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Original Article

# EVALUATION OF PHYTASE PRODUCTION BY *HYPOCREA LIXII* SURT01 IN SUBMERGED AND SOLID-STATE FERMENTATIONS

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**ABSTRACT**

**Objective:** Phytases have important applications in human and animal nutrition because they hydrolyze the phytate present in legumes, cereal grains and oil seeds to release inorganic phosphate. Supplementation of phosphate to the poultry causes a serious problem of eutrophication. This can be reduced by incorporating phytase in poultry feed. Present study explains extracellular phytase production by SmF and SSF from a fungal strain *Hypocrea lixii* SURT01.

**Methods**: Extracellular phytase production by *Hypocrea lixii* SURT01 was evaluated in media containing various refined carbon sources (Fructose, Sucrose, Maltose and lactose in concentration ranging from 1.5% to 7.5%) along with standard medium under submerged fermentation (SmF). At the same time, phytase production was studied under Solid State Fermentation (SSF) with four different substrate such as barley, green gram, bengal gram and black gram.

**Results**: In SmF out of different carbon sources in various concentrations, 6% sucrose showed maximum enzyme production (245U/ml). In SSF, barley showed highest phytase yield (1638 Units/ml) on 5$^{th}$ day of incubation.

**Conclusion**: Evaluation of Solid state fermentation showed enhanced phytase production when compared to Submerged Fermentation.

**Keywords:** Phytase, Submerged fermentation, Solid state fermentation, Enzyme activity.

## INTRODUCTION

Phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6) is a phosphorylated derivative of myo-inositol, important in the storage and retrieval of phosphorus, inositol and ions during plant development and germination [1]. Phytic acid has a strong anti-nutritive effect [2] and this effect is based on the unusual molecular structure of phytic acid. At complete dissociation, the six phosphate groups of phytic acid carry a total of twelve negative charges. Therefore, phytic acid has a strong binding capacity and it effectively binds different mono, di, and trivalent cations and their mixtures, forming insoluble complexes [3]. It forms the fairly stable chelates with almost all multivalent cations which are insoluble at pH 6 to 7, although pH, type and concentration of cations have a tremendous influence on their solubility characteristics [3]. The formation of insoluble phytate mineral complexes in the intestinal tract prevents mineral absorption. This reduces the bioavailability of essential minerals [4]. This study was carried out to evaluate the influence of media composition for phytase production by a fungi *Hypocrealixii* SURT01 through submerged fermentation (SmF) and solid state fermentation (SSF). Submerged fermentation was evaluated by supplementation of various carbon sources such as fructose, sucrose, maltose and lactose at different concentration ranging from 1.5% to 7.5%. Solid state fermentation was evaluated using various sources such as barley, green gram, Bengal gram and black gram.

## MATERIALS AND METHODS

### Fungal Strain

### Organism and culture maintenance

The fungal strain was isolated from poultry field soil by screen plate method, further identified by conventional method and confirmed by 18S rRNA T1 sequencing. The culture was maintained in the Department of Biotechnology, Sathyabama University, Chennai, in PDA slant at 4°C.

### Identification of microorganism

Identification of fungi was done by 18S rRNA sequencing [5]. 18S rRNA T1 sequencing was done by isolation of DNA from the organism and the large fragment of the 18S rRNA gene was amplified by PCR using the universal primers BAC-F-(5'-AGA GTT TGA TC(AC) TGG CTC AG-3') BAC-R (5'AAG GAG GTG (AT)TC CA(AG) CC-3'). The PCR products were purified using a Wizard PCR Preps DNA Purification System according to the manufacturer's instructions. The PCR product after purification is sequenced using a Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and a model 3100 automatic sequencer.

The closest known relatives of the new isolates were determined by performing a sequence database search. The sequences of closely related strains were retrieved from GENBANK and the Ribosomal Database Project (RDP) libraries. Phylogenetic analysis was also performed.

