Research article

Isolation, characterization and optimization of *Cystobasidium minutum* for phytase production

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ABSTRACT

Introduction and Aim: Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6- hexakis dihydrogen phosphate), a storage form of phosphorus is present in legumes, cereals and oilseeds. By forming insoluble complexes with proteins and divalent cations, it serves as an anti-nutrient in animal feed. Phytase (EC 3.1.3.8), a special class of phosphomonoesterases, catalyse the conversion of inorganic phosphate into mono-, di-, tri-, tetra-, and penta-phosphates of derivatives of myo-inositol. It is an important enzyme in food and feed industries. The present study aimed at isolation, molecular identification and optimization of physico-chemical parameters for phytase production by a red pigmented yeast *Cystobasidium minutum.*

Materials and Methods: The phytase activity was estimated by using sodium phytate as substrate and the production of phytase under varied temperature, pH, agitation, incubation time, carbon and nitrogen sources.

Results: The results showed maximum activity of 91.86 and 27.13 U/ml at 35°C and pH 5.5. At an agitation speed of 150 rpm and 120h of incubation time the enzyme activity was 18.9 and 21.4 U/ml respectively. Among the carbon sources tested sucrose served for highest enzyme activity of 26.33 U/ml and ammonium sulphate served the sole source of nitrogen and showed an activity of 50.12 U/ml.

Conclusion: *Cystobasidium minutum* (*Rhodotorula minutum*) produced maximum phytase enzyme in an optimized physical and chemical condition. Hence, from the present investigation, it is found that optimization of physic-chemical conditions may be a promising tool for the growth of organisms and also maximum yield of any metabolite.

Keywords: *Cystobasidium minutum*; phytase; physical; chemical optimization.

INTRODUCTION

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6- hexakis dihydrogen phosphate) is the storage form of phosphorus present in legumes, cereals, and oilseed crops (1). It generates insoluble complexes with protein and divalent cations in animal feed and serve as an anti-nutritional component. such as Fe, Zn, Mg, etc., thus reducing the nutritive value of food (2). About 50 to 80% of the phosphorus in most products of plant origins is found as phytate (3). Phytase (EC 3.1.3.8) is also known as a phytate hydrolyzing enzyme, a class of phosphomonoesterases, that catalyse the hydrolysis of phytic acid into mono-, di-, tri-, tetra-, and penta-phosphates of myo-inositol derivatives and inorganic phosphate (4, 5). Thus, phytase can be used to increase the nutrient quality of feed and/or reduce the quantity of phosphorus that animals release (2).

Phytase enzyme is produced by microorganisms like bacteria, fungi, and yeast (6). Most of the isolates such as *Aspergillus*, *Mucor*, *Rhizopus* and *Penicillium* sp produce extracellular phytases (7, 8). Yeasts are the most dominant strains to produce phytase compared to other groups of microorganisms (9-11). Many yeast species have been identified as phytase producers, including *Schizophyllum commune*, *Schwanniomyces castellii*, and *Saccharomyces cerevisiae* (12). Hence microorganisms are the favoured sources of phytases due to their high yield, easier production, handling, and manipulation of the producing strains. Phytases could be produced commercially from different microbial sources, which are of high significance (6).

Phytase has potential applications in various fields such as the food industry, animal feed and health and environmental-related areas. Since the FDA conferred GRAS (Generally Recognized as Safe) status for the use of phytase in food in the United States, it is commercially available as a food additive (Lee et al. 2005). Phytase production from yeast was successfully led by using submerged fermentation (SmF). Phytate phosphorus is not absorbed by monogastric animals due to the absence of phytase enzymes in their intestines (13).

