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ISOLATION, SCREENING AND OPTIMIZATION OF PHYTASE PRODUCTION FROM NEWLY ISOLATED BACILLUS SP.C43

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ABSTRACT

Rhizosphere soil, Cattle shed soil and poultry farm soil collected from various regions of Hyderabad, Andhra Pradesh were used as source material for isolation and screening of phytase producing bacteria. Out of 162 colonies on media plates, 54 colonies showed positive for phytase production as indicated by clear zones of hydrolysis around them. Hydrolysis efficiency was calculated and 21 isolates with above 50% efficiency were later investigated for phytase production in submerged fermentation. C43 isolate was found to produce significantly ($P<0.05$) high phytase activity and was identified based on preliminary morphological and biochemical tests as Bacillus sp. Production optimization studies showed that maximum enzyme production was obtained in 72 hours at 45^0C and pH 4. Comparisons were made for phytase production by the isolate using different substrates and maximum phytase production was obtained with wheat bran (0.465 U/ml). Wheat bran was mixed with various brans, oil cakes and meals in 1:1 ratio and checked for phytase production. Mixing wheat bran with groundnut oil cake in 1:1 ratio resulted in better phytase production (0.79 U/ml) than using wheat bran alone. Supplementation with nitrogen sources such as Ammonium nitrate, sodium nitrate and ammonium sulphate and carbon sources such as glucose and sucrose resulted in a significant increase in phytase production. On supplementing the medium with metal ions, there was no significant increase in phytase production. However, there was a slight improvement in the phytase production on addition of 5mM Ca^{2+} ions.

KEYWORDS

Hydrolysis zones, Optimization, Phytase, Phytic acid, submerged fermentation.

INTRODUCTION

Phytic acid (*myo*-inositol hexakisphosphate) is an anhydrous storage form of phosphate accounting for more than 80% of the total phosphorus in Cereals and legumes [1, 2, 3]. Under normal physiological conditions phytic acid chelates essential minerals such as calcium, iron, zinc, magnesium, manganese, copper and molybdenum thus preventing their absorption [4, 5, 6, 7, 8, 9]. The organically bound phosphate of phytic acid is not metabolized by monogastric animals such as pig, poultry and fish due to lack of phytase and consequently contributes to the phosphorus pollution problems in areas of intensive livestock production [10, 11, 12, 13, 14]. The anti-nutritive properties and its value as a possible phosphorus source have stimulated researchers to develop phytate hydrolysis methods. Chemical and physical methods to hydrolyse phytate are costly and reduce the nutrient value of feeds and therefore alternative methods for phytate hydrolysis were developed [15]. Therefore the reduction of the phytic acid content of seed meals via its enzymatic hydrolysis is desirable [16, 17]. 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 [18].

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. In addition to use as a feed additive these phytases have medical applications such as in prevention of diabetes complications and anti-inflammatory effects [19, 20] as well as antiangiogenic and antitumor effects through formation of myoinositol phosphate [21]. They are also important in paper and pulp industries [22].

These Phytases are widespread in nature and have been reported in plant and animal tissues and in a variety of microorganisms. Some of the phytase producing microorganisms include bacteria such as *Bacillus subtilis* [23], *Escherichia coli* [24], Yeasts such as *Saccharomyces cerevisiae* [25], *Schwannoiomyces castellii* [26]and fungi such as *Aspergillus niger* [27], *A. oryzae* [28], *A. ficuum* [29] and *Penicillium sp.* [30]. Due to several biological characteristics, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases have considerable potential in commercial applications. 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 there is a continuous search for new phytases with novel properties. Most phytase producing microorganisms from nature were isolated from soils [31, 32] and the rumen flora [33, 34]. The aim of the present study was to isolate phytase producing bacteria from environment and optimize the conditions for maximum production of extracellular phytase.

MATERIALS AND METHODS

Isolation of phytase producing bacteria

Rhizosphere soil, Cattle shed soil and poultry farm soils were collected from various regions of Hyderabad, Andhra Pradesh. One gram of each sample was suspended in 10 ml of sterile distilled water and was serially diluted and appropriate dilutions were spread onto wheat bran extract agar plates containing $(NH_4)_2SO_4$-0.04%, $MgSO_4.7H_2O$-0.02%, casein-0.1%, KH_2PO_4-0.05%, K_2HPO_4-0.04% and agar-2%. The inoculated plates were incubated at 37^{0}C for 1- 3 days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production.

Screening for best phytase producing strain

Microbial colonies capable of hydrolyzing phytate which can be recognized by their surrounding clear halo were obtained by re-plating single colonies [35]. The halo (Z) and colony (C) diameters were measured after 3 days of incubation at 37^o C. Hydrolysis efficiency of all the isolates was determined by the formula Z-C/ C [36, 37]. The isolates with above 50% efficiency were selected and transferred to nutrient agar slants and were then stored at 4^{0}C until use. Further screening was done by subjecting the isolates to shake flask fermentation and assessing the enzyme activity.