### Preparation of spore suspension used as a source of inoculum

7 days old culture used as source of inocula. Sterile distilled water with 0.1% Tween 20 was flooded over the slant surface and scraped with sterile glass rod, filtered through muslin cloth and the resulting suspension was used. The total spore concentration was adjusted to $10^8$ by using hemocytometer [6].

### Submerged Fermentation

Submerged Fermentation medium for phytase production was prepared according to Soni and Khire 2007 and Shieh and Ware 1968 [7], [8] (Table - 1)

**Table 1: Standard Media Composition**

| S. No. | Chemicals | Concentration (g/L) |
|---|---|---|
| 1. | Starch | 50 |
| 2. | Glucose | 25 |
| 3. | Sodium Nitrate | 8.6 |
| 4. | Potassium dihydrogen phosphate | 0.04 |
| 5. | Potassium Chloride | 0.5 |
| 6. | Magnesium Sulphate | 0.5 |
| 7. | Ferrous sulphate | 0.1 |

**Table 2: Supplemented Carbon sources**

| S. No. | Supplemented Sources | Concentration in % |
|---|---|---|
| 1. | Fructose | 1.5, 3.0, 4.5, 6.0 and 7.5 |
| 2. | Sucrose | 1.5, 3.0, 4.5, 6.0 and 7.5 |
| 3. | Maltose | 1.5, 3.0, 4.5, 6.0 and 7.5 |
| 4. | Lactose | 1.5, 3.0, 4.5, 6.0 and 7.5 |

100 ml of fermentation media was prepared in 250 ml of a conical flask and autoclaved. Different carbon sources in various concentration as shown in Table – 2 were filter sterilized and supplemented in fermentation media.0.1 ml ($10^8$spore/ml) suspension was added aseptically. The inoculated flask was kept under shaking (150 RPM) for 5 days at room temperature. 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 supernatant was collected and used as crude enzyme source.

**Solid State Fermentation** [9], [10]

Ten grams of green gram, Bengal gram, black gram andBarley were taken separately as the solid substrate and transferred to 250 mL conical flask. 10 ml of sterile distilled water was added to moisture the content. The flasks were cotton plugged and sterilized at 121°C under 15 psi for 20 min. The flasks were cooled to room temperature and were then inoculated with 1.0 ml fungal suspension($10^8$spores/ml).

The substrate cultures were incubated at 35ºC for 120 hrs. The flasks were shaken twice a day. All the experiments were run parallel in triplicate. After fermentation, 50 ml of 2 % aqueous solution of calcium chloride was added to each flask. Flasks were put in a rotary shaker operated at 200 RPM for 2 hours at room temperature for the extraction of an enzyme from fermented mass [11]. The suspension was squeezed and it was centrifuged at 5000 RPM for 20 minutes at $4^0$C. The clear supernatant was used as crude enzyme source.

**Phytase assay** [12]

Phytase activity was measured in an assay mixture containing 44.1 mM phytic acid and 200 mM glycine buffers (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 400 nm. One enzyme unit was defined as the amount of enzyme liberating 1 µmol of inorganic phosphate in 1 min under the assay conditions. Concentration of protein was determined using the Lowry et al 1951[13] method using bovine serum albumin as standard. 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.

**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 assessstatistical significance

**RESULT AND DISCUSSION**

**Microorganism**

Isolated fungi were subjected to 18sRNA T1 sequencing and showed 95% homology with Hypocrea lixii. Further the sequenced organism was named as *Hypocrealixii*SURT01. This sequence was submitted to Genebank and received an accession number HQ75779.

