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Biocatalyst: Phytase Production in Solid State Fermentation by OVAT Strategy

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Abstract: The phytase-producing *Pichia membranifaciens* S3 (MG663581) was isolated from sugarcane juice using phytase screening medium. The predicted and experimental model showed maximum phytase production of 364 U/ml appearing g/100ml: Jack fruit seed (1.5), peptone (0.15), dextrose (0.50), yeast extract (0.05), malt extract (0.05) pH 5.5 and 28^{0}C) used OVAT strategy by Solid state fermentation through shake flask methodology. The modelling, 3D structure of Phy S3 amino acid sequences was modelled (PhyS3.B99990003) by using Modeler 9.23 and validated results showed that 86.4% in the favoured region by Ramachandran plot.

Keywords: Yeast; jack fruit seed powder; PSM medium; Ramachandran plot; *Pichia membranifaciens*; one source variable at a time.

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1. Introduction

Enzymes are biocatalysts and large macromolecules integrated of polymers of essential or non-essential amino acids connected by amide bonds [1]. Microorganisms are the favoured sources of enzymes, because they are fast growth in large quantities in minimum period and easy availability. In addition, they play an economical role in metabolic and biochemical reactions in the enzyme production [2].Phytase is universally availed in nature like in different microorganisms, in which interest has increased remarkably in the past decades[3].

Phytases (EC 3.1.3.8 ;phosphomonoesterases) primary enzymes having catalyse the hydrolysis of phytate into lower forms of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, in rarely free myo inositol phosphates and inorganic phosphate (Ins P5 to P1) [4]. However, phytases are liberated of P moiety from phytic acid at the position phytases, three types namely (C3) 3-phytase (EC 3.1.3.8), (C5) 5-phytase (EC 3.1.3.72) and (C4) 4-phytase (EC 3.1.3.26) respectively. Hence, phytases are considered to be a noteworthy candidate for use as an enzyme that has great value in enhancing nutrient factors from soil, therefore, phytase enzyme would be an eco-friendly product [4].

Yeasts are known as a potential for producing economical industrial important enzymes, Therefore, *Pichia sp* generally regarded as safe (GRAS) strains are involved in production of extracellular and cell bound phytases and to develop large scale industrial production in economical mode. Yeast like *Zygosaccharomyces priorionus* NCIM 3299 *Zygosaccharomyces bisporus* NCIM 3265 *Schizosaccharomyces octosporus* NCIM 3297 3296, and *Williopsis saturnus* NCIM 3298) has been successfully bring about using

cultivation process of solid state fermentation [5]. Hence, in the past 20 years, several investigators have optimized the nutritional and physical parameters for maximizing the production of yeast phytases [6-9].

In the present investigation,we isolated, identified *Pichia sp* and screened to be a potent phytase producer. Optimal culture conditions and fermentation parameters were assessed for most effective nutrients to enhance phytase production by Solid state fermentation Furthermore, have been identified and purified to near-homogeneity by protein sequence homology, the 3D structure of the protein.

2. Materials and Methods

2.1. Sample collection site description.

Wild yeast strains were isolated from sugarcane juice collected from Dharmapuri Co-operative Sugar Mills, Pallacode, Dharmapuri in a sterile container. The collected juice sample was serially diluted 10^{-5} to 10^{-9} and plated on dextrose (2g), yeast extract (1g), malt extract, peptone (1g), agar (1.7g) in 100ml of sterile distilled water and incubated at 30^0C for 48-72 h respectively. After the incubation period of 48 h, dominative colonies were selected based on shape, color, margin, elevation. Pure culture was prepared by streak plate method. Yeast cultures were maintained on 2% YPMD slants at 4ºC for short period storage [10].

