

## EVALUATION OF PHYTASE PRODUCTION BY *CANDIDA TROPICALIS* ISOLATED FROM FISH GUT AND SUBSEQUENT BIO-PROCESSING OF GROUNDNUT OIL CAKE UNDER SOLID STATE FERMENTATION

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**ABSTRACT**

Extracellular phytase production by *Candida tropicalis* (GenBank Acc. No.JX532154) isolated from the gut of a freshwater teleost, *Anabas testudineus* has been investigated under solid-state fermentation. Among the different oilcakes [Groundnut (GOC), Sesame (SSC), Linseed (LOC), Mustard (MOC) and Soyabean (SOC)] evaluated as substrates, GOC supported maximum phytase activity (38.25±1.02 U/g) resulting 75.25% phytate degradation. Incubation for 8 days at 35°C temperature with 70% moistening media of pH 6 revealed optimum phytase production. Maximum phytase activity was exhibited with 3% (v/w) inoculum size (43.45±1.14 U/g) and 70% initial moisture content (41.3±1.13 U/g). Glucose (2%, w/w) and ammonium nitrate (3%, w/w) supplementation resulted maximum phytase activity (39.45±1.12 and 38.75±1.06 U/g, respectively). Analysis of the fermented oilcake revealed that there was marginal increase (t-value significant at P < 0.05) in crude protein, lipid and minerals (Na, K, Ca, Mg, Zn, Fe, Cu, P, Mn) contents, and reduction of the anti-nutritional factors (e.g., crude fibre, tannin, phytic acid and trypsin inhibitor). The results indicated that application of the gut inhabiting yeasts in bio-processing of oilcakes merits further investigation for possible use of the plant feedstuffs as aqua-feed ingredients.

**Keywords:** Phytase production, solid state fermentation, Ground nut oilcake, *Candida tropicalis*

**INTRODUCTION**

Growing demand, rising cost, declining availability and poor quality of fishmeal have laid emphasis on the alternative protein sources in the diets for fish and farmed animals (Fournier *et al.*, 2004; Ramachandran and Ray, 2007). In this approach, quite a few protein rich oil seed cakes arising out as the agro-industrial byproducts have been extensively tested as low cost feed ingredients (Storebakken *et al.*, 2000; Hardy, 2010). Apart from deficiencies in the essential amino acids the use of oil cakes has been restricted by the presence of some antinutritional factors (ANFs), majority of which are polyphenols, trypsin inhibitors, non-starch polysaccharides and phytic acid (Mandal and Ghosh, 2009). Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6- hexakis dihydrogen phosphate) represents approximately 70-80% of the total phosphorus in plant seeds (Reddy *et al.*, 1982; Lott *et al.*, 2000). It chelates various minerals forming phytates, and forms insoluble complexes with proteins and amino acids, thereby appears as a major ANF diminishing the bioavailability of the essential nutrients (Spinelli *et al.*, 1983; Sabu *et al.*, 2002). Therefore, endogenous phytate compounds reduce feed value of the protein rich oil cakes unless destroyed or inactivated. Phytate-degrading enzymes, commonly known as phytases (myo-inositol hexakisphosphate phosphohydrolase), catalyze breakdown of phytate to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates (Konietzny and Greiner, 2002). Ruminants can utilize phytate with the help of the phytases produced by the ruminal microorganisms. However, for monogastric and agastric animals phytase is considered as an important feed additive to increase the availability of phosphorus and other essential nutrients by virtue of enzymatic hydrolysis of the phytate compounds. Phytases have a wide distribution in plants, microorganisms and in some animal tissues (Konietzny and Greiner, 2002; 2004; Vohra and Satyanarayana, 2003). Microorganisms are the best sources for commercial production of phytases because of their easy cultivation and high yields of the enzyme (Li *et al.*, 2008). Although phytases have been detected in several species of fungi, yeasts and bacteria (Greiner and Konietzny, 2006; Li *et al.*, 2008; Roy *et al.*, 2009; Khan and Ghosh, 2012a), soil fungus, *Aspergillus* sp. is the chief source of commercial phytase used in the animal feeds (Maenz, 2001). In addition, phytate degrading enzymes from yeasts, for instance *Schwanniomyces castellii* (Pandey *et al.*, 2001) and *Pichia anomala* (Vohra and Satyanarayana, 2004) have also received increasing attention. Apart from these environmental sources, numerous studies have reported presence of phytase producing microorganisms

