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Research Article

Ameliorating the phytate-degrading phytase production using an isolated *Bacillus* sp. from agricultural fields

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Abstract

Accumulation of Phosphate in environment can be reduced by activity of Phytase enzyme molecules. Phytases are the enzymes that instigate removal of phosphate form Phytic acid. An indigenous *Bacillus* sp. was screened from the vicinities of agricultural fields for the production of phytase enzyme when grown in phytase specific media. The enzyme was secreted extracellularly and the isolate exhibited high activities. The production media was optimized using statistical techniques and the quantified operational parameters were used to perform experiments in bench-top batch fermenter. The phytase was recovered and purified using ammonium sulphate precipitation, dialysis, and chromatographic techniques. The purified enzyme was characterized for its operational stability under varying pH, temperature, inducers. The purified phytase enzyme was optimally active at pH 5, 30°C and enzyme activity was stimulated by $CuCl_2$. The kinetic parameters were evaluated and purified enzyme had specific activity and molecular weight of 3.49 U/mL and 45 kDa, respectively.

Keywords: Phytase; *Bacillus* species; Optimization; Purification; Characterization; Molecular weight.

Introduction

Phytase (EC 3.1.3.8) are phosphatise enzymes that hydrolyze phytate (*myo*-inositol hexakisphosphate) to *myo*-inositol and inorganic phosphate [1]. Phytate is the major storage form of phosphorus andis not digested by monogastric animals [2]. Phytases are the primary enzymes responsible for the hydrolysis of phytic acid, so that enhancement of availability of phosphorus innutrition of monogastric animals is increased [3]. Phytases showed high optimistic response towards dietary necessities in poultry. Supplementation of antimicrobial for growth performance has been replaced with the addition of phytase [4]. Supplementation of feed with Phytase showed increased growth performance and nutrient digestibility in Pigs and Broiler chicken with linear effects of Calcium and Nitrogen utilization [5]. High levels of phosphorous content were found in manure of swine, poultry, and pre ruminant calves due to their inability to digest phosphorus [6,7]. A combined effect of reduction of phosphorous pollution from broilers waste and retention of non- renewable phosphorous has been reported successfully in feed of Hubbard with the addition of phytase enzyme [8]. And also presence of phytases proved increased plant growth due mineral assimilation when compared with normal chemical fertilizers [9].

During fermentation, Statistical analysis is used for the optimization of phytase production. RSM is widely used for the optimized production of various commercially important enzymes. Response surface methodology (RSM) involves executing the statistically designed experiments, evaluating the coefficients in a mathematical model, and predicting the response and inspecting the suitability of the model [10]. Among the various RSM design, central composite design (CCD) is mostly used. Phytase production is greatly influenced by media components and physical factors such as incubation time, inoculum size, and temperature [11]. In the present work different bacterial isolates were isolated from different sources of soil. *Bacillus* species showed the highest phytase production and was chosen for further

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investigation. Optimal culture conditions and fermentation parameters were optimized by using response surface methodology to enhance phytase production.

Materials and Methods

Isolation of phytase producing microorganism

Soil sample were collected from the Rhizosphere (around the neem tree) near Chennai, India. 1gm of sample was dissolved in 100 ml of sterile distilled water and shaked vigorously and was serially diluted and appropriate dilutions were spread on Luria Bertani media (LB) and Peptone magnesium chloride and magnesium sulphate media (PMM) [12]. The inoculated were incubated at 37°C for 24 hr.

Identification of bacterial isolates

Bacterial isolates were subjected to a series of test and were characterized as *Bacillus* sp. using morphological, physiological properties and biochemical tests [13] and maintained on LB slant at 4°C.

Estimation of crude enzyme and phytase activity

The cell free supernatant (crude enzyme) obtained by centrifugation at 10,000 rpm for 30 min at 4°C was assayed for its total protein concentration using BSA as the standard protein [14].The presence of phytase enzyme was measured by *Heinonen and Lahti* method [15].

