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Immobilised Phytase Production from *Aspergillus foetidus* MTCC 11682 Using an Optimized Media

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Authors' contributions

This work was carried out in collaboration between all authors. Author SA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AVE and JG managed the analyses of the study. Author DS managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Immobilised fungal phytase production from the novel strain *Aspergillus foetidus* MTTC 11682 and optimisation of cultural conditions for a better and continuous economic yield.
Study Design: The study was designed based on the classical method of changing one independent variable while fixing all other at a certain level- one factor at a time, a close ended system for the optimisation of fermentation process.
Methodology: Physical and nutritional parameters were optimised for phytase production and subjected to statistical analysis. Adsorption and Entrapment techniques were employed to immobilise the production strain.
Results: The optimum physical conditions for augmenting the yield up to 6 days incubation period were as follows: pH of 3.5, 30ºC temperatures and 5% inoculum size. Amongst the nutritional parameters, lactose and sodium nitrate were found to be the best carbon and nitrogen sources. K^{++},

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Mg^{++}, Mn^{++} and Fe^{++} ions supported the phytase production. TritonX 100 and tween 80 showed an inducing effect on the secretion of phytase enzyme. Immobilised fungal phytase production resulted in an increased yield of 32.5% with poly urethane foam (PUF) as the matrix. A scale up fermentation resulted in an activity of 52.7 FTU/mL for immobilised cells as compared to 25.5 FTU/mL by its free counterpart.

Conclusion: Phytase produced in an optimised media employing immobilised *Aspergillus foetidus* 11682 on poly urethane foam cubes exhibited better phytase activity, improved stability and long shelf life.

Keywords: Phytase; optimisation; immobilisation; Aspergillus sp; poly urethane foam.

1. INTRODUCTION

The enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the sequential release of phosphate from phytate (myo-inositol hexakisphosphate) thus classed as phosphatases. It is a high molecular weight phosphatase which is found naturally in animals, plants and microorganisms. Microbes are the major natural source for phytase and are exploited for commercial scale production [1]. Among all microbes, fungal production is preferred due to easy culture and harvesting techniques of the enzyme. In general, fungal morphology plays a fundamental and crucial role in determining the overall productivity and its bioengineering results in better activity [3]. Pandey et al. [2] reported the type of strain, culture conditions, nature of substrate and availability of the nutrients as the critical factors determining the yield and needs to be scrutinized for selecting a particular technique. A qualitative symbiotic relationship was observed between *Aspergillus niger* morphology, medium composition and phytase productivity in submerged fermentation (SmF) and solid state fermentation (SSF) [4]. The technology of employing hyphal aggregates by natural accumulation or by artificial immobilisation is being adapted extensively for enhanced enzyme production [5]. Immobilisation generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity [6]. However, the immobilisation support must be conductive to cell viability and have proper permeability that allow sufficient diffusion and transport of oxygen, nutrients, metabolic waste and secretory products across the polymer network [7]. Though, numerous methods have been devised by researchers, no single method and support is appropriate for all the cells. Therefore, it demands the inevitability to select the best matrix for specific organism in specified conditions. Keeping the above background in mind, the optimization of cultural conditions for a new fungal strain *Aspergillus foetidus* MTCC 11682 by submerged fermentation has been reported here. This strain of fungus was recently isolated from soil screening and submitted to MTCC repository (Chandigarh, India). The study was further extended for selection of the best inert matrix that can be used to immobilise the fungi for a scale up fermentation for industrial operation.

2. MATERIALS AND METHODS

2.1 Isolation of Fungi

A phytase producing novel strain *Aspergillus foetidus* MTCC 11682 was isolated from the soil. The culture was maintained on Potato Dextrose Agar (PDA) at 4°C. A 5-day old, fully sporulated plate was used for inoculum preparation. The spore suspension was extracted using 0.1% tween 20 and physiological saline in the ratio of 1:5. The thoroughly homogenised suspension was employed as inoculum throughout the study.

2.2 Medium and Culture Conditions

Aspergillus foetidus MTCC 11682 fungi were cultured on a semi-synthetic media [8] with a slight modification for phytase production in all the experiments. The modified 100 mL media contained maize-1 g, glucose-5 g, $NaNO_3$- 0.86 g, $MgSO_4.7H_2O$- 0.05 g, KCl- 0.05 g and $FeSO_4.7H_2O$- 0.01 g with 5.5 pH. It was cultured at 30ºC at 100 rpm using 1% (w/v) spore suspension (1.8×10^8 spores/mL). The cultural filtrates were removed at specified intervals as per the experimental schedule described below and assessed for phytase activity.