in the gut of ruminants and other animals (pig, poultry and swine). Autochthonous phytase producing gut bacteria in fresh water fishes have been reported very recently (Roy *et al.*, 2009; Khan *et al.*, 2011; Khan and Ghosh, 2012a). In a more recent study, Das and Ghosh (2013) documented phytase-producing yeasts in the gut of freshwater fishes. To our knowledge, phytase-producing fish gut microorganisms are still inadequately studied for enzyme production (Khan and Ghosh, 2012b). It has been advocated that enzymes produced by the gut associated symbiotic microorganisms might help in digestion and assimilation of the plant feedstuffs in fish (Ray *et al.*, 2012). Studies indicated that processing of oil seed meals through physical methods like heat treatment and water soaking, or biological method like seed germination were not effective in reducing the phytate content. On the contrary, solid state fermentation (SSF) by exo-enzyme producing bacteria have been shown to reduce the phytate content in plant ingredients by phytases produced by the bacteria (Bairagi *et al.*, 2002; 2004; Ramachandran *et al.*, 2005; Ramachandran and Ray, 2008; Khan *et al.*, 2012b). Bioconversion of crop residues through biological detoxification of agro-industrial wastes and enzyme production are the most viable economic applications of the SSF (Pandey *et al.*, 2001). After oil extraction, recycling of nutrients in the oil cakes by processing through SSF pretends great economic feasibility to the agro-based oil production sectors. Likewise, efficacy of the fermented oil seed meals for partial or complete substitution of fishmeal has been suggested by several authors (Mukhopadhyay and Ray, 1999; Ramachandran *et al.*, 2005). As likely incorporation of harmful metabolites during the SSF process cannot be ruled out, the use of autochthonous fish gut microorganisms might be justified for processing of plant feedstuffs for possible use in fish feed (Mandal and Ghosh, 2013). In this perspective, the major objectives of the presently reported study were (1) retrieval of protein rich oil seed cakes into value added products and (2) optimization of various process parameters that influence phytase synthesis by a fish gut inhabiting yeast, *Candida tropicalis* (JX 532154) in SSF. Although different oil cakes were initially taken up, considering phytase activity of the studied yeast strain in solid substrates, Groundnut oilcake (GOC) has been utilized as substrate for phytase production and appraised for subsequent value addition in view of its likely use as aquafeed ingredient in future studies.

## MATERIAL AND METHODS

### Microorganism and maintenance of culture

The phytase producing yeast strain used in the present study was isolated from the gut of a freshwater teleost, *Anabas testudineus* and identified as *Candida tropicalis* (GenBank Accession Number: JX532154) by 28S rDNA sequence (D1/D2 region) analysis (Das and Ghosh, 2013). The culture was maintained on selective modified phytase screening media (MPSM) with some modifications (Howson and Davis, 1983). The composition of MPSM was (g l<sup>-1</sup>): glucose, 10; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1; urea, 10; citric acid, 3.0; sodium citrate, 2; MgSO<sub>4</sub>.7H<sub>2</sub>O, 1; sodium phytate, 3; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.01 and agar 20, pH 7. Inoculum was prepared from a freshly raised 5-d-old slant culture in MPSM broth grown at 35°C for 48 h. The inoculant thus obtained contained 6.5 × 10<sup>7</sup> cells ml<sup>-1</sup>.

### Substrate selection

Dried and de-oiled Groundnut (GOC), Sesame (SSC), Linseed (LOC), Mustard (MOC) and Soybean (SOC) cakes were procured from local market and used as solid substrates for the present study. Oilcakes were oven dried at 80°C for 48 h, finely grounded in a laboratory mixer grinder and passed through a fine mesh (400 µm in diameter) sieve to obtain uniform particle size. The powdered samples were stored in a refrigerator (4°C) for use as substrates in SSF. Prior to fermentation, each solid substrate was made moisture free by drying at 100 ± 5°C initially for 30 min and further at 60°C until constant weight was obtained. On the basis of phytase activity by the yeast strain and phytate degradation in the oilcakes finally GOC was selected as substrate for SSF in the later studies.

### Optimization of Solid-state fermentation Parameters

Five g of each substrate was taken in 250 ml Erlenmeyer flasks. The moistening medium was added as per required level for each of the substrate. The mouths of the flasks were covered with non absorbent cotton, autoclaved at 121°C and 15 lbs for 20 min. After proper cooling, the sterilized solid substrate in each set was inoculated with 1 ml of the prepared inoculum under aseptic condition and the flasks were then incubated at 35°C for 72 h, unless otherwise mentioned. Incubation of the sets was carried out in standing condition with mechanical shaking by sterilized glass rods at 12 h intervals. There were three replicates for each assessment. The protocol adopted for optimization of various process parameters was to evaluate the effect of an individual parameter and to incorporate it at the optimized level in the experiment before optimizing the next parameter.