Optimization of phytase enzyme production

The central composite design (CCD) under the response surface methodology (RSM) [16] was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. The independent variables and its levels were chosen based on that could be obtained from the results of one factor at a time experiments. Three major variables namely pH, Temperature (˚C) and incubation time (h) were included in this model. The low, centre and high levels of each variable are designated as -1, 0, and +1, respectively. A matrix of 20 experiments with 3 factors was generated using the software package MINITAB 17.The statistical significance of the model polynomial equation and the goodness of fit of the model were evaluated by the coefficient determination (R^2) and by the F-test analysis of variance (ANOVA). The polynomial equation used to predict the response is given in equation (1).

$$Activity = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC \quad (1)$$

Table 1. Experimental range and levels of independent variables

Independent variables	Design variables	Range and levels		
		-1	0	1
pH	A	6	7	8
Temperature (°C)	B	25	30	35
Incubation Time (hr)	C	14	18	22

Production in bench top fermentor

2000 ml of PSM broth was prepared. The media was poured into the fermentor and silicon tubes were fitted to all port of fermentor. The fermentor along with media, 0.1N H_2SO_4, 0.1N NaOH and antifoam flask were autoclaved together for 121°C at 15 lbs pressure for 15 min. Then the media was allowed to cool and after cooling, 150 ml of inoculum was inoculated. The fermentor was fixed and connected to control panel. The acid, base pump was also connected and the pH was adjusted to 5.5 and temperature at 30°C and the air flow was set at 2 lpm and agitation at 180 rpm. The optical density reading was noted and cell count was taken by using Haemocytometer in 1 hour time intervals. The enzymatic assay for the enzyme activity was measured at 18th, 20th and 22nd hr. The fermentor was stopped, once the stationary phase of the organism was observed.

Purification by ammonium sulphate precipitation

The production media was centrifuged at 10,000 rpm for 12 min and the supernatant was collected. The supernatant after centrifugation was collected and the volume of it was measured. And accordingly 70% ammonium sulphate was measured in order to conduct the salting out procedure under ice cold condition on the magnetic stirrer until complete dissolving takes place then it was kept for overnight in the refrigerator. The pellet was collected and dissolved in 10 ml of 50 mM TrisHCl (pH 8.5). This solution contains the enzymes precipitated by ammonium sulphate [17].

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Purification by dialysis

The dialysis of the salted out crude enzyme requires certain pretreatment for the dialysis membrane. The dialysis membranes are activated by cutting out about 10 cm of the dialysis tube treated with distilled water at 65◦C for 10 minutes to remove glycerol content and were soaked in 100ml of 10 mM EDTA to remove heavy metal. The sulphur molecules were removed by treating with 2% of sodium bicarbonate and finally washed with distilled water. Thus the dialysis bag was pretreated and filled with the enzyme and sealed to avoid air bubbles. The bag was dipped in 500 ml beaker filled with water on a magnetic stirrer in ice cold condition for 8 hours. The water was changed frequently for every hour to avoid equilibration.

Purification by Ion-Exchange Chromatography (DEAE-Sephacryl Column)

The dialyzed sample was removed from the tubing and filtered through a 0.45µm filter and then applied to a packing column (DEAE-Sephacryl) previously equilibrated with 0.01M Phosphate buffer (pH 7.0)slowly percolating large volume of buffer through packed material. A sample of desalted enzyme preparation was loaded onto the column. Flow rate was controlled at 0.5 ml/min by 5 ml of fractions collected and analyzed for protein and enzyme activity.

Molecular weight determination

SDS-Poly acrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of [18]. The distribution of protein on the gel plate was revealed by staining with C Bromophenol blue. Electrophoresis was carried out at 4°C with constant voltage and 20 mA current supply for 2 hr until the tracer dye reached 0.5 cm above the lower end. At the end of electrophoresis, gel was removed and stained with silver staining method [19]. After staining, the gels were stored in 7 % (v/v) acetic acid.