**18S rRNA sequence**

>gi|340343840|gb|HQ875779.1| Hypocrea lixii strain SURT01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

TGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGC
GTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAAATTTT
ATTGTATACCCCCTTGCGGGGTTTTTTATAATTTGAGCCTTTTTGGCGC
CTTTTGTAGGCGTTTTGAAAATGAATCAAAAATTTCAACAACGGATTT
TTTGGTTTTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATTGAATTTTTGAACGCACATTGCG
CCCGCCAGTATTTTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCT
TGAACCCCTCCGGGGGGGTTGGCGTTGGGGATTGGCCCTCCCTTAGGCGG
TGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGC
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ACACCCAACTTCTGAAA

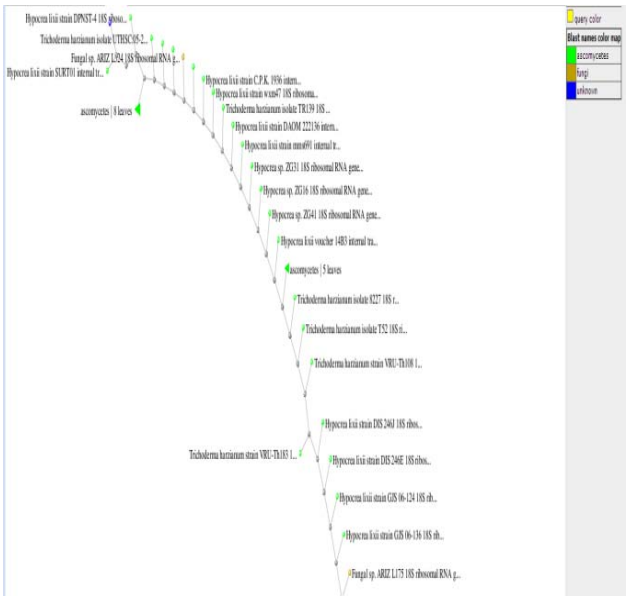


**Fig. 1: Phylogenetic analysis of *Hypocrea lixii* SURT01**

**Effect of Carbon Source Supplementation in Submerged Fermentation**

The effect of various carbon sources on phytase production at different concentration was given in table-3 and figure-2. The results indicated that among the tested carbon sources 6% sucrose supplementation showed maximum phytase production (245 Units/ml) when compared to other carbon sources used in this study. Prasanthakumari *et al* 2011 [14] reported in fungi (PF-3) isolated from soil produced maximum phytase production (47 Units/ml) when sucrose is used in the medium. The effect of various carbon source on protein concentration is shown in Table-4, and Figure-3.

**Table 3: Effect of various carbon sources on enzyme activity**

| S. No. | Concentration in % | Enzyme Activity (units/ml) | | | |
|---|---|---|---|---|---|
| | | Fructose | Sucrose | Maltose | Lactose |
| 1 | 1.5 | 57.5 | 75 | 37.5 | 37.5 |
| 2 | 3.0 | 97.5 | 135 | 57.5 | 57.5 |
| 3 | 4.5 | 132.5 | 170 | 95 | 135 |
| 4 | 6.0 | 152.5 | 245 | 150 | 192.5 |
| 5 | 7.5 | 170 | 227.5 | 132.5 | 172.5 |

**Table 4: Effect of various carbon sources on Protein concentration**

| S. No. | Concentration in % | Protein Concentration (mg/ml) | | | |
|---|---|---|---|---|---|
| | | Fructose | Sucrose | Maltose | Lactose |
| 1 | 1.5 | 6 | 9.5 | 6 | 6 |
| 2 | 3.0 | 9.5 | 13 | 8 | 9.5 |
| 3 | 4.5 | 11.5 | 17 | 10 | 17.5 |
| 4 | 6.0 | 17.5 | 22.5 | 15.5 | 23 |
| 5 | 7.5 | 15.5 | 21 | 13.3 | 21 |