Production of phytase

Each of the isolates were subjected to fermentation in a fermentation medium containing the following (g/L): $(NH_4)_2SO_4$-0.4g, $MgSO_4.7H_2O$-0.2g, casein-1g, KH_2PO_4-0.5g and K_2HPO_4-0.4g dissolved in 1000 ml of wheat bran extract . The pH of the medium was adjusted at 6 before sterilization. It was then inoculated with 10% of the inoculum along with addition of presterilised $CaCl_2$ at a final concentration of 0.2% and incubated in a orbital shaker incubator [REMI, India] at 200 rpm at 37^{0}C for 3 days. After 3 days of fermentation, the fermented broth from the flask was transferred into centrifuge tubes and centrifuged at 6000 rpm for 30 minutes at 4^{0}C. The supernatant was then transferred into clean test tube which was used as crude enzyme solution.

Phytase Enzyme assay

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 using sodium phytate [Hi- Media chemical laboratories, India] as substrate [38]. The number of micromoles of inorganic phosphate produced under the assay conditions were determined

using the standard curve generated (8-80 µg/ml range) 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 (inorganic phosphorus) per minute under standard assay conditions.

Preliminary Identification of C43 isolate

The isolate C43 was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology[39]. Various morphological and biochemical tests were carried out by the techniques described in the Mackie and McCartney Practical Medical Microbiology [40]. Preliminary identification of the isolate was done using morphological tests such as Gram Staining , Spore staining , Capsule staining, hanging drop method and Biochemical tests such as Indole Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilisation Test, Nitrate Reduction Test, Catalase Test, Oxidase Test, Starch Hydrolysis , Gelatin Hydrolysis, Urease test, H_2S Production, Carbohydrate fermentation tests.

Optimization of phytase production

The aim of optimization was to determine suitable conditions and substrates in order to obtain optimum yield of phytase. The effect of various process parameters such as inoculum concentration, inoculum age, incubation time, pH and incubation temperature were tested as follows:

Effect of inoculum size on phytase production

The fermentation medium was taken in different flasks and inoculated with different concentrations of 24 hr old inoculum and incubated at 37^{0}C for 3 days. The various inoculum concentrations used were 1,5,10, 15, 20 and 25%. At the end of incubation, the enzyme samples were extracted and assayed for enzyme activities.

Effect of Age of Inoculum on Phytase Production

The fermentation medium adjusted to a pH of 6 was taken and inoculated with 10% culture having different age 12, 18, 24 and 36 hrs and incubated at 37^{0}C for 3 days. At the end of incubation, the enzyme samples were extracted and assayed for enzyme activities.

Effect of Incubation time on phytase production

Culture medium was inoculated with 10% of 24 hr old inoculum and incubated at 37^{0}C with 200 rpm shaking to induce the cells to produce phytase. For determination of optimum time for phytase production, 3 ml of the culture was sampled every 24 hours for 6 days, centrifuged and the supernatant collected was estimated for phytase activity by the method as described above.

Optimization of temperature and pH for phytase production

The wheat bran extract media was prepared in 7 different flasks adjusted in the range of pH 3-9 using 1N HCl and 1N NaOH. The appropriately labeled flasks with media adjusted to different pH were then inoculated with 10% of 24 hr old inoculum and kept in a shaker incubator at 200 rpm for 3 days at different temperatures of 30^{o}C, 35^{o}C, 37 ^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C and 60^{o}C. At the end of 3 days the fermented broth was collected, centrifuged, supernatant collected and phytase activity estimated by the routine method.

Optimization of substrates for phytase production

Different agricultural residues - wheat bran, rice bran, Bengal gram bran, red gram bran, groundnut oil cake, sesame oil cake, coconut oil cake, cotton oil cake, soyabean meal, oatmeal, corn meal and barley meal were used as substrates in submerged fermentation in order to study their effect on phytase production by the isolate. Different substrate extracts were prepared and stocked. Separate media were prepared by dissolving the ingredients of phytase production media in each of the substrate extracts. Each of the media flasks were then inoculated with 10% of 24 hr old inoculum and kept in a shaker incubator at 200 rpm at 37^{o}C for 3 days. After 3 days the fermented broth from each flask was centrifuged and supernatant collected and estimated for phytase activity.

Wheat bran which served as the best substrate was then mixed with other substrates in 1:1 ratio and used in preparation of media. Each media flask was then inoculated with 10% of 24 hr old inoculum and kept in a shaker incubator at 220 rpm at 37^{o}C for 3 days. After 3 days the fermented broth from each flask was centrifuged and supernatant collected and estimated for phytase activity.

Effect of supplementation of substrate with different nitrogen and carbon sources

Wheat bran extract media was prepared in different flasks and each flask was supplemented with a different nitrogen source at 1% (w/w). The various nitrogen sources used were ammonium nitrate, sodium nitrate, ammonium sulphate, peptone and urea. Each media flask was then inoculated with 10% of 24 hr old inoculum and kept in a shaker incubator at 200 rpm at 37^0C for 3 days. After 3 days the fermented broth from each flask was centrifuged and supernatant collected and estimated for phytase activity. Similarly the effect of different carbon sources such as glucose, maltose, sucrose, lactose and starch at 1% (w/w) were evaluated for phytase production in submerged fermentation.