2.2. Primary screening of phytase producing isolate.

One loop full of dominative isolate was plated onto phytase screening media (PSM) plates containing (g/100ml):1.5 Dextrose, 0.5 phytic acid, 1 $NaNO_3$, 0.5 $MgSO4.7H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$, 0.01 $MnSO4.6H_2O$, 1.5 agar, pH 5.5.Plates were incubated at 30°C and checked for phytase production on the basis of the halo zone of hydrolysis around the colony (Kłosowski et al. 2018). The halo zoned isolate was carried for further work.

2.3. Sequence and deposit.

In genotypic characterization, extract genomic DNA from yeast cells, quantitate the genomic DNA and polymerase chain reaction (PCR) was carried out using the applied biosystem research thermal cycler. After amplification purify the amplified product using a PCR purification kit (orange). The sample was loaded on the machine and the data in form A, C, T, and G were released. The 16S rRNA sequencing was carried out using the applied biosystem. The isolates' genes were sequenced using primer **(5′-3′)** FP - GCCTGTCTCAAAGATTAAGCC**,** RP- CACCTACGGAGACTTTGTTAC**.** The sequence result was identified based on the percentage (%) similarity obtained from above was compared with known sequences in the Genbank using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). The identified sequence was submitted to blanket-NCBI (https://www.ncbi.nlm.nih.gov/BankIt) submission tool and also sequence has been deposited in the GenBank nucleotide sequence databases under Accession No. A phylogenetic tree was made in robust tree software using the Neighbor-joining method with Bootstrap analysis to obtain evolutionary relationships of taxa [11].

2.4. Phytase production on Shake flask conditions.

The initial optimization step was carried out (OVAT) shake flask conditions with different sources including peptone and dextrose were used as a carbon source in different concentrations of (g/100ml) (0.15-0.55), (0.25-1.25). Yeast extract and malt extract were used as nitrogen source in different concentration of (0.05- 0.25), (0.05-0.25) with the Jackfruit seed powder concentration (0.5-2.5) pH to (5.5-9.5), temperature at 28,30,32,37 and 40°C.

2.5. Phytase production and assay.

Solid-state fermentation was carried out optimized sources of peptone 0.15, dextrose 0.5, yeast extract 0.05, malt extract 0.05, substrate with jackfruit seed powder (1.5 g/100ml) and add 1 ml *Pichia sp* S3 (10^8cells mL^{-1}) of culture, condition was maintained at pH 5.5 and temperature 28°C [12]. The shaker was run with agitation 150 rpm for 72h. 20 ml of sample was withdrawn at 72h and analyzed for phytase activity assay using (trichloroacetic acid, Taussky shorr color reagent) [13] and protein estimation using folin ciocalteu reagent [14].The activity of the enzyme was expressed as units per ml for the reaction.

2.6. Partial Purification of Phytase by APS, Dialysis, DEAE- Column chromatography.

All three purification processes were carried out at 4^0C in laboratory. The collected crude extract of Proteins was precipitated using ammonium sulphate [15]. They were fractioned 20 ml crude extract with the addition of ammonium sulphate until different saturation levels were reached (20-80%) under magnetic stirrer. The collected precipitate was transfer to centrifuge tube and pelleted by centrifugation at 15,000 rpm for 20 min at 4°C and pellets resuspended in a 20 mM and pH 7.5 Tris-HCl buffer. and assayed activity of phytase.

The highest phytase activity suspension was desalted in a 12 cm dialysis bag against the Tris-HCl buffer overnight under magnetic stirring with a frequent change to remove the residual $(NH_4)_2SO_4$. The dialyzed protein was again centrifuged 10000 rpm for 5 min to remove any insoluble particulate matter [16].The dialyzed solution was then poured to DEAE-Cellulose anion exchange column (2 cm 10 cm), equilibrated with 20 mM Tris-HCl buffer and eluted with a (0.5 and 1.0 M) NaCl. The fractions were pooled, concentrated [17]. At three stage of purification process, total protein content of the fractions by Lowry's method with BSA was used as the standard curve at 595 nm and phytase activity were analysed.