Characterization of purified enzyme

The stability of phytase was studied under different parameters such as pH, temperature and varying substrate concentration by incubating the purified enzyme with varying pH ranging from 3.0 - 9.0 using 0.1M Phosphate buffer. The enzyme samples were added to 1 ml of the buffer and incubated at 37°C for 10 minutes and specific phytase assay was carried out. The thermal stability of the enzyme was analyzed by incubating at 10, 20, 30, 40 and 50◦C for 10 minutes. The activators and inhibitors were known to affect different classes of phytase [20]. To determine whether the activity of phytase could be affected, 1mM Concentration of EDTA, $MnCl_2$, $ZnCl_2$, $CaCl_2$, $CuCl_2$ and $MgCl_2$was added with extracellular supernatant and incubated at 37˚C for 10 minutes followed by enzyme assay that showed the residual activity.

Evaluation of kinetic parameters

Determination of single-substrate catalyse reaction was carried out by measuring the enzyme activity at 10-70 mM of Sodium phytate as substrate concentration. The examination was done by incubating 0.01µl of enzyme with 0.01µl of substrate for 10 min at room temperature and phytase assay was carried out. The K_m and V_{max} values were calculated using the effect of Sodium phytate on enzyme activity using the software, Hyperbolic Regression Software.

Results and discussion

Analysis of hydrolysis of phytic acid

In the present study, when the isolated and identified microorganisms (*Bacillus sp.*) were streaked on to the phytase specific media and incubated at 37˚C for overnight, it was found that there was a clear zone with 0.5 cm diameter formed around the streaked area for the bacterial organism showing highest phytase activity.

Response surface estimation

The observed experimental values given in the table 2 were subjected to multiple linear regression analysis. The significance of regression coefficient were determined by performing student's t-test and represented in table 3 and represents all terms involving Temperature were not statistically significantly. The statistical analysis results including the regression co efficient and p values for linear quadratic and interaction effects were given in the table 4 with 95% significance levels. Low p value indicates more significance of the corresponding coefficient and its effect on phytase activity (U/mL). Significance of each coefficient and the interaction between the variables were evaluated by the p-value. The

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estimated response surface model is specified by equation (2).

$$Activity = 0.35801 - 0.00811\,A - 0.00303\,B + 0.03208C + 0.00571A^2 + 0.0007\,B^2 - 0.03094C^2 - 0.00188AB - 0.00489BC - 0.00013AC \quad (2)$$

Table 2. Comparison of the values of phytase enzyme activity obtained experimentally and predicted from RSM

Run	pH	Temperature (°C)	Incubation time (hr)	$Activity_{expt}$ (U/mL)	$Activity_{pred}$ (U/mL)
1	6	25	14	0.095982	0.093172
2	8	25	14	0.088315	0.092136
3	6	35	14	0.085759	0.093172
4	8	35	14	0.085191	0.086243
5	6	25	22	0.142412	0.144173
6	8	25	22	0.132757	0.128157
7	6	35	22	0.143690	0.142682
8	8	35	22	0.115150	0.120774
9	6	30	18	0.143690	0.138333
10	8	30	18	0.132757	0.126860
11	7	25	18	0.128923	0.130751
12	7	35	18	0.140140	0.127059
13	7	30	14	0.095414	0.085940
14	7	30	22	0.130485	0.128706
15	7	30	18	0.124380	0.128131
16	7	30	18	0.124380	0.128131
17	7	30	18	0.124380	0.128131
18	7	30	18	0.124380	0.128131
19	7	30	18	0.124380	0.128131
20	7	30	18	0.124380	0.128131

Table 3. Estimated regression co-efficients for response surface model

Term	Coefficient	Standard Error of the Coefficient	P-Value
Constant	0.35801	0.00368	0.000
A	-0.00811	0.00338	0.038
C	0.03208	0.00338	0.000
C^2	-0.03094	0.00645	0.001
AC	-0.00489	0.00378	0.025

Table 4. Analysis of variance (ANOVA) for response surface model for Phytase enzyme activity

Sources of variation	Sum of squares	Degree of freedom	Mean square	F-value	Probability>F
Model	0.006700	9	0.000744	13.52	0.000
Residual	0.000551	10	0.000055		
Lack of fit	0.000551	5	0.000110		
Pure error	0.000000	5	0.000000		
Total	0.007251	19			
$R^2 = 0.924$					