Effect of metal ions on phytase production

To determine the effect of different metal ions on phytase production, the fermentation medium was supplemented with 5mM, 10mM and 15mM concentrations of different metal salts such as $CaCl_2$, $MgCl_2$, $CoCl_2$, $FeSo_4$, $NiCl_2$, KCl, NaCl, $CuSO_4$, $MgSO_4$ and $ZnCl_2$ and inoculated with 10% overnight culture and incubated at 37^0C in shaker incubator at 200 rpm. At the end of 3 days the fermented broth was collected, centrifuged, supernatant collected and phytase activity estimated.

Statistical analyses

Studies were performed in triplicates on two different occasions (n=6) and mean value was calculated. For statistical analysis, all the data were analysed 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

In the present study, phytase producing bacteria were isolated from Rhizosphere soil, Cattle shed soil and poultry farm soil collected from various regions of Hyderabad, A.P. A total of 162 colonies showed growth on media plates, out of which 54 colonies showed positive for phytase production as indicated by the clear zones of hydrolysis around them (**Fig 1**). Among these, 19 were from rhizosphere soil samples and were designated as R1 to R19, 27 were from cattle shed soil samples which were designated as C 20 to C 46 and 8 were from poultry farm soil samples and designated as P 47 to P 54. All the 54 isolates were replated and their halo (Z) and colony (C) diameters were measured after 3 days of incubation at 37^0C (**Fig 2**). Hydrolysis efficiency of all the isolates was calculated which ranged from 4% to 200%. The results are tabulated in **Table 1**. Twenty one isolates showed hydrolysis efficiency of above 50%. They were selected and further screening was done by subjecting them to shake flask fermentation and assessing the enzyme activity. C43 isolate was found to produce phytase with significantly ($P<0.05$) higher activity (**Fig.3**) and was selected for further studies. Identification of the strain C43 was carried out by Gram staining, microscopic examination and biochemical tests. The organism was seen as a Gram-positive rod. Results of biochemical tests are presented in **Table 2**. Based on the identification scheme in Bergey's Manual of Systematic Bacteriology, C-43 was identified as *Bacillus* sp.

While developing a production medium, an important factor to be considered is the cost effectiveness of the medium. This can be achieved by employing cheaply available agroindustrial residues as substrates for microbial growth and enzyme production[46,47]. Any variation in the environmental conditions of the microorganisms such as change of temperature, P^H, substrates, moisture, supply of air, inoculum concentration, carbon and nitrogen sources etc. influence the metabolic activity of the microorganism. The effect also varies widely from species to species for each of the organism. Hence it is necessary to optimize the fermentation parameters for the maximum production of phytase with a view to develop economically feasible technologies. Incubation time plays an important role in maximum enzyme production.[46,47] The present results showed that the maximum enzyme activity was obtained when 10% of 24 hr old inoculum was added to the fermentation media and was significantly high at $P<0.05$ level

(**Fig. 4 and Fig. 5**). There are reports showing that the maximum enzyme production was obtained in 72 hrs in *Enterobacter* sp. 4 [41] and *Pseudomonas* sp. [42]. Present results also indicate that the phytase activity reached a maximum at 72 hrs (3 days) of fermentation (**Fig 6**). Extending the fermentation resulted in a slight decrease in phytase activity, which might be due to proteolytic degradation of the enzyme. The effect of some physical factors such as temperature and pH on the production of crude enzyme was investigated. Reported studies showed that the maximum enzyme production was obtained when temperature was maintained at 37^{0}C in case of *Enterobacter* sp.4. The maximum phytase production was reported at 5.5 pH**.** The present results showed the optimum temperature for production of phytase was 45^{0}C and pH 4(**Fig 7**). There was a progressive decline in the enzyme production above 50^{0}C and the enzyme production dropped at pH above 7.

Table 1: 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 2: Microscopic and biochemical characteristics of the isolate C43

S.No:	Identification Characteristics	Results
1.	**Morphology :**	
	a) Form	Rods
	b) Gram Staining	Gram Positive
	c) Motility	Motile
	d) Spore	+
	e) Capsule	-
2.	**Biochemical Tests:**	
	a)Indole Test	-
	b)Methyl Red Test	-
	c)Voges Proskauer Test	+
	d) Citrate Utilisation Test	+
	e) Nitrate Reduction Test	+
	f) Catalase Test	+
	g) Oxidase Test	+
	h)Starch Hydrolysis	+
	i) Gelatin Hydrolysis	-
	j)Urease test	-
	k) H_2S Production	-
	l) Carbohydrate Fermentation Tests:	
	Glucose Fermentation	A, NG
	Lactose Fermentation	A, NG
	Mannitol Fermentation	A, NG
	Maltose Fermentation	A, NG
	Sucrose Fermentation	A, NG
	Xylose Fermentation	NA, NG
	Galactose Fermentation	A, NG

a) + positive; b) - :Negative; c) A:Acid production; d) NA :No acid production; e) G:Gas production; f) NG No gas production

