-4} dilutions of each sample were spread onto wheat bran extract agar plates. The media consisted of 0.04% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 0.1% Casein, 0.05% KH_2PO_4, 0.04% K_2HPO_4 dissolved in wheat bran extract. The pH was adjusted to 6.5 using 1N HCl and 2 % agar was added before autoclaving at 121°C for 15 minutes (Powar and Jagannathan, 1982). The inoculated plates were incubated at various temperatures of 20°C, 37°C and 45°C for 1-3 days. After incubation, the plates were observed for the growth of colonies and the clear zones of hydrolysis around them. Each such colony was picked up and maintained on nutrient agar medium till further use.

Screening for best phytase producing isolate

Phytase activity of the isolated strains was screened by re-plating each of the single colonies on wheat bran extract agar media plates and observing their surrounding clear halo **(**Chunshan *et al.,* 2001**)**. The halo (Z) and colony (C) diameters were measured after 3 days of incubation at 37°C and the hydrolysis efficiency of all the isolates was determined (Rodrigues and Fraga, 1999; Stephen and Jisha,2008). The isolates with above 50% efficiency were selected and transferred to nutrient agar slants and were then stored at 4°C until use. Further screening was done by subjecting the isolates to fermentation (SmF) and assessing the enzyme activity.

Submerged fermentation (SmF) and estimation of phytase activity

Each of the isolates was inoculated at a concentration of 10% into a different flask containing sterilized wheat bran extract media. Before inoculation, presterilised $CaCl_2$ was added at a final concentration of 0.2%. The contents of the flasks were thoroughly mixed and then incubated in a shaker incubator at 200 rpm at 37°C for 72 h. The fermented broth was centrifuged at 6000 rpm for 30 minutes at 4°C and the supernatant collected was used for phytase activity determination. Phytase activity was determined by quantification of the phosphate released from phytate during the enzymatic reaction. The enzymatic activity was measured by a modification of the Heinonen-Lahti

method (1981) as described by Yoon *et al.* (1996). 1 ml of crude enzyme solution was mixed with 200 µl of 10 mM of sodium phytate and 200 µl of sodium acetate buffer (pH 5.5) and volume made up to 2 ml with sterile distilled H_2O. The mixture was then incubated at 37°C for 1 hour. After 1 hr of incubation 4 ml of acetone-acid-molybdate (AAM solution) was added to the enzymatic reaction sample. The contents were mixed carefully and 0.4 ml of 1M citric acid solution was added and left at room temperature for 15 minutes. The colour developed was read against blank in a visible spectrophotometer at 410 nm. The number of micromoles of inorganic phosphate produced under the assay conditions was determined using the standard curve generated and then the enzyme units calculated. One unit of phytase activity is defined as the amount of enzyme required to liberate 1 µmol of Pi per minute under assay conditions.

Statistical analyses

Studies were performed in triplicates on two different occasions (n=6) and mean value was calculated. The data was statistically analyzed by One-way Analysis of Variance (ANOVA) and Post-Hoc Multiple comparison test (LSD) using SPSS (Statistical Package for Social Sciences), version 19.0, IBM Corporation, Somers, NY, USA.

Results and discussion

Preliminary isolation of phytase producing bacteria

The technique of isolating microorganisms varies according to the nature and physiological properties of the microbe to be isolated. The more classical method to isolate new bacteria is direct isolation on solid media. Enrichment culture is also frequently used in order to isolate microorganisms having special growth characteristics. It allows selective cultivation of one or more bacterial strains obtained from a complex mixture such as that found in most soils. The choice of the medium and the conditions used in the enrichment culture favours the growth of the desired forms. The most useful plate technique for screening phytase producing microorganisms is based on the production of clear zones of hydrolysis around the colonies, which later are subjected to fermentation and estimated for phytase activity.

Rhizosphere region of agricultural crops especially legumes is a rich source of phytase producing bacteria (Scott and Loewus, 1986; Sutardi and Buckle ,1986; Scott ,1991; Greiner *et al.,*1997;Hegeman and Grabau ,2001; Mittal *et al,.* 2011;Mukesh kumar *et al.,* 2011; Sasirekha *et al.,* 2012). Monogastric animals such as pig and poultry do not carry bacteria that produce phytase and the undigested phytic acid is excreted in the feces, thus attracting a large no: of phytase producing bacteria in these areas (Kim *et al.,* 2002; Baharak Hossein khani *et al.,* 2009; Mittal *et al.,* 2011).

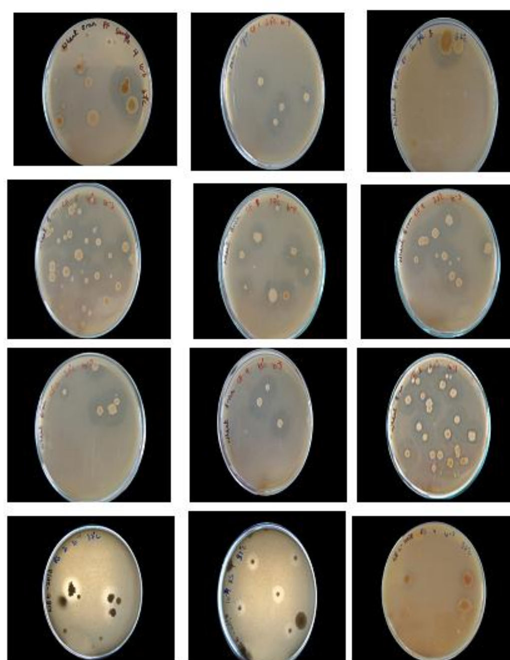

Fig. 1. Bacterial isolates with zone of hydrolysis on wheat bran extract media plates.