2.7. Homology modelling Phy S3 protein.

Homology 3D protein modeling of selected Phy S3 protein *Pichia membranifaciens* (MG663581)sequence was performed, using its suitable best-matched homologous sequences for building the 3D structure was searched against PDB using BLAST-RCSB and alignment file was used to build the model using the automated homology modeling tool MODELER 9.23.Evaluation of the predicted protein model was done by SAVES server (http://servi ces.mbi.ucla.edu/SAVES/).Based on the generated result, the best built model was finally submitted to Protein Model Database (PMDB) and the PMDB ID was acquired [18].

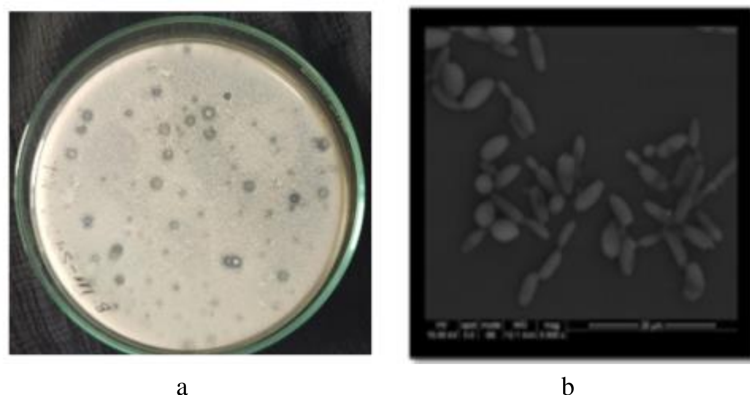
3. Results and Discussion

3.1. Isolation and screening of phytase producer.

A total of ten yeast strains were isolated in YPMD medium from the sugarcane juice samples collected at Dharmapuri Co-operative Sugar Mills. All the isolates were screened on phytase producing medium containing 0.5% phytic acid as the substrate. Among the ten yeast isolates, one isolate S3 was picked on the basis of the clearing zones around the colonies in PSM medium (Figure 1a). Accordingly, this isolate was chosen for further investigations.

3.2. Phenotypic characterization.

Strain S3 (Figure 1b) showed that the arrangement of few buds but many prominent bud scars on individual cells rounded or slightly elongated had a smooth surface and interconnecting processes. It depicted bipolar young budding yeast cells under 3000x magnification.



a b

Figure 1. *Pichia membranifaciens S3* a) Clear zone around S3, b) SEM image: intact colonies.

3.3. Genotypic characterization.

Yeast isolate S3 was subjected to molecular identification using 16S rRNA. The 16S rRNA gene was amplified by PCR. The amplified product, with reference to the marker used, was estimated to be amplicon approximately 290 bp. The 16S rRNA sequence of the S3 shows the phylogenetic relationship and closest similarity {100%} with *Pichia membranifaciens* available in NCBI-BLAST database, the optimal tree with the sum of branch length = 0.1501 (Figure 2). The partial sequence of 16S rRNA gene was submitted in Blankit tool and accessed the GenBank accession number MG663581.Several authors reported the production of phytase using variety of yeast [19]. However, few studies made an attempt for the *Pichia sp* used for phytase production by *Pichia membranifaciens*, *Pichia membranifaciens* [20]. However, there is no report on potential phytase producing *Pichia membranifaciens* S3 isolated from sugarcane juice streams utilizing jackfruit powder as potential substrates.

3.4. Phytase production optimization by SSF.

During ssf, all the nutrient sources were supplied for the microbial growth and phytase production. Thus, different five concentrations of two carbon sources and two nitrogen sources were investigated for their efficacy on phytase production. Among different concentrations of peptone sources, 0.15g/100ml produced a highest phytase activity and biomass {364± 0.71

U/ml} {1.76 ± 0.32} than other concentrations similarly, dextrose in {364± 0.24 U/ml} {1.31 ± 0.18} activity in 0.15g/100ml (Figure 3). Similarly reported that supplementation of carbon sources increased significant phytase production [21].