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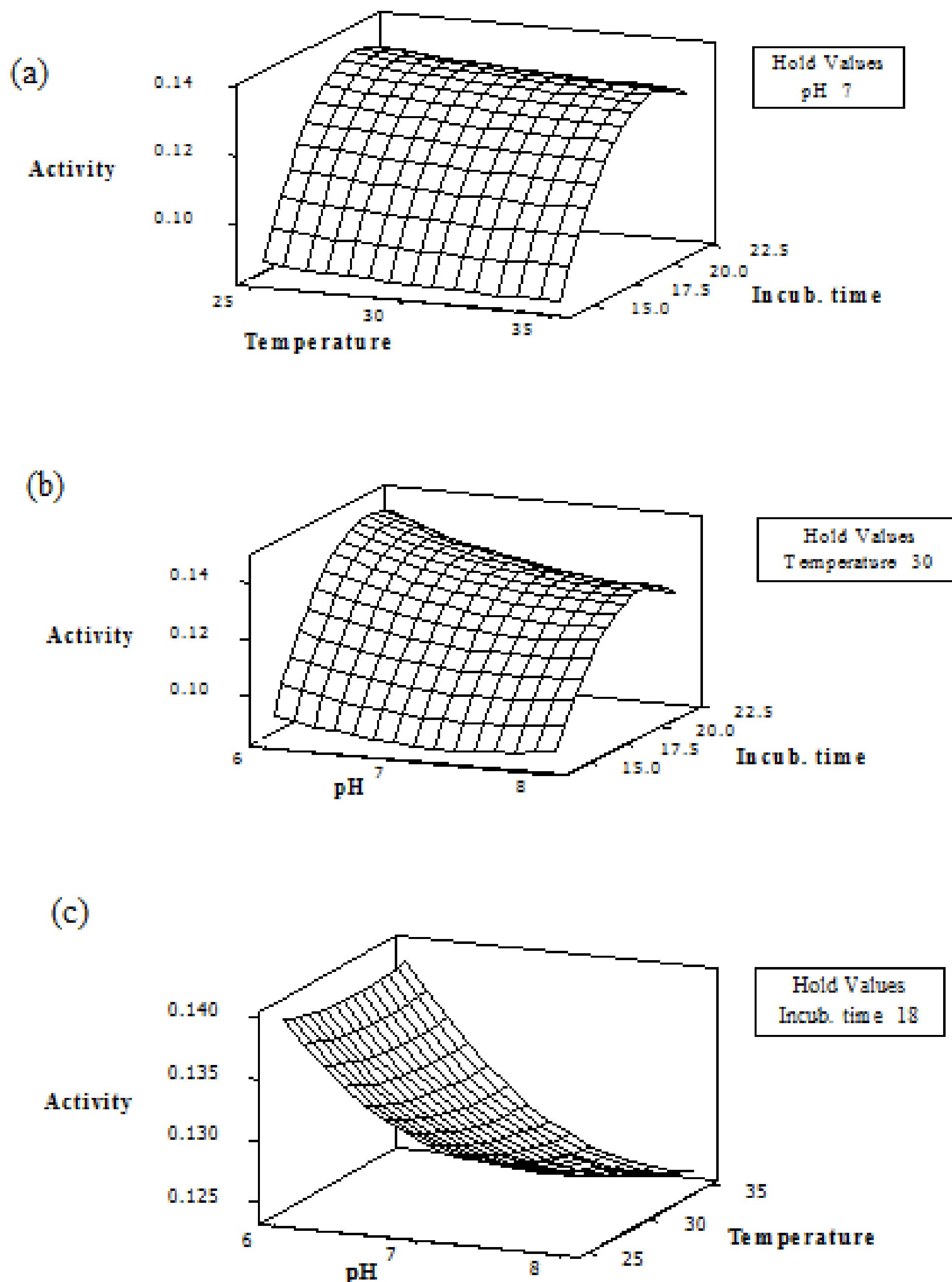

Fig. 1. Response surface for phytase enzyme activity as a function of (a) Temperature and incubation time, (b) pH and incubation time, (c) pH and Temperature