### Effect of initial moisture content

A salt solution containing (g l<sup>-1</sup>) K<sub>2</sub>HPO<sub>4</sub> (0.5); KH<sub>2</sub>PO<sub>4</sub> (0.5); MgSO<sub>4</sub>. 7H<sub>2</sub>O (0.5); NH<sub>4</sub>Cl (1), CaCl<sub>2</sub> (0.01) having a pH of 6 was used as the moistening media for SSF. Optimum initial moisture content for phytase production was determined by varying the percentage of the moistening media (10-90% v/w) added to the solid substrates.

### Effect of pH of the moistening media

The most suitable pH of the moistening media for phytase production was determined by adjusting the pH of the media at different levels in the range of pH 3-9 with an interval of pH 1 by addition of HCl or NaOH solutions.

### Effect of incubation temperature

In order to determine the effective temperature for phytase production fermentation was carried out at 5°C intervals in the range of 25°C to 50°C.

### Effect of inoculum size

Fermentation medium (5g) was inoculated with 1% to 5% (v/w) of 48h culture (containing 6.5 × 10<sup>7</sup> cells ml<sup>-1</sup>) to optimize the inoculum size for phytase production.

### Effect of different surfactants

Different surfactants, namely, Tween-20, Tween-40, Tween-80, Dimethyl sulfoxide (DMSO, 1% v/w) were added to the production medium to establish its effect on phytase production.

### Effect of NaCl

To study the effect of NaCl on phytase production solid substrates were supplemented with different concentrations of NaCl (1%-6%, w/w).

### Effect of supplementation of different carbon and nitrogen sources

The SSF medium was supplemented with different carbon sources (1%, w/w) (glucose, sucrose, lactose, maltose, starch) and organic/inorganic nitrogen sources (1%, w/w) (peptone, tyrosine, tryptophan, ammonium sulfate, ammonium nitrate) to explore their effects on phytase production. Further, the selected carbon and nitrogen sources (glucose and ammonium nitrate, respectively) were varied within a narrow range (1%-5%) to optimize phytase production.

### Effect of incubation period on phytate degradation

After optimizing various parameters, a time course experiment was conducted for 10 days with a SSF batch with all optimum parameters. Phytase production by the yeast strain and phytate degradation in the substrate was measured at an interval of 2 days. Reduction of phytic acid in the raw (dried ground substrate) and fermented oilcakes were examined by extracting the phytic acid from 1g of sample using 2.4% HCl, with continuous shaking (180 rpm) for 16 h. After extraction, the suspension was centrifuged (10,000×g, 20 min) and the supernatant was used for phytate determination according to Vaintraub and Lapteva (1988) using sodium phytate as standard.

### Enzyme extraction

To evaluate extracellular phytase production by the yeast (*Candida tropicalis*) during SSF, enzyme extraction was carried out from the fermented material. The fermented material arising out from the 5g of solid substrate was mixed thoroughly with the required amount of distilled water containing 0.1% (v/v) Tween-80 (so that the final extraction volume was 50 ml) by keeping the flasks on a rotary shaker (Lab. Companion, SL-300R) at 150 rpm for 1 h. The solids were then separated from the solution by filtration through filter paper (Whatman No. 1). The filtrate was centrifuged at 10,000 g for 10 min at 4°C in a refrigerated centrifuge and the supernatant thus obtained was collected as crude enzyme for phytase assay.

### Phytase assay

Quantitative phytase assay of the crude enzyme was done after Yanke *et al.* (1999) using sodium phytate as the substrate. The colour that developed due to phytase activity was determined with a spectrophotometer (Shimadzu UV1800) at 700 nm. One phytase unit (U) was defined as the amount of enzyme per milliliter of supernatant that released 1 µg of inorganic phosphorus per minute. Enzyme yield was expressed as U/g dry substrate.

### Analysis of proximate composition, minerals and antinutrients

Proximate composition of raw and fermented GOC were analysed following the standard methods of AOAC (1990): crude protein (N%×6.25) by micro Kjeldahl digestion and distillation, lipid was determined by extracting the residue with 50-60°C petroleum ether in a Soxhlet apparatus, crude fiber was determined as loss on ignition of dried lipid free residue after digestion with 1.25% H<sub>2</sub>SO<sub>4</sub> and 1.25% NaOH. Total free amino acids and fatty acids were measured according to Moore and Stein (1948) and Cox and Pearson (1962), respectively. The mineral elements were analysed by atomic absorption spectrophotometer (Perkin Elmer Analyst 700) using standard reference chemicals. Na, K, Ca were analysed by flame photometry. Calcium and phosphorus were estimated by biochemical methods as described by Oser (1971).

