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PARTIAL PURIFICATION OF PHYTASE FROM *Hypocrea lixii* SURT01, A POULTRY ISOLATE

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ABSTRACT

In the present study, *Hypocrea lixii* SURT01, a isolate from poultry soil was subjected to 18s rRNA analysis was submitted to NCBI – Genbank. The strain was induced in minimal media which was found to produce the phytase enzyme. The characterization of extracellular phytase showed optimum activity at pH of 6.5 and temperature of 50ºC after an incubation for 120 hours. Different nitrogen sources were screened and peptone showed more enhancements in the enzyme production. Time course study was done on the culture media and that showed 120 hours to reach maximum activity. The enzyme was partially purified by 60% by ammonium sulphate precipitation and gel permeation chromatography was done to get different fractions. SDS PAGE was carried out to understand the molecular weight of the protein.

KEYWORDS:Phytase, Purification, Ammonium sulphate, Gel permeation chromatography, SDS PAGE.

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INTRODUCTON

Phytase (myo-Inositol hexakisphosphate phosphohydrolase) is enzyme that catalysis the release of phosphorous from phytic acid, a major storage form of phosphorous in cereals, legumes, oil seeds and nuts[1]. Phytates are considered as anti-nutritional 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[2,3,4,5,6,7]. Phytase are either absent or present at very low level, in the gastro-intestinal tract of mono gastric animals such as swine and poultry,[8] consequently the phytate in animal feed is discharged in the faeces without phosphorus absorption and led to its non-availability. Plant based feed ingredients due to its high phytic acid content enhance both nitrogen and phosphorous discharge thereby increasing the pollution level[9]. To overcome this, feed with phytase supplementation will increase the bioavailablity of phosphorus and decreases phosphorous pollution[10,11,12]. The present study reports the optimization of nutritional conditions, extraction and purification of phytase from a poultry soil isolate *Hypocrea lixii* SURT01 using submerged fermentation.

MATERIALS AND METHODS

(i) Chemicals

Phytic acid, sodium salt was purchased from Sigma Chemical Company, USA. All other chemicals used were of analytical grade and obtained from manufacturers including Himedia, Merck and Loba chemie.

(ii) Fungal Strain

The fungal strain used throughout the work was an isolate from poultry field soil and preliminary identification of the isolated strain was carried out on the basis of morphological analysis and various biochemical tests[13]. Further the identification of the species was done by 18S rRNA sequencing by Chromous biotech, Bengaluru, India. Fungal strain was maintained on Potato Dextrose Agar (PDA) slants. PDA contains (per litre distilled water: PDA 39 g; Agar 5 g; Antibiotic Chloramphenicol).

(iii) Medium and culture condition

Submerged fermentation medium for phytase production[15], contains (per litre of distilled water) starch 50 g; glucose 25 g; sodium nitrate 9 g; potassium dihydrogen phosphate 0.04 g; potassium chloride 0.5 g; magnesium sulphate 0.5 g; ferrous sulphate 0.10 g; pH 5.5. Fermentation medium (100ml in 250ml Erlenmeyer flask) was inoculated with 1% (v/v) of spore suspension (5×10^7 spores per ml) prepared by suspending the spores from 7 days old sporulated slant of *Hypocrea lixii* SURT01 grown on PDA in 10ml of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30° C at 120 rpm. Samples were removed after every 24h and checked for pH, growth, total protein and phytase activity.

(iv)Phytase assay

Phytase activity was measured in an assay mixture containing 44.1 mM phytic acid and 200mM glycine buffer (pH 2.8) and suitably diluted enzyme. Reaction mixture is incubated at 37°C for 30 minutes, colour reagent was added and the developed colour was read colorimetrically at 400nm. One enzyme unit was defined as the amount of enzyme liberating 1µmol of inorganic phosphate in 1min under the assay conditions[16,17]. Each experiment was carried out in triplicate and the values reported as the mean of three such experiments in which a maximum of 3–5% variability was observed. Concentration of protein was determined using Lowry method[18] using bovine serum albumin as standard.

(v) Nitrogen sources

The following refined nitrogen sources such as peptone, urea, ammonium nitrate and ammonium phosphate was evaluated.100ml of inoculum media was prepared in 250ml of conical flask with supplemented individual nitrogen sources at different concentration (0.5%, 1%, 1.5%, 2% and 2.5%). After sterilization 0.1ml (10^8spore/ml) of the spore suspension was added aseptically. The inoculated flask kept under shaking (150rpm) for 4 days at 30^oC. After the incubation the media was filtered through muslin cloth to remove mycelia debris and the collected filtrate was centrifuged at 10000 RPM for 10

minutes. The suspension was collected and used as crude enzyme source[19].