Agroindustrial wastes are generally considered the best substrates for the cultivation of microorganisms to produce different enzymes [43]. In the present study Comparisons were made for phytase production using different substrates - wheat bran, rice bran, Bengal gram bran, red gram bran, groundnut oil cake, sesame oil cake, coconut oil cake, cotton oil cake, soyabean meal, oatmeal, corn meal and barley meal. Brans such as wheat and rice bran are considered excellent substrates for the production of extracellular phytate-degrading enzymes in microorganisms [44].The most commonly used substrates for phytase production was reported to be wheat bran and oil cakes in solid state fermentation by *Mucor racemosus* NRRL 1994 [45]. Present results are in good accordance with previous studies showing maximum enzyme production (0.465

U/ml) when wheat bran was used as substrate (**Fig 8**). Wheat bran was used as mixed substrate with various brans, oil cakes and meals in 1: 1 ratio and checked for phytase production (**Fig 9**). Mixing wheat bran with rice bran and groundnut oil cake resulted in better phytase production (0.50 and 0.79 U/ml respectively) than using wheat bran alone.

Wheat bran which was chosen as the best substrate for phytase production was then supplemented with different carbon sources at 1% and evaluated for phytase production. The results showed a mixed trend on enzyme production (**Fig 10**). There was an increased production of the enzyme with glucose and sucrose which was 0.52 and 0.59 U/ml respectively. While starch did not show any impact on enzyme synthesis, lactose and maltose marginally inhibited the enzyme production. This finding was compatible with studies of Nampoothiri et al. (2004) and Sreeramulu et al. (2006) who found that addition of glucose was useful for enzyme production. Nampoothiri et al. studied thermostable phytase production by *Thermoascus auranticus* in submerged fermentation, wheat bran was used as a carbon source supplemented with different mono, di and polysaccharides such as glucose, sucrose, starch etc. Addition of glucose and starch was found to be useful for enzyme production; addition of Tween 20 (2%) also resulted in higher enzyme titres. Sreeramulu et al. used lactic acid bacteria (*Lactobacillus* and *Streptococcus* sp) and found *L. amylovorus* B4552 as the best strain, which produced 125-126 units phytase /ml in a glucose medium supplemented with inorganic phosphorus .

Fig. 1: Some of the bacterial isolates showing zone of hydrolysis on wheat bran extract media plates