2.3 Phytase Assay

Phytase activity was estimated calorimetrically by monitoring the release of inorganic phosphorous from phytic acid sodium salt (Sigma Chemical

Company, St Louis, MO, USA) using Kim and Lei's method [9]. About 200 µL of culture supernatant was incubated at 37ºC in 0.2 M sodium citrate buffer (pH 5.5) containing 1% 9mM sodium phytate. The reaction was terminated by addition of 400 µL of 15% trichloroacetic acid (w/v). The liberated inorganic phosphorus was measured by incubation with 2 mL of freshly prepared color reagent (1 M sulphuric acid, 2.5% ammonium molybdate (w/v), and 10% ascorbic acid (w/v) mixed in the ratio of 3:1:1) at 50ºC for 15 min. Absorbance of 820 nm incident light through the colored solutions was measured. One unit of phytase was expressed as the amount of enzyme required to release 1µmol of inorganic phosphorous/min from sodium phytate at 37ºC. Phytase was expressed as FTU/mL.

2.4 Optimisation of Physical and Nutritional Parameters for Phytase Production

The entire study was performed in triplicates using 25 mL production medium in 50 mL Erlenmeyer flask and the parameters were investigated using one variable at a time.

2.4.1 Optimisation of physical parameters

Temperature, pH, incubation days and the inoculum size were optimised under physical parameters. The effect of pH on phytase production was studied in the range of pH 3.0 to 10.0. The varying incubation temperatures were within the range of 28- 40°C. At every 24 hours (h) interval, the culture was filtered through Whatmann No.1 filter paper to determine the time period of fermentation and fungal biomass. The filtered mycelium was dried at 60°C for 24 h in a pre-weighed dry Petriplate and expressed as g dry Wt/L medium. The study was continued until a decline in phytase production was observed. To understand the optimum inoculum concentration, the media was seeded with different inoculum percentage (1,2,3,4, 5,6,7,8,9,10 and 15%) w/v and filtrates were assayed in every 48h for enzyme activity check.

2.4.2 Optimisation of nutritional parameters

Different carbon source, nitrogen source, metal ions and surfactants were tested and the filtrates were assayed for phytase activity on the 6^{th} day. Glucose, sucrose, lactose, galactose, mannitol and glycerol were tested as carbon sources at 5% w/v level. Various organic (casein, peptone, urea, yeast extract) and inorganic (ammonium sulphate, ammonium oxalate, sodium nitrate) sources of nitrogen were tested at 0.86% w/v concentration. The chloride or sulphate salts of Ba, Ca, Co, Fe, Mg, Mn, K, Pb, Hg, Na, Zn and Ni were tested at 0.05% w/v. Similarly surfactants such as ethylene di-amine tetra acetic acid (EDTA), glycerol, mercaptoethanol, sodium dodecyl sulphate (SDS), toluene, triton X 100, tween 20 and tween 80 were tested at 0.1% w/v level of incorporation in the production media. All the studies were carried out against a control which was devoid of the factor under the study and done in triplicates. Solutions of carbon source and surfactants were filter sterilized (0.22 µm) separately and were added in the fermentation medium prior to inoculation. The optimisation influence was studied by comparing the phytase yield between the basal media and optimised media incorporating the optimum physical and nutritional parameters.

2.5 Immobilisation of Fungus for Phytase Production

The *Aspergillus foetidus* MTCC 11682 was immobilised by employing adsorption [10] and entrapment techniques under strict aseptic conditions. All studies were carried out in triplicates and the productions were carried out in 25 mL of optimised media.

2.5.1 Immobilisation by adsorption

The immobilisation matrices used for adsorption were areca sheath, wood shavings and poly urethane foam (PUF). All matrices were subjected to pretreatment as described by Krishnaprasad et al. [10]. The PUF was cut into 1x1x1 cm^3 cubes. 5 %w/v spore suspension was inoculated into 25 mL of growth media, potato dextrose broth (PDB) containing 1.25 g of treated matrices. The flasks were incubated in a rotary shaker at 30°C for 5 days until fungi grew profusely on the matrix. The growth media was decanted and replaced with production media and cultured at similar condition.