Among the antinutritional factors, tannin and phytic acid were determined by biochemical methods described by Schanderi (1970) and Vaintraub and Lapteva (1988), respectively. Trypsin inhibitor activity was determined according to Smith *et al.* (1980).

### Statistical analysis

All experiments were performed in triplicate and the mean values were reported along with standard error (mean ± SE, n=3). Statistical analyses [t-test, One-way ANOVA, Two-way ANOVA and student Newman-keuls (SNK) test] of the data were performed according to Zar (1999) using SPSS Ver10 (Kinnear and Gray, 2000) software.

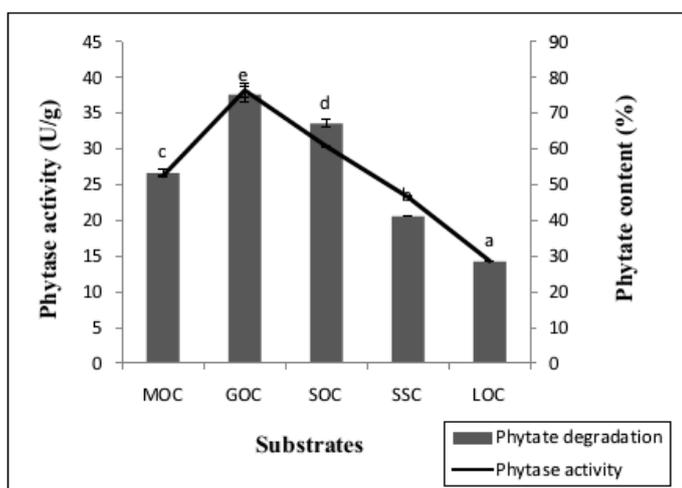
## RESULTS AND DISCUSSION

SSF is a bio-process where microorganisms are grown on solid substances with minimum water level (Pandey, 1992a; Van de Lagemaat *et al.*, 2001). An effort has been made in the presently reported study to optimize the important physical and nutritional parameters that influence extracellular phytase production by a yeast strain, *Candida tropicalis* (GenBank Accession No. JX532154) isolated from fish gut. Previous studies have reported *Candida* spp. as normal component in the gut of both marine and freshwater fishes (Gatesoupe, 2007). Although to the authors' knowledge, phytase producing ability of a *Candida* sp. isolated from

fish gut has not been addressed previously. In addition, SSF was carried out using nutritionally rich de-oiled GOC as solid substrates in view of processing of the ingredient for possible use as an aqua-feed ingredient. In vitro processing of plant ingredients by microbial enzymes has been recommended by several authors to diminish ANFs and augment nutrient accessibility (Antony and Chandra, 1998; Ramachandran and Ray, 2007). In the present study, an autochthonous yeast strain isolated from fish gut was utilized for bio-processing of a plant ingredient. Although fermentative nutrition in fish is less understood (Esakkiraj et al., 2009), it has been opined that even an apparently negligible number of yeasts in fish gut may be of physiological importance (Gatesoupe, 2007).

**Evaluation of substrates**

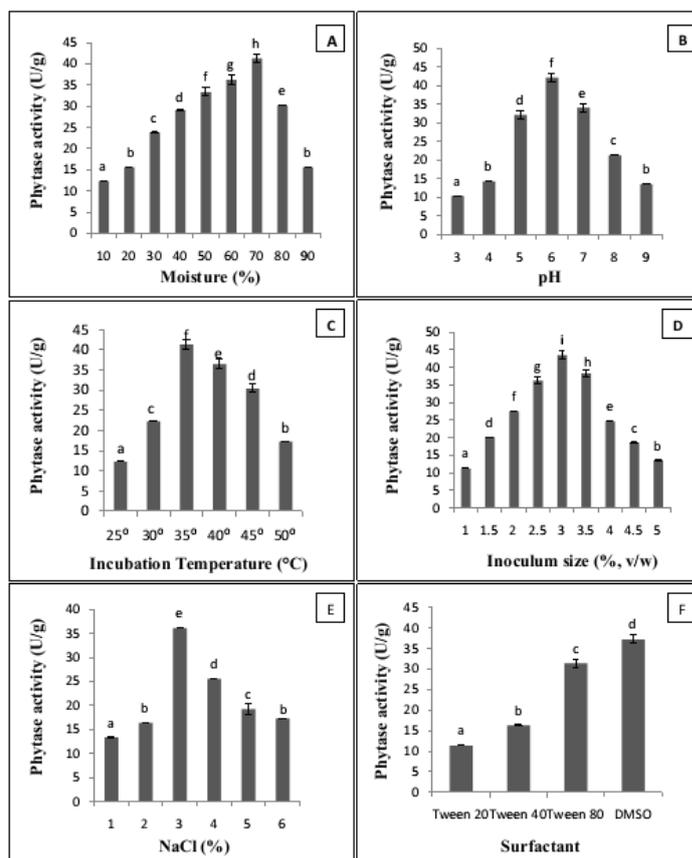
Yeasts mainly grow at the surface of substrate molecules by adhesion (Sabu et al., 2002). Five different oilcakes were tested as substrates in SSF. The results are presented in figure 1. It was evident that the use of GOC as substrate resulted both, highest phytase activity (38.25± 1.02 U/g) as well as phytate degradation (75.25%), which was followed by SOC (30.41± 0.09 U/g and 67.18%, respectively). Two-way ANOVA revealed that the result was statistically significant (P< 0.05). Hence, GOC was used as substrate for further studies. Being rich in nutrient content, GOC has been used by several workers as solid substrate to provide carbon, nitrogen or energy sources in the SSF process during the production of different enzymes (Immanuel et al., 2010; Kranthi et al., 2013).