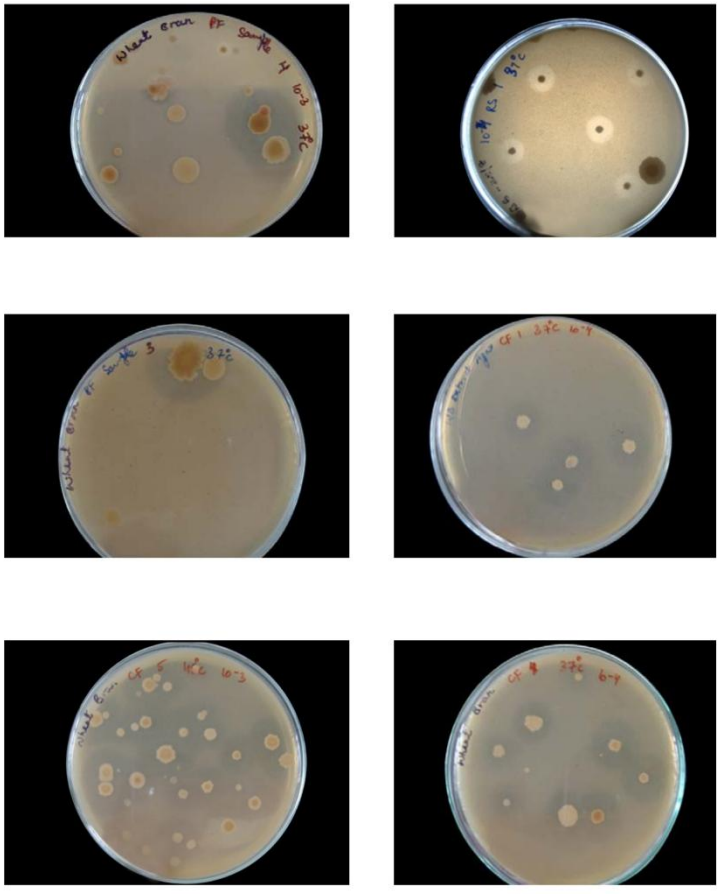


Fig. 2: Screening for Bacterial isolates and measuring hydrolysis zones

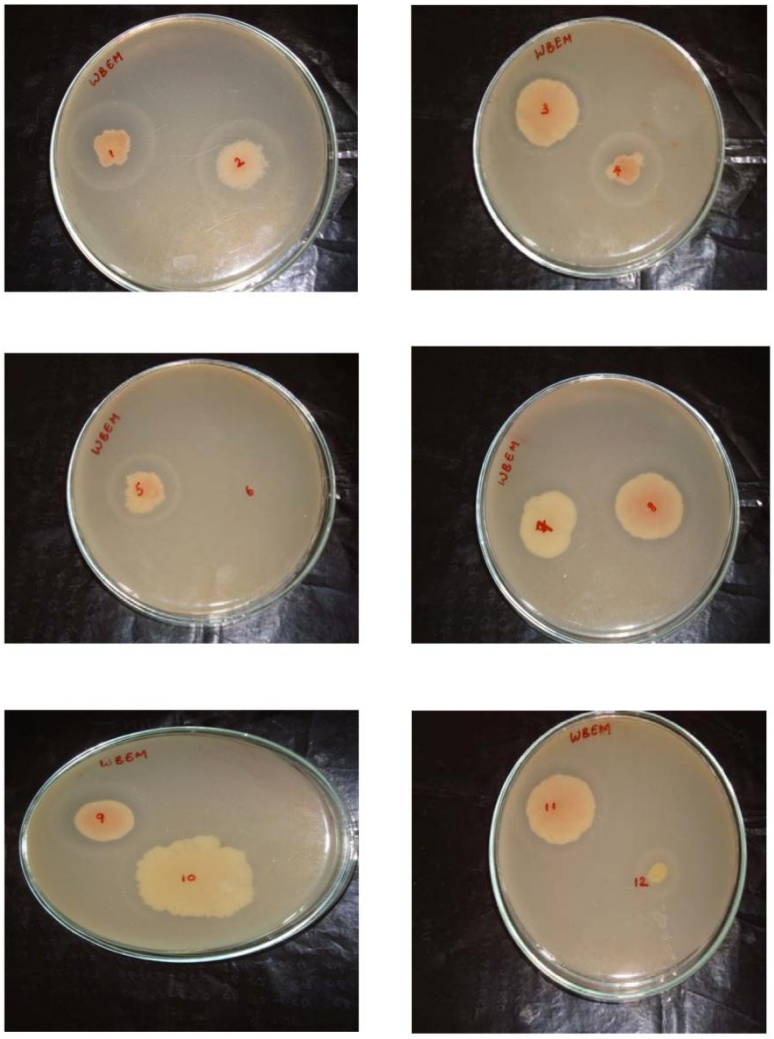
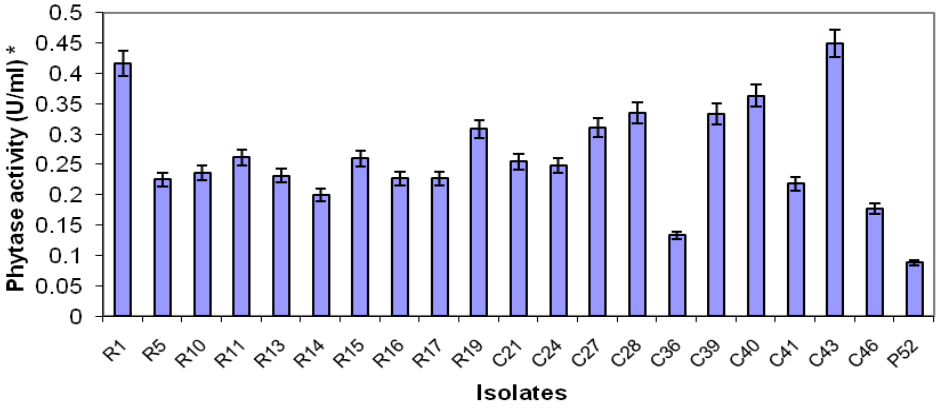
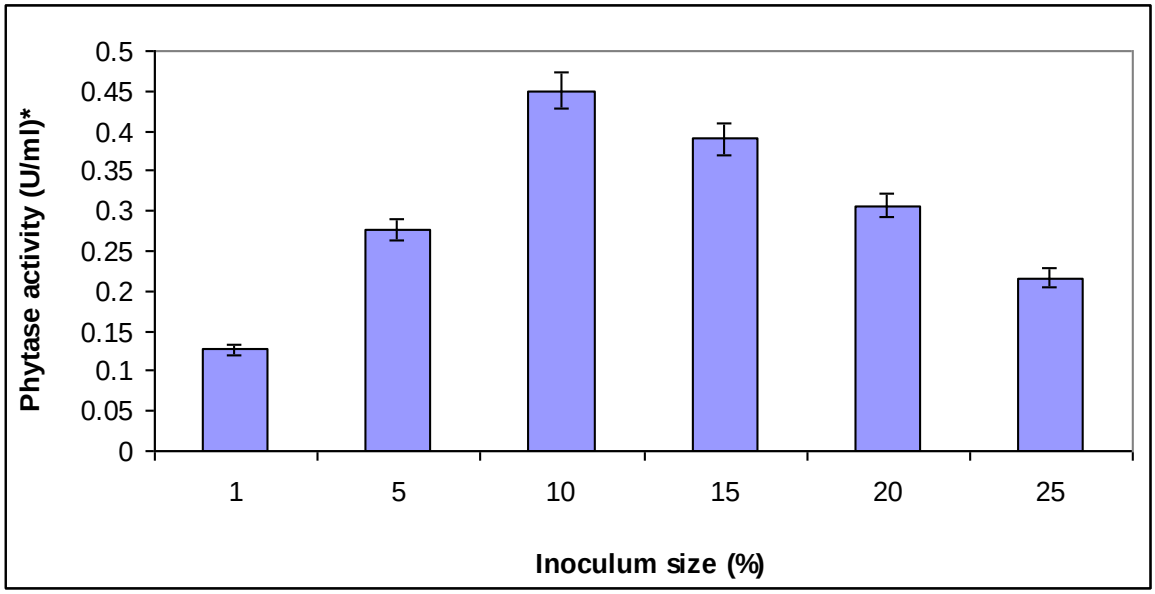