(vi) Effect of pH on phytase production

The phosphate buffer with different pH values ranged from 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9 was prepared and 0.5ml of each was added to the 0.5ml of crude enzyme and the reaction mixture was incubated at room temperature for 1 hour. The enzyme assay was carried out as mentioned earlier[20].

(vii) Effect of temperature on phytase production

To 0.5ml of crude enzyme, 0.5ml of optimum phosphate buffer was added in a test tube. The reaction mixture was incubated at 30, 40, 50, 60 and 70ºC for 1 hour. The enzyme assay was carried out as mentioned earlier[20].

(viii) Partial Purification of Enzyme

After 5 days incubation, the fermented broth was filtered to remove mycelium and subjected to centrifugation at 10,000rpm for 30min at 4°C and the clear supernatant was collected. The cell free supernatant was collected and used as crude enzyme for purification[21].

(ix) Ammonium sulphate precipitation

Fifty milliliters (50 mL) of crude enzyme was precipitated (fractional) with $(NH_4)_2SO_4$ (Analytical grade, Hi-media) at 70% (w/v) saturation for 24 h at 4ºC. The precipitate was centrifuged at 10,000 rpm for 10 min. The precipitate was re-dissolved in Tris-HCI buffer (pH 7.8) and dialyzed against several volumes of the same buffer for 24 h at 4°C using acetylated cellophane tubing prepared from dialysis tube (Hi-media)[22].

(x) Gel electrophoresis

Sephadex G-150 (Sigma, Aldrich) was packed into a column (1.5 ×25 cm) and equilibrated .

with Tris-HCI buffer (pH 7.8).The column was eluted with the same buffer at a flow rate of 20 mL/h. A fractions of 2.0 mL were collected at interval of 30 min and the absorbance at 280 nm was read using UV-VIS spectrophotometer (Varion – Cary 100)[23].

(xi) SDS- PAGE

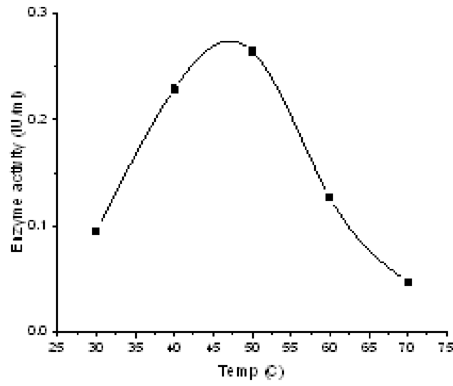
SDS PAGE was performed according to the Laemmli[24] with the 4% Acrylamide stacking gel and 10% Acrylamide separating gel to determine the molecular mass and purity of the protein. Staining was carried out with CBB (coomassie brilliant blue) staining method.

(xii) Statistics

Values in the figure are expressed as mean ± (SE). The Student t-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p < 0.05$ was considered to assess statistical significance

RESULTS

Fungi was isolated from poultry soil, and purified as single strain by multiple streaking. The isolated organism was subjected for 18S rRNA sequencing and identified as *Hypocrea lixii,* subsequently named as *Hypocrea lixii* SURT01. The sequence generated was then aligned with reference sequences obtained from GenBank using BLAST program. Further the obtained sequence was submitted to National Centre for Biotechnology Information (NCBI) and received accession number HQ875779[14]. *Hypocrea lixii* SURT01 was cultured and preserved on Potato Dextrose agar plate and slant. Production of phytase enzyme by the organism was checked initially by using Czapox Dox media supplemented with 2% of sodium phytate.



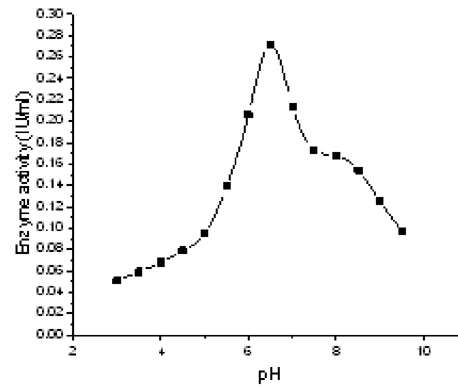
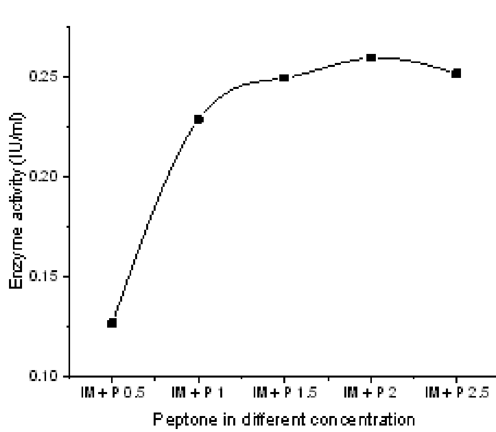


Figure -1 ***Effect of temperature on enzyme activity*** **Figure-2** ***Effect of pH on enzyme activity***

The optimum temperature for enzyme activity is recorded at 50° C (Fig - 1). Effect of pH on phytase activity of *Hypocrea lixii* SURT01 was conducted in different pH range starting from 3 to 9.5. In which phytase activity is recorded maximum at pH 6.5 of about 0.272 IU/ml (Fig - 2).