Purification and molecular weight determination analysis

The Phytase enzyme from PMM medium by *Bacillus sp.* was purified to electrophoretic homogeneity by a three-step process , and carried out at room temperature except for centrifugation and precipitation (at 4˚C).It was purified by a combination of ammonium sulphate precipitation (80%), dialysis and subsequent DEAE Sephacryl ion exchange chromatography to homogeneity, which yielded washed-out fraction with high activity. The supernant of 18 h grown culture of *Bacillus sp.* was used as the crude source of enzyme. As summarized in table 5, the culture supernant of *Bacillus sp.* had an initial specific activity of 0.409 U/ml and the final specific activity of 3.491 U/ml after purification respectively.It shows the purification will result in the increase in enzyme specific activity. At the final stage of purification,the recovery of the enzyme activity was rather low.

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Table 5. Comparison of enzyme before and after purification

Enzyme Production	Concentration of protein (mg/ml)	Enzyme activity(U/ml)	Enzyme Specific Activity(U/ml)	Purification Factor
Crude Enzyme	0.35	0.1434	0.4097	1
Ammonium Sulfate	0.101	0.1013	1.0037	2
Dialysis	0.053	0.0868	1.6395	3
Chromotography	0.017	0.0593	3.4911	7

After the combination of various purification techniques,finally the eluted protein fractions from the source were subjected to SDS-PAGE. Only one band was observed in the purified sample. The molecular weight of the purified enzyme was determined nearly to be 45 kDa (Fig. 2).

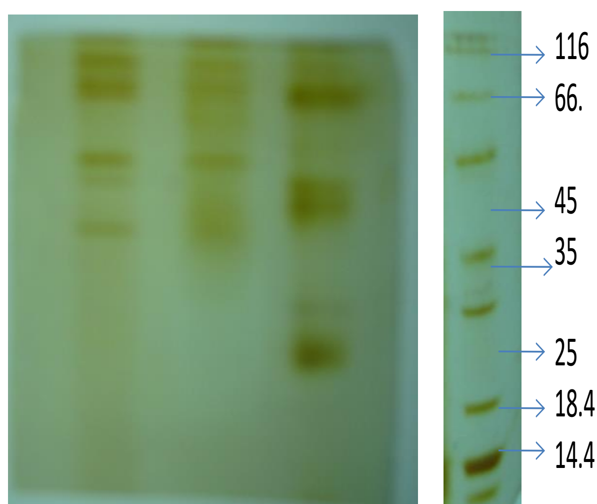

Fig. 2. The band shows the molecular weight of the phytase enzyme is 45kDa

Characterization of phytase

The results on the effect of pH on enzyme activity are presented in Fig. 3. The pH kinetics of the enzyme suggests that the enzyme activity increased sharply at pH 5, followed by a sudden decline thereafter.

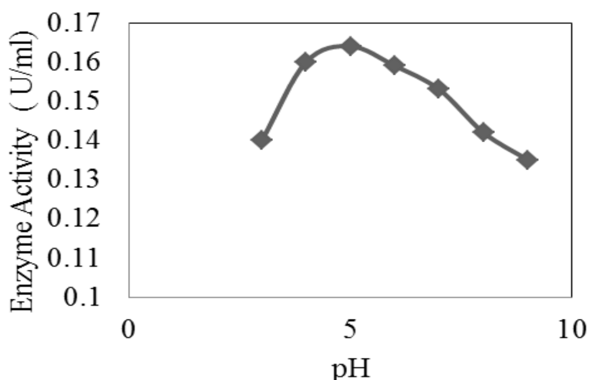

Fig. 3. Effect of pH on the enzyme activity

The results on the effect of temperature on enzyme activity are presented in Fig. 4. The temperature kinetics of the enzyme suggests that the enzyme activity increased sharply at 30°C, followed by a sudden decline thereafter. The experimental results showed in Fig. 5 that effect of chemical compounds and activity of phytase enzyme from *Bacillus* sp. which shows $CuCl_2$ acts as a enhancer.