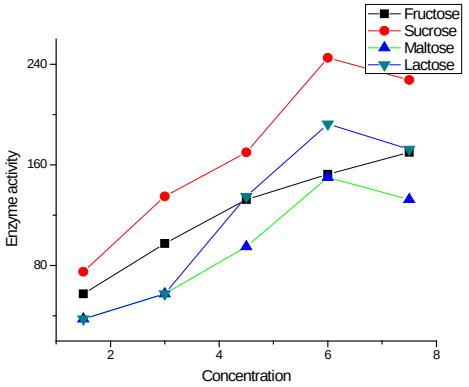


**Fig. 2: Effect of various supplemented carbon sources on phytase activity**

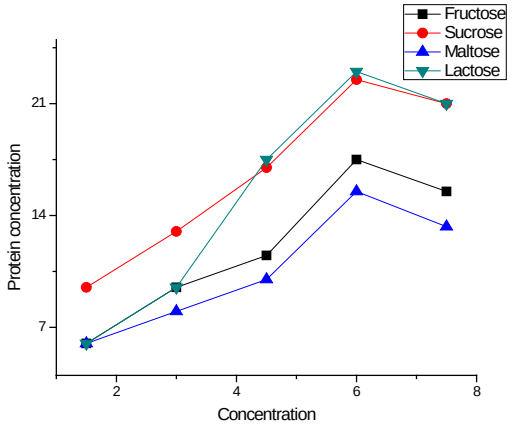


**Fig. 3: Effect of Carbon Source Supplementation on protein.**

### Solid State Fermentation

Substrates used in solid state fermentation are protein rich also provide carbon source, vitamins and minerals. Different solid sources like barley, green gram, black gram and bengal gram were explored for phytase production. Results showed that barley recorded maximum phytase activity (1638 Units/ml) (Fig .4). It might be due to the reason that barley provides adequate amount of nutrient. Spier*et al* 2008 [15] showed that *A. ficuum*NRRL 3135 produced 26 U/gby using citric pulp and other residues of the agroindustry.

**Table 5: Effect of various substrates of Enzyme activity**

| S. No. | Solid Substrate | Enzyme Activity (U/ml) |
|---|---|---|
| 1 | Barley | 1638 |
| 2 | Green gram | 658 |
| 3 | Bengal gram | 1106 |
| 4 | Black gram | 336 |

According toNgo Thanh Xuan et al., 2009 [16] reported that phytase gene from *Aspergillur niger* XP was cloned in *P. pastoris* which showed higher expression of phytase production. Chen, *et al.*,2004 [17] reported E. coli appA gene in *P. pastoris* with the maximum phytase activity after an induction period of 96 h was 118 to 204 IU/ml at the flask scale and 1880 – 4946 IU/ml for high cell-density fermentation.

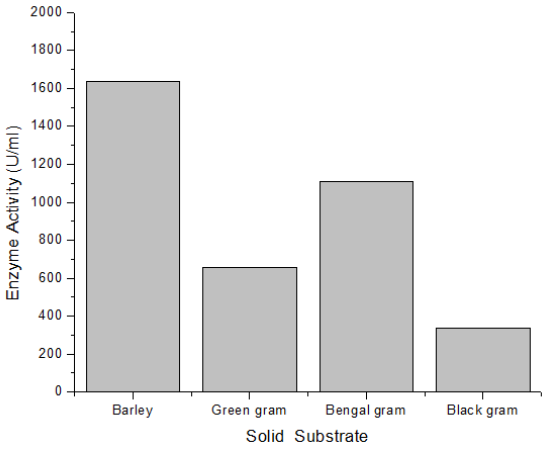


**Fig. 4: Effect of various substrates of Phytase activity**

### CONCLUSION

Phytase are eco-friendly enzymes that are widespread in nature. Their supplementation to animal feed is an effective way to increase the availability of phosphorous to animals, thus improving their performance and reducing manure-born phosphorous pollution. The advantages of solid state fermentation due to the culture medium is not free flowing, depth of the medium is usually shallow, fungal growth involves penetration of the hyphae deep into solid substrate particles.

In this study optimization of phytase production was carried by using a fungi *Hypocrea lixii* SURT01 (Poultry soil isolate) through submerged fermentation and solid state fermentation. Out of various carbon sources used 6% sucrose recorded maximum phytase production. Maximum increase in Phytase activity was observed in barley under solid state fermentation.

### CONFLICT OF INTEREST

Declared None

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