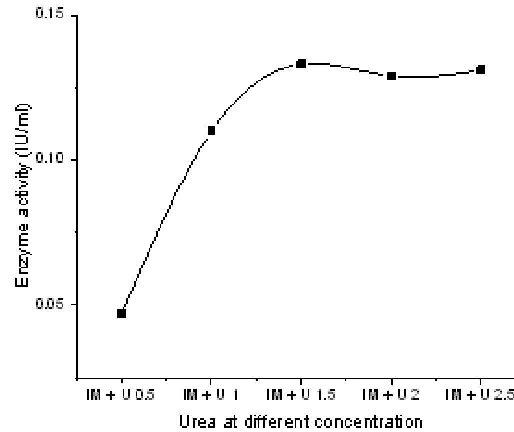
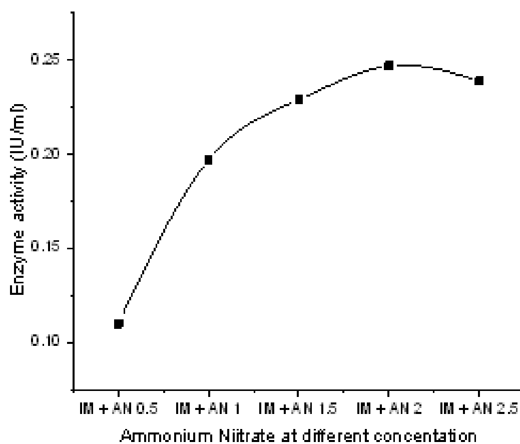


Figure -3 ***Effect of peptone on phytase activity*** **Figure-4** ***Effect of urea on phytase activity***



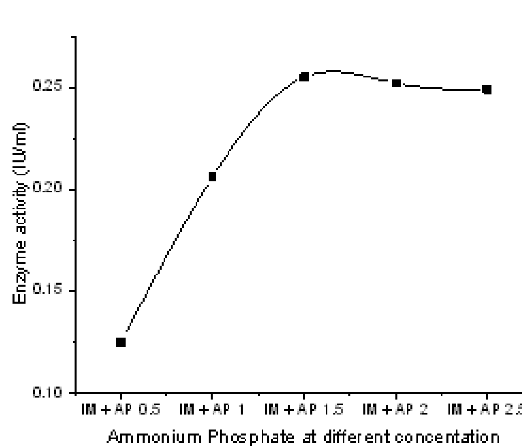


Figure -5 ***Effect of NH_4NO_3 on activity*** **Figure-6** ***Effect of $NH_4H_2PO_4$ on activity***

The activity of the phytase enzyme (units/ml) under various nitrogen sources as well as under various concentrations of nitrogen are shown in Fig3-6. Peptone showed higher activity at 2 mg/ 100ml , Urea at 1.5 mg/100 ml Ammonium nitrate at 2 mg/100 ml and ammonium phosphate at 1.5 mg/100 ml. Among the nitrogen source analyzed peptone demonstrated the maximum activity. Present studies on phytase production under submerged fermentation conditions by *Hypocrea lixii* SURT01 using different nitrogen source at different concentration, shows that the 2% peptone supplementation shows maximum activity of about 0.265 units/ml (Fig - 3), which is followed by 1.5% ammonium dihydrogen phosphate supplementation 0.255units/ml (Fig - 6). When compared with the standard medium present studies show 64% increase in enzyme production.

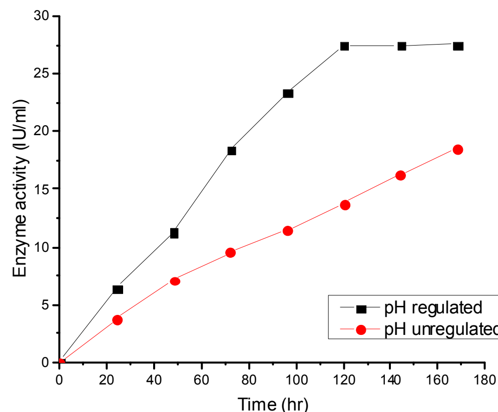


Figure 7
Time course of phytase production from Hypocrea lixii SURT01.
Media were sampled at different time intervals and phytase activity was determined

Two fungal cultures were maintained, one with regulated pH and another without regulation of pH. Samples were taken at different time interval and analyzed for enzyme activity. The results were shown in Figure 7. In the regulated pH, the activity of enzyme was comparatively more than the unregulated culture. During the submerged fermentation an attempt was made to evaluate the activity of phytase from 0 to 7 days by assaying the harvested supernatant. In the period between 72 -120 hrs there was maximum phytase production. Maximum phytase activity of 27.5 U/ ml was achieved at 120hrs.