Yeasts attributed to the genus *Cystobasidium* Harrison are basidiomycetes fungi that are unicellular yeasts, characterized by a high rate of growth. The strain may appear as yellow, red, or orange colour and is known as carotenogenic yeast or red yeast due to its production of carotenoids (14). It is widely distributed in soil, freshwater, air, seawater, plants, compost soil and dairy products, and also shown to

be present in phylloplane and decaying plants (15). So many studies have been discussed about the importance of the genus *Cystobasidium* (earlier called *Rhodotorula*), and various applications in the fields of biotechnology. Naturally, by-products of the agri-food industry are capable of being bio-transformed into primary and secondary metabolites of value addition. These have been suggested to serve as a source of pigments and metabolites with applications in food and medicinal industries (16). The current study concentrated on isolation, molecular identification, and optimization of the cultural conditions for phytase production by *Cystobasidium minutum* (*Rhodotorula minutum*).

MATERIALS AND METHODS

Source of *Cystobasidium minutum*

Soil samples were obtained from areca nut agricultural fields in Chikka Aluvara, Kodagu, Karnataka, India.

Media used

Sabouraud's Dextrose Agar (SDA): Dextrose 40 g/l; Peptone 10 g/l: Agar 15 g/l; Malt Yeast Extract agar (MYE): Peptone 5 g/l; Yeast extract 3 g/l; Malt extract 3 g/l; Glucose 10 g/l; Agar 20 g/l.
All the chemicals and reagents used in this investigation were obtained from Hi-Media, Mumbai, India, SRL Pvt. Ltd. India.

Isolation of pigmented yeast

Pigmented yeast used in the present investigation was isolated from areca nut agricultural soil through standard serial dilution method. Appropriate dilutions such as 10^{-3}, 10^{-4}, and 10^{-5} were inoculated onto SDA medium (17). The plates were incubated at 30°C for 72 hr and the isolate was stored at 4°C in MYE slants for further experiments.

Quantitative screening of *Cystobasidium* sp. for phytase production

The isolated strain was inoculated in 100ml of MYE broth (found to be a suitable medium for hyper phytase production through our preliminary studies) and was incubated for 5 days at 30°C and 150 rpm in an orbital shaker. After incubation, the culture broth was spinned at 6000 rpm for 15 minutes at 4°C. The supernatant obtained was used for the determination of crude extracellular phytase activity with some modification in the procedure using sodium phytate as substrate (18). The procedure in brief: 0.5 ml of 15 mM sodium phytate and 0.25 ml of supernatant made up the reaction mixture was incubated at 37°C, for 30 minutes. The reaction was terminated by adding 4 ml of AAM solution (10 mM Ammonium molybdate: 5N H2SO4: Acetone) in the ratio of 1:1:2. The reaction mixture was vortexed and the absorbance was measured at 400 nm. One unit of phytase enzyme is defined as the quantity of enzyme required to release 1 µmol of inorganic phosphate in 1 minute under assay conditions.

Molecular identification of *Cystobasidium minutum*

One of the hyper phytase yielding isolates was explored for its physiological and morphological properties using the techniques of Zhao *et al.*, (15). The entire genetic code of the isolate was retrieved using the CTAB kit technique. The quantity and purity of DNA were assessed by measuring the absorbance at 230, 260, and 280 nm and determining the ratios A260/A280 and A260/A230. Throughout amplification studies, the purified DNA was kept at -20°C (19). The forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for the ITS sequencing (15). The PCR was performed under the following conditions: 94°C for 10 min, then 30 cycles of 92°C for 1 min, 52°C for 1 min, 72°C for 1 min, and final synthesis at 72°C for 5 min. The PCR products were purified for sequencing after being separated by agarose gel electrophoresis. By using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), the resulting sequences were compared to rDNA sequences from the GenBank. Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align the ITS segments received from the GenBank database and MEGA 11 was used to create the phylogenetic tree using the neighbour-joining method (20, 21).