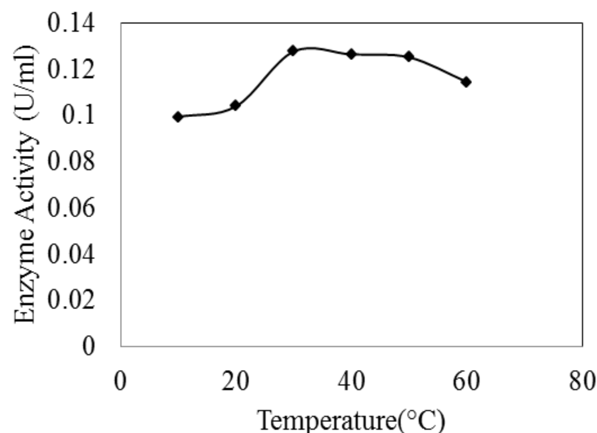

Fig. 4. Effect of temperature on the enzyme activity

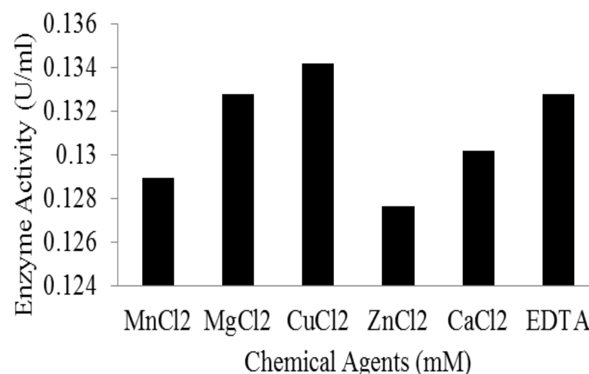

Fig. 5. Effect of Chemical Agents on the enzyme activity

Kinetic analysis

The effect of substrate concentration on reaction rate was studied as shown in Fig. 6. The kinetic parameters were estimated using

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hyperbolic regression software and tabulated in table 6.

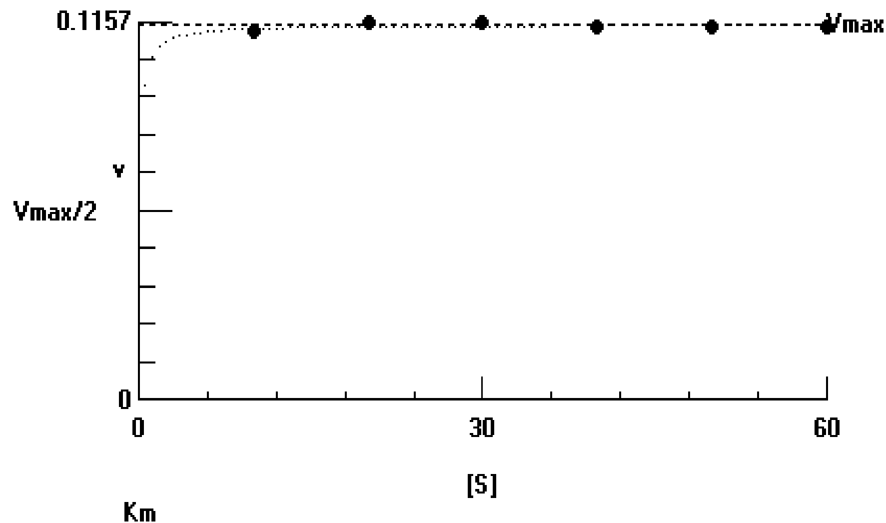

Fig. 6. Reaction rate versus substrate concentration

Table 6. Kinetic parameters

S. No.	Name of the plot	Kinetic Parameters	
		K_m (mM)	V_{max} (g/min L)
1	Line-weaver Burk	1.693	0.1202
2	Eadie-Hofstee	1.693	0.1202
3	Hanes-Woolf	1.722	0.1203

Conclusions

The phytase producing bacteria were isolated from rhizosphere and cell growth of *Bacillus sp*. was investigated in shake flask culture. The maximum value of units of phytase and specific phytase activity was observed. The optimization of production media was carried out by RSM and optimized parameter was carried out in 5 L fermentor using batch fermentation process. The enzyme thus produced was purified by DEAE Sephacryl Ion Exchange Chromatography. The molecular Weight was found to be 45 kDa. The optimum temperature for the enzyme produced from the bacteria was found to be 30° C and pH 5. The enzyme kinetics of single-substrate-catalysed reactions for activator was determined.