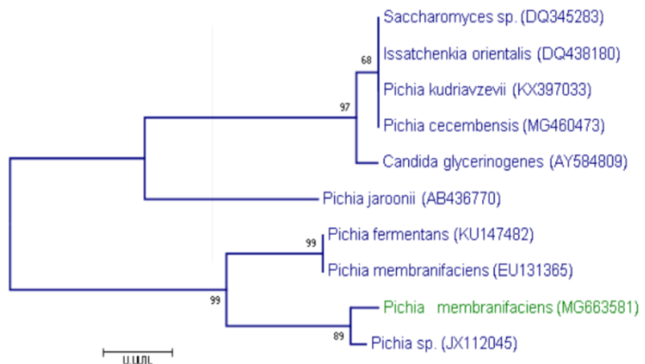


Figure 2. Phylogenetic and evolutionary relationships taxa of *Pichia membranifaciens S3* (MG663581).

The medium supplemented with yeast extract and malt extract respectively,0.05g/100ml produced a highest phytase activity and biomass {364± 0.57 U/ml} {1.56 ± 0.32},{364± 0.27 U/ml} {1.82 ± 0.51} than the other concentrations. Many authors reported that the same concentration which produces highest phytase activity [22].The inoculated flasks were kept for incubation at five different temperatures discrete from {28–40^{0}C}. The results showed that the highest activity of phytase {364± 0.34 U/ml} was obtained at 28^{0}C. After that, the phytase activity decreased gradually with the increase in incubation temperatures. A similar result was reported by author [22]. Results showed that the shift of pH backward the acidic side resulted in a highest phytase productivity.

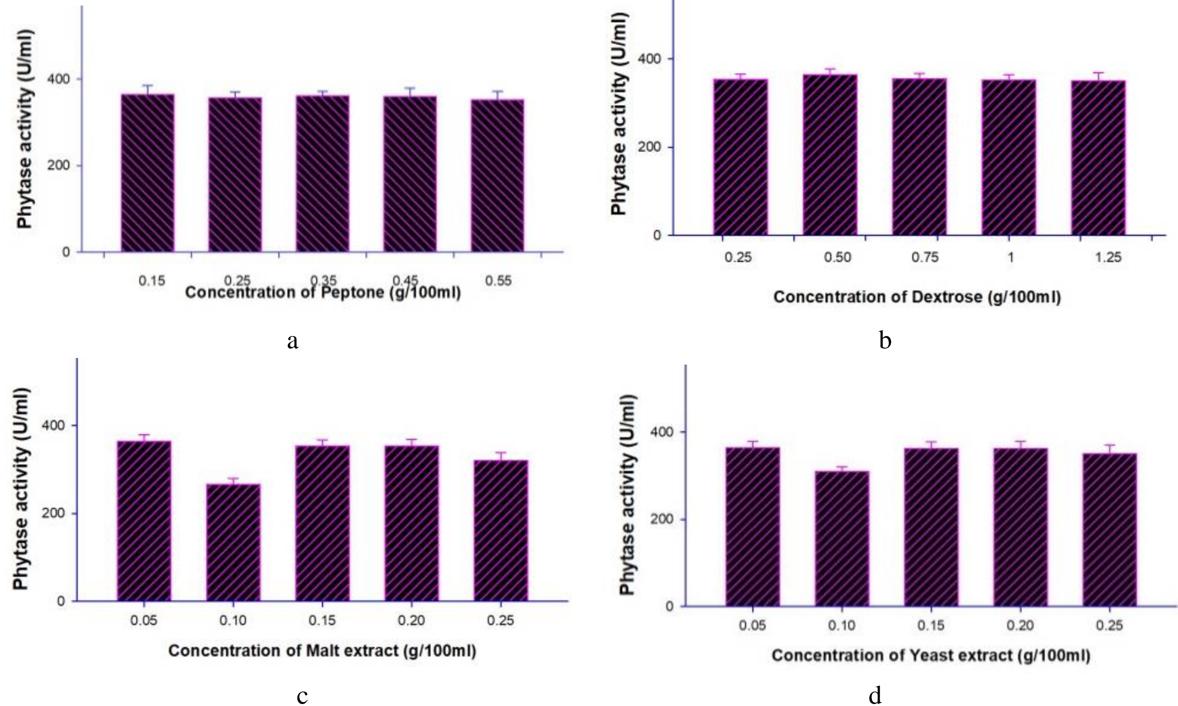


Figure 3. Effect of different concentration of sources for production of Phytase **a)** Peptone **b)** Dextrose **c)** Yeast extract **d)** Malt extract