Effect of temperature on phytase production from *Cystobasidium minutum* through submerged fermentation

The isolate was cultivated in MYE medium with 1% inoculum and incubated in a rotary shaker at various temperatures, ranging from 20 to 40°C with an interval of 5°C at 150rpm for 5 days, in order to determine the optimum temperature for culture growth and phytase synthesis. After incubation, the culture broth was centrifuged at 6000 rpm and 4°C for 10 min. The culture filtrate was used as crude phytase preparation.

Effect of pH and agitation on phytase production from *Cystobasidium minutum* through submerged fermentation

The effect of pH and agitation on the production of phytase from *Cystobasidium minutum* was studied by cultivating the isolate in MYE broth adjusted with different pH such as 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 at an interval of 0.5 by using 1N NaOH or 1N HCl before sterilization. The flasks were incubated at 35°C and 150 rpm for 5 days. For determining the effect of agitation on phytase production, the isolate was incubated at different speeds of agitation ranging from 100 to 250 with an interval of 50 rpm. Other conditions such as pH, temperature and incubation

time were kept constant at 5.5, 35°C and 5 days respectively.

Effect of incubation period on phytase production from *Cystobasidium minutum* through submerged fermentation

To optimize the incubation period for phytase production, *Cystobasidium minutum* was grown in MYE broth at 35°C and 150 rpm for different incubation time such as 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h. After every incubation time, the culture broth was centrifuged and the supernatants were assayed for phytase content.

Optimization of cultural conditions

For confirmation of the optimum growth condition and production of phytase by *Cystobasidium minutum* various physical parameters such as different pH, temperatures, agitation and incubation time (h), and chemical parameters by replacing the carbon and nitrogen sources used in the basal media (9).

Impact of various carbon and nitrogen sources on phytase production by *Cystobasidium minutum*

The isolate was cultured in MYE broth to determine how different carbon and nitrogen sources affected the ability of *Cystobasidium minutum* to produce phytase with carbon sources like sucrose, lactose, maltose, glucose, fructose and xylose, and different nitrogen sources such as yeast extract, potassium nitrate, urea, ammonium sulphate, sodium nitrate and ammonium nitrate, incubated for 5 days at 35°C in an orbital shaker.

RESULTS

Isolation of pigmented yeasts and their characterization

A hyper phytase producing yeast strain was isolated from areca nut field soil. On the basis of color, colony morphology and microscopic examination the cells were ovoidal and elongated in shape and were identified as *Cystobasidium* sp., as shown in (fig 1).

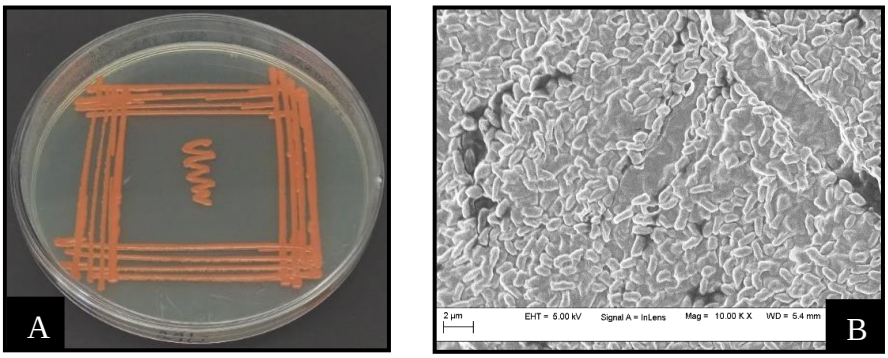

Fig. 1: Isolation of *Cystobasidium* species from agricultural soil A- *Cystobasidium minutum* on MYE agar medium; B- SEM image of *Cystobasidium minutum*

Identification of pigmented yeast

The amplified Internal Transcribed Spacers (ITS) of the ribosomal DNA sequence were used to describe the isolate (22). The strain GS-MY11 showed 96.97% similarity to *Rhodotorula* sps when the ITS sequence of the present isolate was checked. GS-MY11 (GenBank ON307085) was compared to the sequences in the GenBank database and found the ITS nucleotide sequence matched with *Rhodotorula* sps. as precisely shown in the phylogenetic tree. (Fig. 2 and 3).