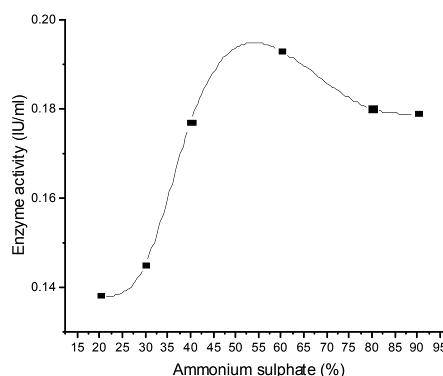


Figure 8
Effect of NH_4SO_3 on phytase activity

Table 1
Ammonium sulphate precipitation of crude enzyme

S.No	% of Ammonium sulphate	Enzyme Activity (IU/ml)
1.	40	17.7
2.	60	19.3
3.	80	18.0

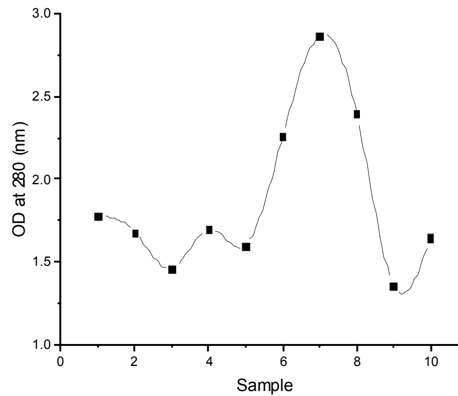


Figure 9
Effect of NH_4SO_3 on phytase activity

Table 2
Purification of Enzyme by Gel Filtration Chromatography

S.No	Sample	OD at 280nm
1	1	1.46
2	2	1.6
3	3	1.68
4	4	1.7
5	5	1.78
6	6	2.26
7	7	2.87
8	8	2.4
9	9	1.65
10	10	1.36

Table 3
Phytase Activity in purified sample

S.No	Sample Number	Enzyme Activity (units/ml)
1.	6	0.5
2.	7	30.2
3.	8	27.0

Table 4
Purification of phytase from Hypocrea lixii

Steps	Enzyme Activity UmL^{-1}	Protein Activity Uml^{-1}	Specific Activity $Uml^{-1}mg^{-1}$
Crude extract	27.5	0.302	91
Ammonium sulphate precipitation	19.3	0.12	160.8
Gel filtration	30.2	0.08	377.5

This study reports biological production and purification of phytase from *Hypocrea lixii* SURT01. Enzyme production was found to be maximum in 120 hours that is on fifth day 27.5 U/mL. The specific activity of the crude enzyme as shown in Table-4 was 91Uml^{-1}mg^{-1} protein and this was increased to 160.8Uml^{-1}mg^{-}1 protein by ammonium sulphate precipitation and further increase in specific activity of 377.5Uml^{-1}mg-1protein was recorded after gel filtration chromatography. The molecular mass of purified phytase was found to be 56kDa, by SDS–PAGE analysis. The obtained enzyme, would find application in animal feed industry for improving nutritional status of the feed and combating environmental phosphorus pollution. Shah and Ratna Trivedi [25] reported in *Aspergillus tamari* the molecular weight of phytase enzyme was 85kDa. Fujita *et al.,* [26] reported phytase from *Aspergillus oryzae* under solid state fermentation conditions which showed an optimum pH of 5.0, and stability at 50°C and molecular weight 56 kDa. Extracellular phytase with two pH optima (2.5 and 5.0) has been reported from *Aspergillus niger*[23] and commercial *Aspergillus niger phytase* (Natuphos)[27].

CONCLUSION

In the present communication, phytase from *Hypocrea lixii* SURT01 produced under submerged fermentation and its biochemical properties including pH, temperature were standardized. By maintaining pH at 6.5 and temperature around 45ºC, different carbon sources were screened and peptone showed more enhancements in the enzyme production. Time course study was done on the culture media and that showed 120 hours to reach maximum activity. The enzyme was partially purified by 60% by ammonium sulphate precipitation and gel permeation chromatography was done to get different fractions. SDS PAGE was carried out to understand the molecular weight of the protein.

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