**Figure 1** Phytase production by fish gut inhabiting *C. tropicalis* using different oil cakes as substrates. Degradation of phytate in the substrates used has also been shown. Bars with different alpha plates are statistically significant (P< 0.05; SNK test).

**Effect of initial moisture content**

Initial moisture content is a vital factor for growth of the organism and enzyme production in SSF (Sabu et al., 2006). New cell creation necessarily requires certain quantity of water (Pandey, 1992a, b). There was gradual increase in enzyme production with increase in the moisture content up to certain level (figure 2A), and maximum phytase was obtained at 70% moisture level for GOC (41.3 ± 1.13 U/g). Enzyme production was reduced because of further increase in moisture content. The reason of which might be decrease in air content of the substrate with increase in water content at constant substrate volume (Gautam et al., 2002). At the lowest and the highest water contents the decomposition rate of the total organic matter decreases, and this in turn affects the enzyme production (Pandey et al., 2001; Pandey, 1994). Usually bacteria entail a higher moisture content to sustain better growth and metabolic activities. On the contrary, fungi have been accounted to have a wider moisture range (20–70%) to yield improved production (Sabu et al., 2006). The capability of the organism for maximum yield at low moistening media could led to reduction in overall cost of enzyme production. One-way ANOVA revealed that variation in phytase production at different initial moisture contents were statistically significant (P< 0.001).



**Figure 2** Effect of initial moisture content (A), initial pH of the moistening media (B), different incubation temperature (C), inoculum size (D), NaCl (%) (E) and surfactant (F) on phytase production in SSF. Bars with different alpha plates are statistically significant (P< 0.001; SNK test).

**Effect of different pH and temperature**

Optimum initial pH of the moistening media required for maximal phytase production by *Candida tropicalis* was evaluated under various pH levels (3-9). The present study indicated that the yeast isolate needed slightly acidic to neutral pH (5-7) for phytase activity. However, maximum phytase activity was recorded with pH 6.0 (42.28 ± 1.13 U/g). Previous reports have shown that the optimum pH for phytase production may vary within acidic range (pH 5–6) in different yeasts (Aoki et al., 1976) and fungi (Batra and Saxena, 2005; Rajkumarand Nandy, 1983; Barthomeuf et al., 1994). Although, optimum phytase production achieved in the present study with the initial pH 6 might be due to the fact that the yeast strain used in the SSF was isolated from the intestine of a stomach bearing fish (*Anabas testudineus*) and it was adapted to the slightly acidic pH therein. The study registered sharp decline in enzyme production when initial pH of the moistening media was higher or lower than pH 6.0 (figure 2B). A pH other than the optimum level may interfere with the amino acid composition of the enzyme and thereby decreases the enzyme activity (Esakkiraj et al., 2009). Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum (Sabu et al., 2005). Any alter in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability (Vania and Gabriela, 2007).

The temperature is one of the most vital parameters that have to be controlled in a bioprocess (Sharma et al., 2000). Result on the effect of temperature on phytase production by *C. tropicalis* indicated 35°C as the optimum temperature showing highest phytase activity (41.38 ± 1.11 U/g) (figure 2C). Further increase in temperature resulted in decrease in the enzyme yield. Though *C. tropicalis* was isolated from fish gut, results of incubation temperature on enzyme activity revealed typical mesophilic nature of the yeast. Most of the biological processes are operated in relatively narrow range of temperature (Sabu et al., 2006). Temperature plays important role in determining protein denaturation, enzymatic inhibition, promotion or inhibition on production of a particular metabolite, cell death, etc. Results of one-way ANOVA revealed that variation in phytase production at different temperature and pH was statistically significant (P< 0.001).