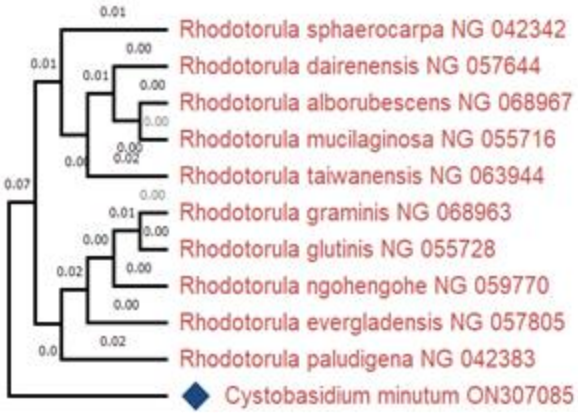

Fig. 2: Phylogenetic tree of GS-MY11 through neighbor-joining analysis of ITS region of rDNA

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   1 aagcatatca ataagcggaa cgtgaaacta acaaggattc ccctagtaac ggcgagtgaa
  61 gtgggaaaag ctcaactttg aaatctggca ccttcggtgt ccgaattgta gtctcaagaa
 121 gtgttttctg tgctagtcca tgtatgagtc tgttggaaca cagcgtcata gagggtgaca
 181 accccgttca tgacatggat actagtgctc tgtgatacac tctcgaagag tcgagttgac
 241 gtaatgcagc tcaaattggg tggtaaattc catctaaagc taaatattgg cgagagaccg
 301 atagcaaaca agtaccgtga gggaaagatg aaaagcactt tggaaagaga gttaacagta
 361 cgtgaaattg ttgaaaggga aacgattgaa gtcagacgtg cgtgatgcgg ttcagctctg
 421 gttcgccagg gtgtattccg tatctttgca ggccaacatc gggttagtcg gataaagatt
 481 agttgaatgt ggcatcttcg ggtgtgttat agcttctaat tgaatacgat tgatgagacc
 541 gaggaacgca gcgcgccgca aggcaaaggt tccgacccgc gcttaggatg ttggtgaaat
 601 ggctttaaac gacccgt//
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Fig. 3: Nucleotide sequence of the isolate GS-MY11 obtained through 18S rDNA molecular characterization

Effect of temperature on phytase production from *Cystobasidium minutum*

The phytase production under the temperature range of 20 to 40°C at intervals of 5°C was studied for 5 days. At the isolate *Cystobasidium minutum* showed maximum phytase activity of 91.85 U/ml when assessed with glucose and yeast extract as the carbon and nitrogen sources in the basal medium. Hence 35°C was found to be optimal for the synthesis of phytase (fig. 4).

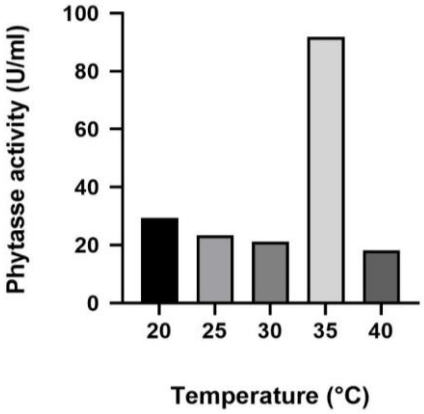

Fig 4: Phytase activity by *Cystobasidium minutum* grown at different incubation temperatures

Effect of pH on phytase production from *Cystobasidium minutum*

In the present study a pH of 5.5 was found to be optimum for maximum phytase activity of 27.13 U/ml (Fig 5).