Fig. 3: Phytase Activity of various isolates screened



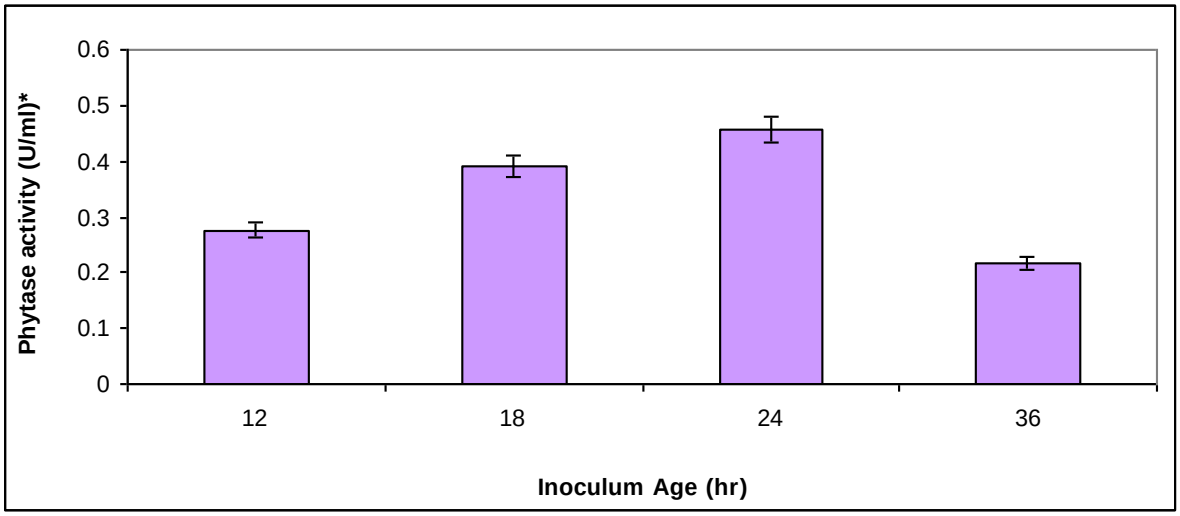
*Values are Means of two experiments, each with three replicates (n=6)

Fig. 4: Effect of Inoculum size on Phytase production



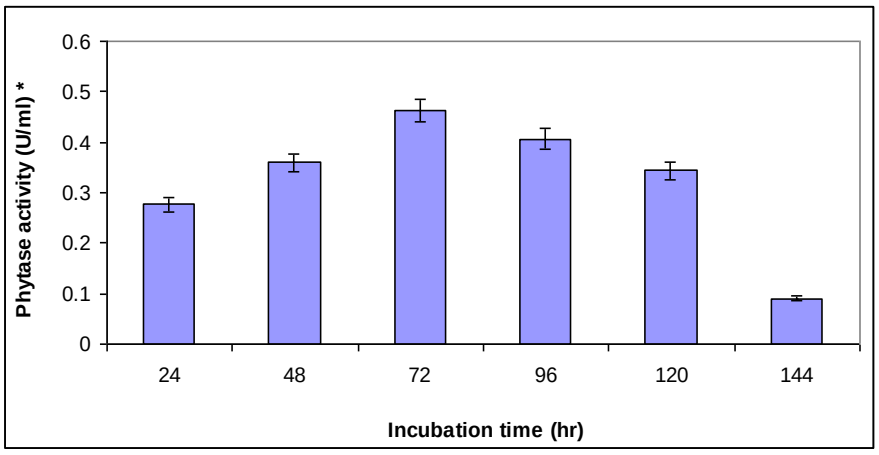
*Values are Means of two experiments, each with three replicates (n=6); P< 0.05

Fig. 5: Effect of Inoculum age on Phytase production



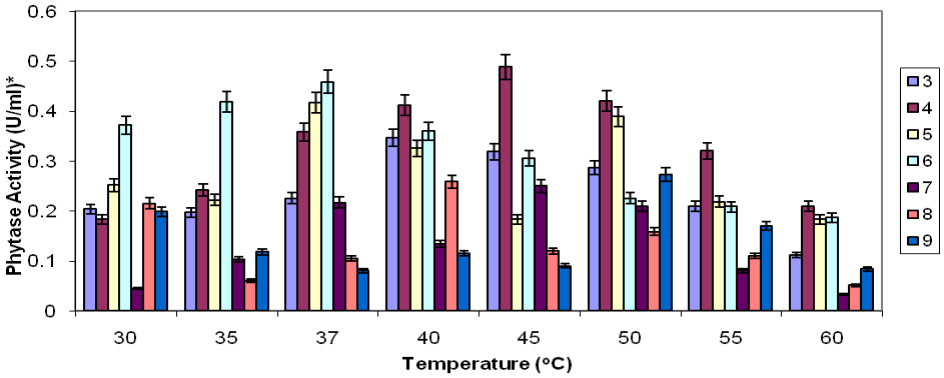
*Values are Means of two experiments, each with three replicates (n=6) ; P< 0.05