### Effect of inoculum size

The inoculum size (volume) plays a significant role in the production of metabolites under SSF. Phytase activity gradually increased with increase in inoculum size up to 3% (v/w), and thereafter declined. It was revealed that maximum phytase activity ( $43.45 \pm 1.14$  U/g) complied with 3% of inoculum (figure 2D). Higher concentrations of inoculum were inhibitory for phytase production giving minimum enzyme yield (Sabu *et al.*, 2002) at highest inoculum size (5%). Reduced enzyme production at higher concentrations might be due to increased competition for nutrient uptake and exhaustion of nutrients creating nutrient imbalance (Ramachandran *et al.*, 2005; Roopesh *et al.*, 2006). On the contrary, lower concentrations may not be sufficient to achieve maximal enzyme production (Sabu *et al.*, 2002). A balance between the proliferating microbial biomass and available substrate material should be maintained to yield maximum enzyme (Pandey *et al.*, 2000).

### Effect of NaCl on phytase production

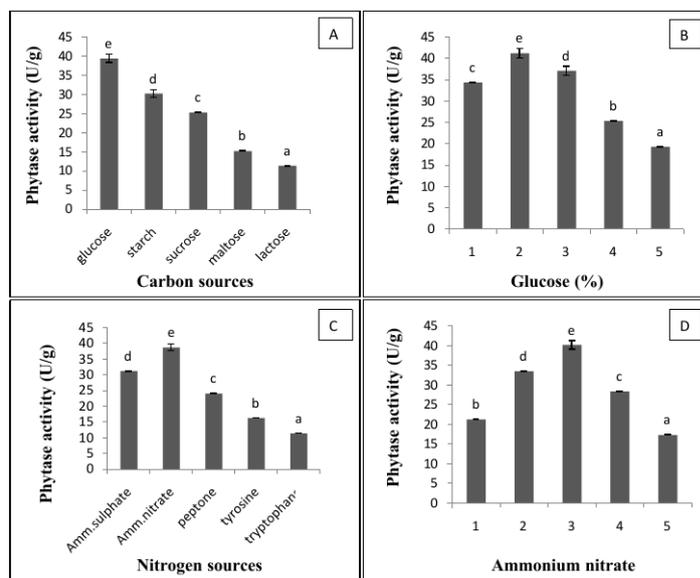
It is very important to examine the effects of different concentrations of NaCl (w/w) in the production medium. Li *et al.* (2008) mentioned that added NaCl was the most suitable for phytase production by the marine yeast and noticed that the marine yeast strain could produce a high level of phytase in the production medium prepared with seawater. In the present study, 3% NaCl supplementation showed highest phytase activity ( $36.18 \pm 0.07$  U/g) and at higher concentration phytase activity was decreased (figure 2 E).

### Effect of different surfactant

Surfactants have been reported to influence the growth and extracellular enzyme production of the microorganisms. However, their effects might vary from enzyme to enzyme, even from organism to organism. In the present study, effects of different surfactants (1%, v/w) such as Tween 20, Tween 40, Tween 80, and DMSO showed variation in phytase activity. These surfactants were added to the moistening media and highest phytase activity ( $37.29 \pm 0.024$  U/g) was shown on supplementation of DMSO into the medium (figure 2F). Several researchers have shown that incorporation of surfactants could induce the formation of smaller pellets leading to increase in the extracellular enzyme synthesis and hence higher yield of the phytase (Sasirekha *et al.*, 2012). The surfactants could also increase the cell wall/cell membrane permeability that might lead to the simultaneous augmentation in the secretion of biomolecules (Das *et al.*, 2013).

### Effect of various carbon and nitrogen sources

Type and nature of carbon and nitrogen sources are among the most important factors for any fermentation process. Carbon is a major constituent of the cell. The rate at which a carbon source is metabolized can often influence the production of metabolites as it represents the energy source that will be available for the growth of the microorganism (Roopesh *et al.*, 2006). The requirement of additional carbon sources in the fermentation medium for enhanced enzyme production was evaluated by incorporating different carbon sources (1%, w/w) into the media. The results indicated that among diverse carbon sources tested, glucose supplementation supported highest phytase activity ( $39.45 \pm 1.12$  U/g) than the other tested carbon sources (figure 3A). Further study revealed 2% (w/w) glucose supplementation as an optimum condition for phytase production (figure 3B). To initiate growth and metabolism, microorganisms necessitate carbon sources at easily available form. Glucose, being the easily metabolizable sugar, has been reported to increase phytase production from thermophilic mold *Sporotrichum thermophile* (Singh and Satyanarayana, 2005). In the presently reported study, starch was the second best carbon source for phytase production ( $30.25 \pm 1.05$  U/g). Reports are also available on starch supplementation to augment phytase production by *Aspergillus niger* (Vats and Banerjee, 2002) and *Mucor racemosus* (Roopesh *et al.*, 2006). Further, Li *et al.* (2008) documented inclusion of starch to enhance phytase production in submerged culture with the marine yeast strain *Kodamea ahmeri* BG3 isolated from the gut of a marine fish, *Hexagrammes otakii*.