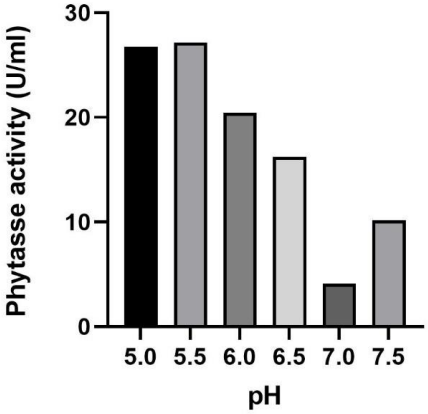

Fig. 5: Phytase activity by *Cystobasidium minutum* grown at different pH

Effect of agitation on phytase production from *Cystobasidium minutum*

The effect of agitation on phytase production from *Cystobasidium minutum* was determined and the result showed a maximum phytase production of 18.9 U/ml at 150 rpm (Fig. 6).

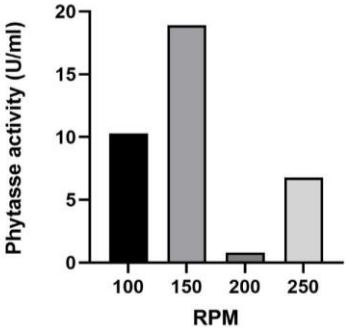

Fig. 6: Phytase activity by *Cystobasidium minutum* grown at different agitation speed

Effect of fermentation time on the phytase production from *Cystobasidium minutum*

Study on the incubation time for phytase production from *Cystobasidium minutum* for 120 h (5 days) at an interval of 12 hr. Phytase production was found to be highest (21.4 U/ml) at 120 h of incubation time (Fig. 7).

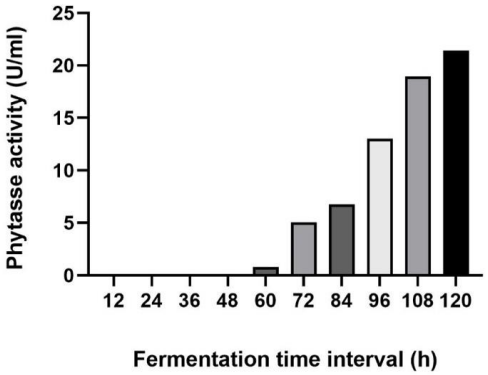

Fig. 7: Phytase activity by *Cystobasidium minutum* grown at different incubation time

Optimization of cultural conditions of chemical parameters

The optimization of carbon and nitrogen sources used for chemical parameters, and pH, temperature, agitation and incubation time used as physical parameters for phytase production from

Cystobasidium minutum was carried out. *Cystobasidium minutum* showed highest phytase activity of 26.33 U/ml with sucrose (fig. 8), while ammonium sulphate used as one of the nitrogen sources showed maximum phytase activity of 50.12 U/ml (fig. 9).

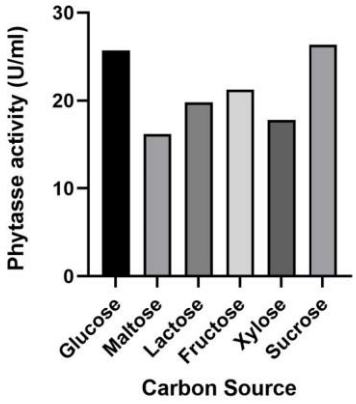

Fig. 8: Phytase activity by *Cystobasidium minutum* grown in different carbon sources

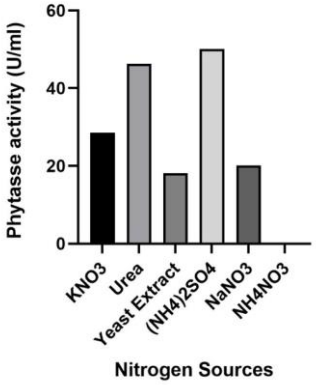

Fig. 9: Phytase activity by *Cystobasidium minutum* grown in different nitrogen sources

DISCUSSION

Based on the colony morphology, the isolated pigmented yeast GS-MY11 was identified as *Cystobasidium minutum* (ON307085) based on its physiological properties and ITS sequencing. Similar identification has also been carried out by (15).