Fig. 6: Effect of Incubation time on enzyme production



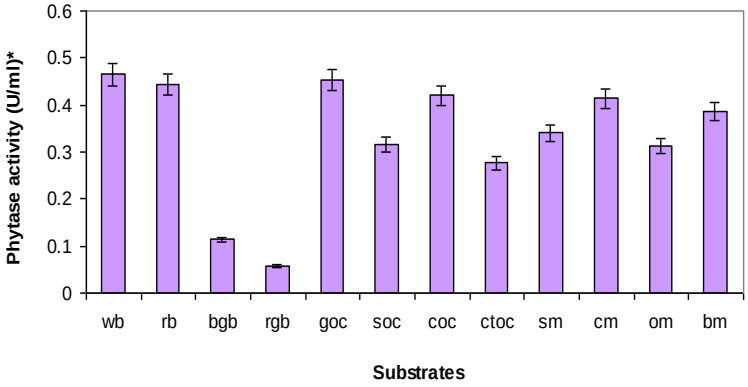
* Values are Means of two experiments, each with three replicates (n=6); P< 0.05

Fig. 7: Effect of temperature and pH on phytase production



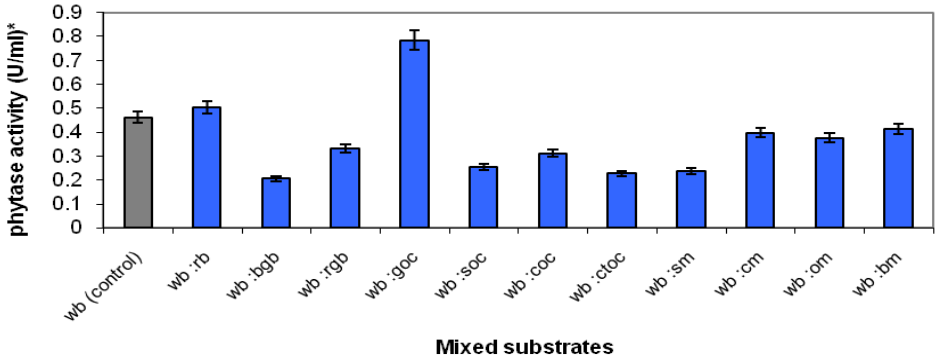
*** Values are Means of two experiments, each with three replicates (n=6); P< 0.05**

Fig. 8: Optimization of substrates for phytase production



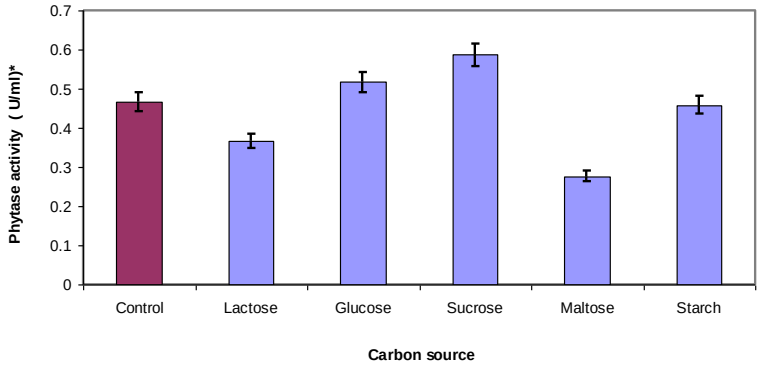
***Values are Means of two experiments, each with three replicates (n=6); P< 0.05**

Fig. 9: Mixed substrates for phytase production



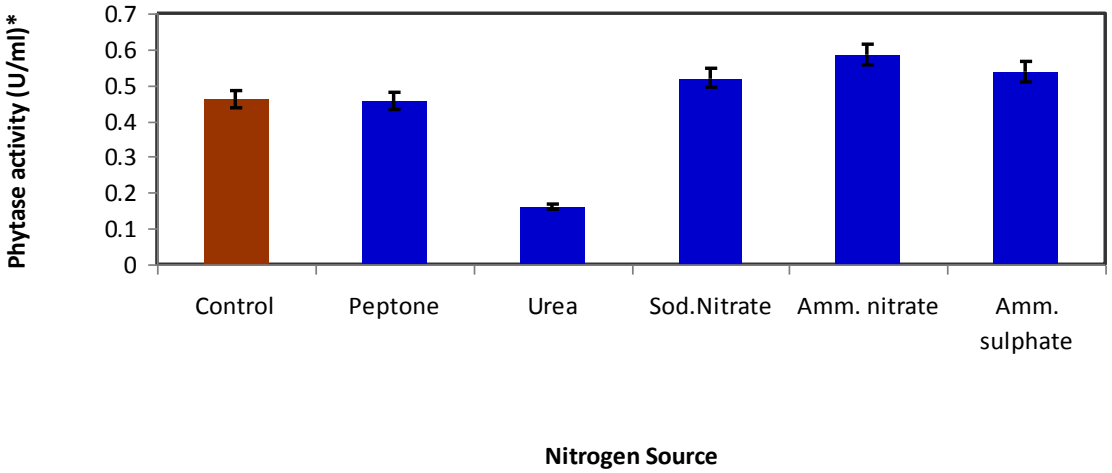
*Values are Means of two experiments, each with three replicates (n=6); P< 0.05