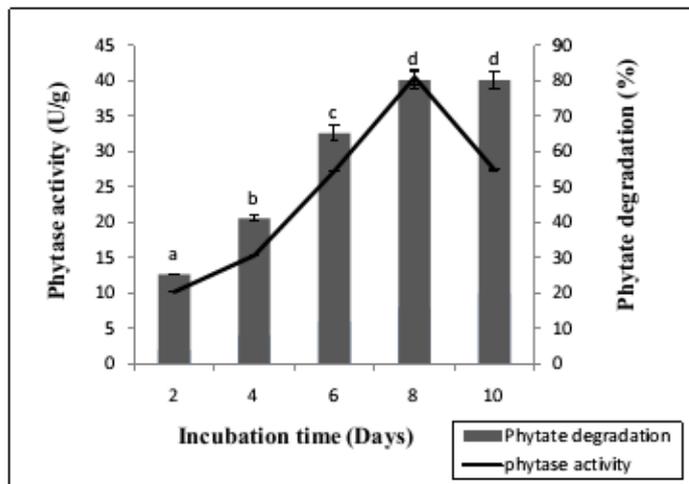


**Figure 3** Effect of carbon sources (A), varying levels of the selected carbon source (B), nitrogen sources (C) and varying levels of the selected nitrogen source (D) on phytase production in SSF. Bars with different alpha plates are statistically significant ( $P < 0.001$ ; SNK test).

Similarly, nitrogen sources are important nutrients for enzyme production. The impact of additional nitrogen sources on phytase production was evaluated by incorporating different organic and inorganic nitrogen sources (1%, w/w) in the medium. Among all the nitrogen sources tested, ammonium nitrate supplementation resulted maximum phytase production ( $38.75 \pm 1.06$  U/g), which was followed by ammonium sulphate ( $31.28 \pm 0.09$  U/g) (figure 3C). Further study revealed 3% (w/w) ammonium nitrate as optimum concentration for phytase production (Fig. 3D). In comparison to the organic nitrogen sources used, our study revealed optimum phytase production by *C. tropicalis* with supplementation of inorganic nitrogen sources. The high level of free amino acids in organic sources might have repressed phytase production (Lan *et al.*, 2002). It has been postulated that many enzymes could be repressed by rapidly utilizable amino acids, and thus inadequacy of these amino acids in fermentation growth media usually favour enzyme synthesis (Ward, 1989). Singh and Satyanarayana (2005) and Li *et al.* (2008) also documented inorganic ammonium sulfate as the best nitrogen source to increase phytase production by the thermophilic mold *Sporotrichum thermophile* and the marine yeast strain *Kodamea ohmeri* BG3, respectively. One-way ANOVA revealed that variation in phytase production at different carbon and nitrogen sources were statistically significant ( $P < 0.001$ ).

### Time course study

After optimizing the various process parameters, a time course study was conducted to see the cumulative effect of various parameters. With GOC as substrate, maximum enzyme activity ( $40.53 \pm 1.12$  U/g) was recorded after 8 days under optimized conditions (figure 4). However, phytase production declined with further increase in the incubation period and almost ceased after 10 days, which could have been due to the reduced nutrient level of the medium (Sabu *et al.*, 2005). Otherwise, it could also be the effect of poisoning and denaturation of the enzyme by interference of the other components in the medium (Ramesh and Lonsane, 1987). Concentration of phytate in the substrate reduced gradually as fermentation progressed. The concentration of phytate decreased from  $2.62 \pm 0.03$  to  $0.85 \pm 0.04$  g/100g dry weight (80.21% reduction) after 8 days. Phytate degradation did not increase further during the last two days of incubation. Therefore, it was apparent that apart from phytase production, *C. tropicalis* was also effective in removing phytate, an anti-nutrient, from the solid substrate used in the present study.



**Figure 4** Effect of incubation period on phytase production in SSF. Bars with different alpha plates are statistically significant ( $P < 0.001$ ; SNK test).