Conflict of interest

Authors declare there are no conflicts of interest.

References

[1] Maurya AK, Parashar D, Satyanarayana T. Bioprocess for the production of recombinant HAP phytase of the thermophilic mold *Sporotrichum thermophile* and its structural and biochemical characteristics. International Journal of Biological Macromolecules. 2017;94:36-44.

[2] Salome KR, Beazley MJ, Webb SM, Sobecky PA, Taillefert N. Biomineralization of U(VI) phosphate promoted by microbially-mediated phytate hydrolysis in contaminated soils. Geochimica et Cosmochimica Acta. 2017; 197:27-42.

[3] Barrientos L, Scott JJ, Murthy PP. Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. Plant Physiol. 1994;106:1489-1495.

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[4] Cromwell GL. Antimicrobial and promicrobial agents. In: Lewis AJ, Southern LL (Editors). Swine nutrition. 2nd edition. Boca Raton FL: CRC Press;2001;401-421.
[5] McCormick K, Walk CL, Wyatt CL, Adeola O. Phosphorus utilization response of pigs and broiler chickens to diets supplemented with antimicrobials and phytase. Animal Nutrition, 2017; 3(1):77-84.
[6] Rezaei M, Borbor S, Zaghari M, and Teimouri A. Effect of phytase supplementation on nutrients availability and performance of broiler chicks. International Journal of Poultry Science. 2007;6(1):55-58.
[7] Remus J. Poultry and environment reap the benefits of new generation phytase. Feedtech: International Feed Production and Applied Nutrition. 2005;9:22-25.
[8] Megeed A and Tahir A. Reduction of phosphorus pollution from broilers waste through supplementation of wheat based broilers feed with phytase. Journal of Chemistry. 2015:67:1-3.
[9] Gujar PD, Bhavsar KP, Khire JM. Effect of phytase from Aspergillus niger on plant growth and mineral assimilation in wheat (Triticum aestivum Linn.) and its potential for use as a soil amendment. J Sci Food Agric. 2013;93 (9):2242-2247.
[10] Mittal A, Singh G, Goyal V, Yadav A. and Kumar N. Optimization of medium components for phytase production on orange peel flour by *Klebsiella* sp. DB3 using response surface methodology. Innovative Romanian Food Biotechnology. 2011;9:35-44.
[11] Vohra A, Satyanarayana T. Phytase production by the yeast, *Pichia anomala*. Biotechnol Lett. 2001;23(7):551-554.
[12] Chanderman A, Puri AK, Permaul K, Singh S. Production, characteristics and applications of phytase from a rhizosphere isolated *Enterobacter* sp. ACSS, Bioprocess Biosyst Eng. 2016;39(10):1577-1587.
[13] Sneath PHA. Endospore-forming Grampositive rods and cocci. In: Bergey’s Manual of Systematic Bacteriology Williams & Wilkins., Baltimore, MD. 1986.
[14] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Bio Chem. 1951;193:264-275.
[15] Heinonen JK, Lahti RJ. Analytical Biochemistry. 1981;113:313-317.
[16] Vohra A, Satyanarayana T. Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochemistry. 2002;37:999-1004.
[17] Porto TS, Monteiro TIR, Moreira KA, Lima-Filho JL, Silva MPC, Porto ALF, Carneiro-da-Cunha MG. Liquid–liquid extraction of an extracellular alkaline protease from fermentation broth using aqueous two-phase and reversed micelles systems. World J Microbiol Biotechnol. 2005;21:655-659.
[18] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-686.
[19] Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987;8:93-99.
[20] Choi YM, Suh HJ, Kim JM. Purification and Properties of Extracellular Phytase from *Bacillus* sp. KHU-10. Journal of Protein Chemistry. 2001;20(4):287-292.

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