Sandhya *et al.,* (23) reported a study on the effect of temperature for phytase production from *Aspergillus niger* through submerged fermentation and showed 30°C as optimum temperature for the growth and production of phytase (23.8 U/ml). In another study, 50°C was shown to be an appropriate temperature for a bacterial strain named DR 6 to produce 387 U/ml of phytase (9). The optimum temperature for phytase activity in *Cyberlindnera jadinii* CJ2 was found to be 50°C with a highest activity of 51.95 mU/mgdw, while the activity decreased with increase in temperature (24). Capusoni *et al.,* (24) have shown pH 4.5 as optimum for highest phytase activity by *Cyberlindnera jadinii* CJ2**.** According to Alves *et al.,* (25) *Muscodor* sp. was shown to synthesize phytase at pH 5.0. The impact of pH on marine yeast *Kodamea ohmeri* BG3 to produce phytase was tested and found that an initial pH of 5.0 was found optimum (26). All microorganisms are sensitive to pH for their metabolic activities, similarly the production of phytase is also affected by variation in the pH level in comparison to the optimum. 4Phytase producing bacteria such as *Lactobacillus, Escherichia, Pseudomonas,* and *Klebsiella* sp. were also found to be more effective at pH 6.0-6.5 and 60°C (9). Alves *et al.,* (25) have shown 125 rpm as suitable agitation speed for phytase production from *Muscodor* sp., with increasing the speed of agitation, the yeast biomass was higher but enzyme activity decreased. In another study, at 170 rpm, marine yeast *Kodamea ohmeri* BG3 produced the highest phytase (26). However, in the present investigation 150 rpm was the optimum agitation for highest phytase activity. Using *Muscodor* sp., the highest phytase activity (26.51 U/mg) was attained after 144 hours of fermentation (25). In other investigations, *Rhizopus microsporus* var. *microspores*, *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA also produced the highest enzymes. (27, 28). Li *et al.,* (26) reported a study on the fermentation time for the production of phytase by marine yeast *Kodamea ohmeri* BG3 shown at 72 h of fermentation showed higher phytase activity of 557.9 mU/ml. *Aspergillus niger* production of phytase was influenced by the incubation period, with the fourth day of incubation showing the highest levels (25.6 U/ml) of both growth and phytase production (23). In the present study the phytase enzyme activity showed an increasing trend with increase in the incubation time and a highest activity was found at 120h. Li *et al.,* (26) reported that different carbon and nitrogen sources significantly influenced the marine yeast *Kodamea ohmeri* BG3 produces phytase. They found that the best carbon and nitrogen sources for phytase production were glucose (30.0 g/l) and ammonium sulphate (15.0 g/l), which produced 500 and 534.8 mU/ml, respectively. It is also shown by Sano *et al.,* (29) that ammonium sulphate served as sole source of nitrogen for increased accumulation of phytase by a yeast strain *Arxula adeninivorans,* However, in the present study yeast extract used in the basal medium itself served as the best nitrogen source for highest phytase yield.

CONCLUSION

Microorganisms including bacteria, fungi and yeast produce phytase enzymes. Yeasts are the most dominant strains to produce phytase compared to other groups of microorganisms. Many yeast species have been identified as phytase producers, including *Schizophyllum commune, Schwanniomyces castellii,* and *Saccharomyces cerevisiae*. Hence microorganisms are the favoured sources of phytases due to their high yield, easier production, handling and manipulation of the producing strains.

Cystobasidium minutum produced maximum phytase enzyme in an optimized physical and chemical condition. Hence, from the present investigation, it is found that optimization of physico-chemical conditions may be a promising tool for the growth of organisms and also maximum yield of any metabolite.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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