**Effect of fermentation on proximate composition of GOC**

The main advantage for the production of enzymes in SSF using agroindustrial residues is the waste utilization through the production of value added products (Immanuel *et al.*, 2010). Analysis of composition in the GOC after fermentation at optimal conditions by the fish gut isolate *Candida tropicalis* (JX532154) revealed that there was marginal increase in the contents of protein, lipid, and minerals (table 1). Fermentation was effective in reducing the crude fibre content and the anti-nutritional factors such as tannins, phytic acid and trypsin inhibitor, and enhancing available free amino acids and fatty acids.

**Table 1** Proximate composition and concentration of different mineral ions in raw and fermented GOC

Parameters	Raw	Fermented	% Increase (↑) / Reduction (↓)
Crude protein	42.51± 0.04	48.30± 0.03	13.62 ↑
Crude Lipid	7.5± 0.05	8.7± 0.03	16.0 ↑
Crude Fibre	4.52± 0.05	2.31± 0.04	48.89 ↓
Total free fatty acid	1.06± 0.03	1.8± 0.02	69.81 ↑
Total free amino acid	0.88± 0.04	1.17± 0.02	32.95 ↑
Tannin (mg/g)	2.5±0.02	0.96±0.22	61.6 ↓
Phytate (g %)	2.62 ±0.03	0.85±0.04	80.21 ↓
Trypsin inhibitor (mg/g)	10.29± 0.05	5.11±0.04	50.34 ↓
<b>Minerals</b>			
Na (mg /g)	1.18±0.51	1.40±0.35	18.64 ↑
K (mg /g)	12.84±1.02	14.02±0.93	9.19 ↑
Ca (ppm)	1.23±0.45	1.42±0.34	15.44 ↑
Mg (mg /g)	4.33±0.31	5.03±0.21	16.16 ↑
Zn (mg /g)	1.65±0.62	1.82±0.55	10.30 ↑
Fe (ppm)	11.25±0.41	14.71±0.35	30.75 ↑
Cu (mg/g)	0.19±0.02	0.25±0.01	31.57 ↑
P (mg/g)	4.25±0.34	4.62±0.42	8.70 ↑
Mn (ppm)	5.45±0.41	7.51±0.46	37.79 ↑

Values are means ± SE of three determinations (t-values are significant at 0.05 level).

The efficacy of fermented oil seed meals for partial or complete substitution of fishmeal has been suggested by several authors (Mukhopadhyay and Ray, 1999; Ramachandran *et al.*, 2005). Although raw GOC contains good quantities of protein, lipid and minerals, presence of certain anti-nutritional factors restrict their regular nutritional utilization. Tannins, phytates and trypsin inhibitors are some of the common anti-nutritional substances in the oil seeds (Tacon and Jackson, 1985). Results of the present study suggest that tannin, phytic acid and trypsin inhibitor could be significantly reduced through SSF by *C. tropicalis*. Therefore, adoption of SSF as a processing technique might contribute to enhance the nutrient value of the GOC. Fermentation is a simple and cheap process where there may be an increase in the nutrient level through microbial synthesis (Wee, 1991). In the present study, increased level of crude protein, free amino acids and free fatty acids in fermented GOC in comparison to the raw GOC is in agreement with the findings of Mandal and Ghosh (2013).

**CONCLUSION**

Supplementation of microbial phytase or pre-treatment of plant ingredients by microbial phytase has been proved to improve phytate phosphorus bio-availability, and thereby reduce the use of inorganic phosphorus supplements (Sardar *et al.*, 2007; Cao *et al.*, 2008). Therefore, in the present study, emphasis has been given on optimization of the culture conditions for phytase production by wide variety of microorganisms, as well as deactivation of plant phytate through fermentation (Sabu *et al.*, 2002; Singh and Satyanarayana, 2005; Roopesh *et al.*, 2006). Results of the present study might suggest that the phytase-producing microbial symbionts from the fish gut can be effectively used to achieve both of these objectives. On account of its potential in value addition, yeasts may be useful for bio-conversion of seed meals and/or agricultural wastes for possible use as animal feed ingredients in consideration of ensuing feed related waste outputs and trim down the feed cost (Mandal and Ghosh, 2013). Further, *in vitro* processing by autochthonous microbiota might be assumed as an effective strategy as the organism itself and their metabolites would not cause harm to the fish providing the basis for mutual relationship (Khan and Ghosh, 2012b). However, further research should be accomplished to conclude whether inclusion of the yeast phytase or the bio-processed oilcake, indeed, endow with some benefit to the fish prior to advocate their use in aquafeed.

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