Rhizosphere soil, Cattle shed soil and poultry farm soils were collected from various localities in Hyderabad and used as source material to screen for phytase producing bacteria. The main criterion to identify bacterial species as producers of an extracellular phytase was a significant growth showing a clear zone on the screening agar media. A total of 162 colonies showed growth on wheat bran extract agar media plates on incubation at 20°C, 37°C and 45°C. Out of the 162 colonies, 54 colonies were

positive for phytase production as indicated by the clear zones of hydrolysis around them (Fig. 1). Most of the colonies showing hydrolysis zone were obtained on incubation at 37°C and a few at 45°C. At 20°C a few colonies appeared but without zones of hydrolysis (Table 1). Among them, nineteen were from rhizosphere soil samples and designated as R1 to R19, twenty seven were from cattle shed soil samples and were designated as C20 to C46 and eight were from poultry farm soil samples designated as P47 to P54.

Table 1. Preliminary isolation of phytase producing bacteria.

Sample	Incubation Temperature (°C)	Colonies	Zone of Hydrolysis
PF1	20	–	–
	37	+	–
	45	–	–
PF2	20	+	–
	37	–	–
	45	+	+
PF3	20	+	–
	37	+	+
	45	–	–
PF4	20	–	–
	37	+	+
	45	+	+
CS1	20	–	–
	37	+	+
	45	–	–
CS2	20	–	–
	37	+	+
	45	–	–
CS3	20	–	–
	37	+	+
	45	+	+
CS4	20	–	–
	37	+	+
	45	+	+
CS5	20	–	–
	37	–	–
	45	+	+
RS1	20	–	–
	37	+	+
	45	–	–
RS2	20	–	–
	37	+	+
	45	–	–
RS3	20	–	–
	37	+	+
	45	–	–
RS4	20	–	–
	37	+	+
	45	+	+
RS5	20	–	–
	37	+	+
	45	–	–

+ : present; – : absent

PF : Poultry farm soil; CS : Cattle shed soil; RS : Rhizosphere soil

Table 2. Hydrolysis Efficiency of isolates.

Isolate	Colony diameter, C (mm)	Halo diameter, Z (mm)	Hydrolysis Efficiency, Z-C/C (%)
R1	7	21	200
R2	40	42	5
R3	23	27	17
R4	30	33	10
R5	17	21	65
R6	14	19	36
R7	26	35	35
R8	29	34	17
R9	22	27	23
R10	8	13	63
R11	11	17	55
R12	26	32	23
R13	20	31	55
R14	19	34	79
R15	11	21	91
R16	11	23	109
R17	20	34	70
R18	8	9	12.5
R19	19	29	52.6
C20	19	28	47
C21	15	25	67
C22	21	27	29
C23	33	36	9
C24	11	21	91
C25	20	24	20
C26	30	32	7
C27	10	21	110
C28	11	22	100
C29	30	32	7
C30	24	29	21
C31	15	21	40
C32	35	38	9
C33	26	27	4
C34	23	26	13
C35	24	30	25
C36	15	32	113
C37	20	27	35
C38	20	24	20
C39	12	30	150
C40	12	27	125
C41	18	29	61
C42	22	30	36
C43	8	24	200
C44	28	33	18
C45	30	35	17

C46	8	22	175
P47	36	40	11
P48	25	27	8
P49	29	31	7
P50	30	33	10
P51	15	20	33
P52	10	19	90
P53	25	29	16
P54	12	15	25

Table 3. Phytase activity of various isolates screened.

S.No:	Isolates	Phytase activity (U/ml) *	± SEM
1	R1	0.4166	0.004
2	R5	0.225	0.006
3	R10	0.2366	0.004
4	R11	0.2616	0.011
5	R13	0.2316	0.006
6	R14	0.2	0.004
7	R15	0.26	0.006
8	R16	0.2266	0.004
9	R17	0.2266	0.004
10	R19	0.3083	0.003
11	C21	0.255	0.003
12	C24	0.2483	0.003
13	C27	0.3116	0.004
14	C28	0.335	0.003
15	C36	0.1333	0.003
16	C39	0.3333	0.002
17	C40	0.3633	0.005
18	C41	0.2183	0.005
19	C43	0.45	0.002
20	C46	0.1766	0.003
21	P52	0.0883	0.003

*Values are Means of two experiments, each with three replicates (n=6); $P < 0.05$
SEM: Standard Error of Mean

Screening for phytase producing bacteria

All the 54 isolates were replated and their halo (Z) and colony (C) diameters were measured after 3 days of incubation. Hydrolysis efficiency of all the isolates was calculated which ranged from 4% to 200% (Table 2). Twenty one isolates showed above 50% hydrolysis efficiency. They were selected and further screening was done by subjecting them to shake flask fermentation and assessing the enzyme activity (Table 3). Isolate C43 was found to produce 0.45 U/ml of phytase which was significantly ($p< 0.05$) higher than the other isolates (Figure 2). Thus isolate C43 was selected for further identification and characterization studies.

Conclusion

An efficient phytase producing bacterial isolate C43 was obtained from cattle shed soil samples of Hyderabad. Studies were conducted to identify the strain and optimize the phytase production and characterize phytase from the selected strain to evaluate its effectiveness as feed additive.

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