The maximum phytase activity of 364±0.32 was obtained at pH 5.5, after that there was a gradual decrease in phytase production. The results in the present study indicated that maximum phytase activity of 364± 0.16 was obtained phytic acid concentration of 0.15 g/ml.

In earlier studies reported that similar results by Hussin et al.2010.The present work was started by the effect of production phytase by using 5 concentrations of agro industrial residues. The results in Figure 4 showed that Maximum phytase productivity {394± 0.17 U/ml} was achieved with 0.20 g/ml.

3.5. Partial Purification.

The phytase activity of the crude culture supernatant was highest when medium obtained after optimization of nutritional parameters {peptone,dextrose ,yeast extract,malt extract} and culture conditions {pH-5.5,Temperature 28^{0}C} incubated for 72h.Thus,enzyme was first concentrated by ammonium sulphate fractionation and the aimed protein was detected in the precipitate saturation after 30% salt cut process. After removal of ammonium sulphate by dialysis membrane for overnight with stirrer, followed by protein was purified by DEAE anion exchanger with elution buffer. Table 1 showed that phytase activity and protein fold. At the end of the purification process, the phytate was purified 3.67 fold with a recovery of 43.6 %. The specific activity was increased up to 41.8 U mg-1 of total protein, in reported similar results [23].

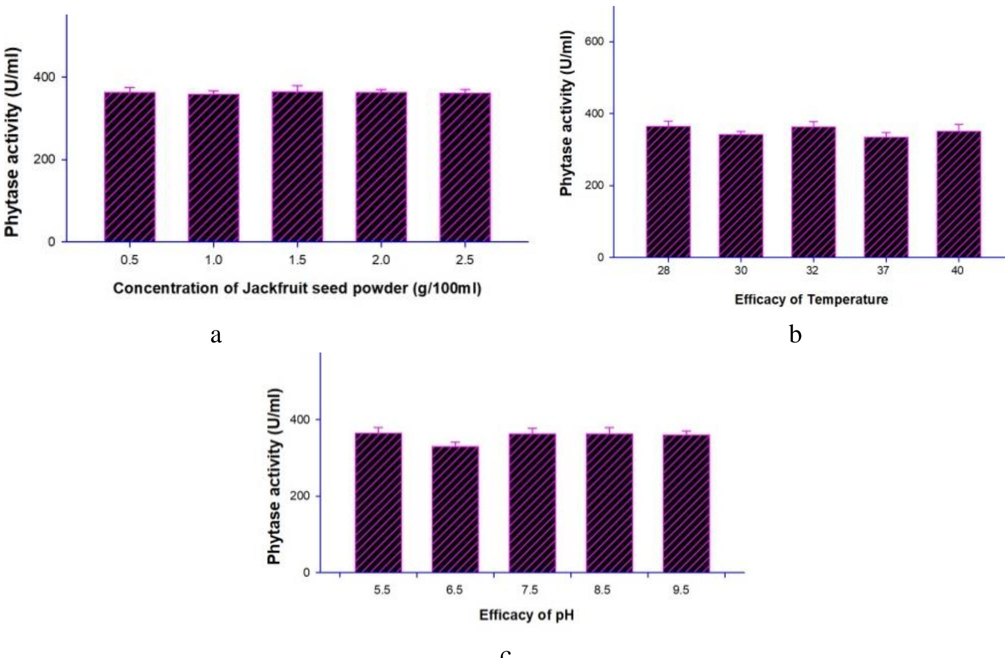


Figure 4. Effect of different concentration and conditions for production of Phytase **a)** Jack fruit seed powder **b)** Temperature **c)** pH.