Fig. 10: Effect of carbon sources on Phytase Production



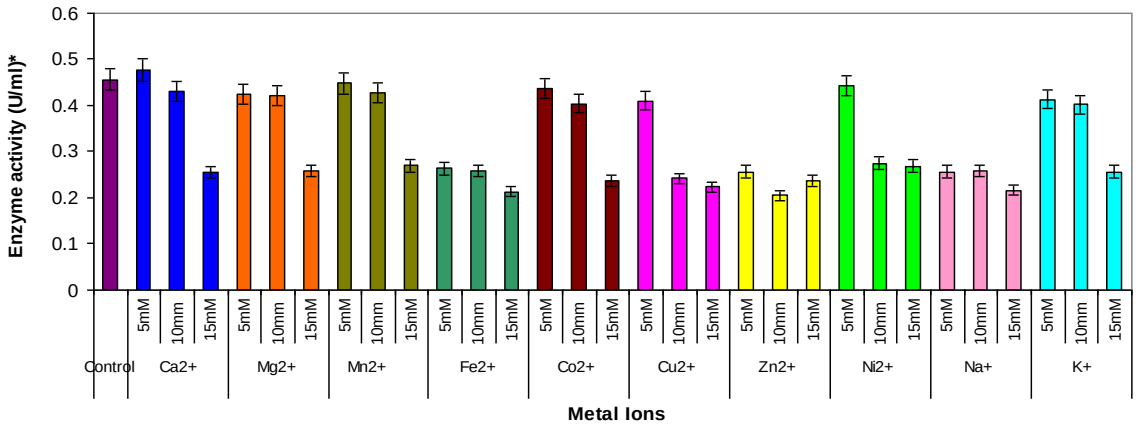
*Values are Means of two experiments, each with three replicates (n=6); P< 0.05

Fig. 11: Effect of nitrogen sources on Phytase Production



*Values are Means of two experiments, each with three replicates (n=6); P< 0.05

Fig. 12: Effect of metal ions on phytase production



*** Values are Means of two experiments, each with three replicates (n=6); P< 0.05**

Nitrogen source and the content also significantly affected phytase production (**Fig 11**). When no nitrogen source was added in the growth medium, growth and phytase production were greatly affected. Supplementation with a nitrogen source resulted in a better phytase production compared to the control without supplementation. Maximum phytase production was observed when ammonium nitrate was used as a nitrogen source. Supplementation with sodium nitrate and ammonium sulphate also resulted in a significant increase in phytase production compared to the unsupplemented controls. This is in agreement with previous studies using *A. niger* van Tieghem as the fermentation organism and *Sporotrichum thermophile Apinis* [48, 49]. A high yield of extracellular phytase was found from marine yeast strain in a simple oat-based medium which contained 2.3% of ammonium sulphate [50].

Role of influence of metals on the enzyme production was observed by the addition of metallic salts to the production medium. Not much information is available regarding effect of these trace elements on phytase production. On supplementing the medium with metal ions, there was no significant increase in phytase production (**Fig 12**). Among the different cations, only Ca^{2+} ions at a concentration of 5mM significantly (P< 0.05) improved the secretion of phytase into the medium; this may be attributed to its property of acting as effluxing agent and helps in membrane permeabilization and acting as ion channels. All the other metal ions at various concentrations used considerably inhibited the enzyme production. However, there was no significant (P>0.05) effect upon addition of 5mM of Mn^{2+} in the production media. The decrease in the production of phytase in the presence of other metal ions may be attributed to their inhibitory effect on growth or possibly inhibition or inactivation of enzyme itself by these metals ions. Whereas the actual mechanism of stimulation or inhibition of metabolites is still not known.

The results presented here demonstrate that among many methods, optimization of medium components and cultivation conditions remains a feasible way to enhance enzyme activity as well as yield.

CONCLUSION

In conclusion, the phytase production from newly isolated *Bacillus* sp.C43 is economical due to the use of cheap substrates. Further studies of biophysical and biochemical characterization of phytase are in progress to determine enzyme characteristics like molecular weight, temperature and pH stabilities and optima, modulators of enzyme activity, substrate

specificity. Thus effort will be made to develop cost effective phytase with improved properties for the animal feed industries.

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