3.6. In silico method for prediction of secondary structure.

The predicted secondary arrangements of alpha-helix {Hh-40.63%}, random coils {Cc-45.24%}, extended strands {Ee-8.65%} and beta turns {Tt- 5.48%} were found in *Pichia membranifaciens* S3 results by NPS@ SOPMA secondary structure prediction. Previously also reported that alpha-helical content {42.76%} was found 3-phytase B, respectively [24].

Table 1. Partial purified Phytase and protein concentration.

Purification conditions	Total Protein mg/ml	Total phytase activity U/ml	Specific activity U/mg Protein	Purification (fold)	Yield (%)
Crude supernatant	32	364	11.37	1	100
Ammonium sulphate precipitation	8	318	39.75	3.5	87.3
Dialysis	7.2	298	41.3	3.63	81.8
DEAE celluloose ion exchange chromatography	3.8	159	**41.8**	**3.67**	**43.6**

3.7. Homology modelling.

Modeller 9.23 was used to develop the 3D structure by providing the sequence alignment file {PhyS3- *Pichia membranifaciens* S3}, template file {pdb_95}, and target file {1qwo, 1qfx, 2gfi, 1sk8, 3k4p} (Figure 5). A similar type of homology model validation was also studied previously [25] while working with *Aspergillus* phytases. After the construction of 3D{.pdb} model, the evaluation and quality estimation of the model were performed. The satisfactory of the generated model {PhyS3.B99990003} was assessed by using the general stereo chemical parameters by SAVES server.

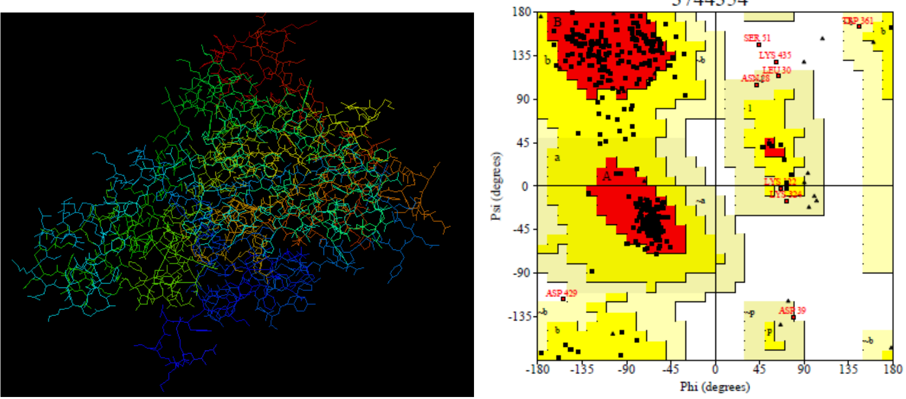


Figure 5. Wireframe diagrams of the modelled Phytase (ID: PM0083054) and Ramachandran plot.

Ramachandran 448 plot of energy minimized model of phytase structures had been generated. The result showed 86.4% of the residues within the most 344 favorable region, 11.3% within the moreover allowed region (Figure 5). After that, the model {in .pdb format} was deposited in PMDB database and received accession number ID: PM0083054.This structural finding was also studied some earlier reports. The presence of more than 97% residues in the most favoured region of Ramachandran plot was the characteristics of the good quality model [26].

4. Conclusions

In this study, we have concluded that *Pichia membranifaciens* S3 (MG663581) produces a uniquely stable phytase using substrate Jack friut seed powder under SSF with optimized design of shake flask. It has been suggested that our research introduced a low cost medium and very simple technique. In silico and homology model of this study states that 3D structure of protein model designs. Our work will have concluded that high benefits and suitable for large scale production.

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Conflicts of Interest

The authors declare no conflict of interest.

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