2.5.2 Immobilisation by entrapment

Calcium alginate beads and carrageenan cubes [11] were employed for immobilisation by entrapment. Sodium alginate (3%) and carrageenan (4%) were suspended in PDB and sterilized at 110ºC for 10 min at 10 lbs pressure. On cooling, it was mixed with 5% (w/v) spore

suspension to homogeneity. Calcium alginate beads were synthesized by drop wise extrusion of sodium alginate spore suspension, into chilled $CaCl_2$ solution using hypodermic syringe. Carrageenan cubes were prepared by transferring carrageenan spore suspension on sterile petriplate. On gelation, cubes of 1x1x1 cm^3 were cut, cured for 2 h in cold 0.3M KCl to enhance their mechanical stabilities. The spore immobilised matrices were collected, rinsed with sterile distilled water thrice and 1.25 g of it was transferred to production media for phytase production.

2.6 Enzyme Production by Repeated Batch Process

A scale up fermentation was done from 25 mL media to 100 mL in a 250 mL Erlenmeyer flask employing the best inert matrix obtained from the immobilisation experiment. The production was carried out as described above and the cultural filtrates were examined for the phytase activity. Replenishment of the production media was performed at periodic intervals of each working cycle. To understand the retention of phytase activity, the filtrates were collected on alternate days from the 6th day till 25th day of fermentation.

2.7 Characterisation of Immobilised Fungal Phytase

The physiochemical properties of the phytase produced by immobilised fungus and its free counterpart were studied. The effect of pH was determined by pre incubation of the enzyme in buffers over the pH range 2.5 to 7.5, using 0.2 M glycine-HCl buffer (pH, 2.5– 3.5), sodium acetate buffer (pH, 4.5–5.5) and Tris-HCl buffer (pH, 6.5–7.5) for a period of 30 min. The effect of temperature was determined by pre incubating the enzymes at temperatures 4, 30, 37, 50, 60, 70 and 80ºC for 30 min. After the pre incubation at respective pH and temperature, the samples were analyzed for phytase activity as described in section 2.3. The shelf life of immobilised fungal phytase was evaluated in spent medium stored at -20ºC over a period of 1 year. The phytase activity was measured quarterly until the end of the experiment.

2.8 Statistical Analysis

The data of optimisation were subjected to a one-way analysis of variance (ANOVA) and tested for significance employing post hoc Duncan's multiple range test (SPSS, 2010 version 18.0).

3. RESULTS AND DISCUSSION

3.1 Optimisation of Physical Parameters for Phytase Production

3.1.1 Optimum pH

Phytase production was higher ($P< 0.001$) in acidic environment within the pH range of 3.5 to 6.5 as compared to the alkaline pH ranged between 7.5 to10. The pH of 3.5 was optimal for phytase yield on 6th day of fermentation with an activity of 21 FTU/mL (Fig. 1). This proved the general adeptness of fungal physiology to grow best at acidic pH and the ability of *Aspergillus* sp. to tolerate high acidic medium. This is in accordance with findings of Gargova and Sariyska [12] who reported acidic medium to be best for fungal phytase production. *Aspergillus niger* has a pH optimum at 3.0 for phytase [13]. *Aspergillus ficcum* has pH optima, 2.0 and 5.5 in the acidic ranges [14]. Likewise pH 4.0 - 4.5 was reported as the optimum for an unidentified fungal strain NSF 9 [15]. Interestingly, an increase in phytase production was observed on 8th day in neutral to alkaline pH medium. This phenomenon could be due to the dip of pH by production of organic acids in prolonged fermentation period favoring the phytase production. However, further investigation is required, as the facts about the impact of fermentation on media pH at different days were not recorded.

3.1.2 Optimum incubation temperature

On 6th day, 28ºC and 30ºC of temperatures were found to be better for the phytase yield (16 and 17 FTU/mL) when compared to the lower and higher temperatures selected for this study (Fig. 2). The production of enzyme decreased gradually with the increase in temperature, confirming the mesophilic nature of the sample organism. The optimum range of most phytase producing microorganisms lies within a temperature range of 25- 37ºC [16]. This finding has close resemblance to the findings of Gulati et al. [17] wherein 30ºC was reported to be optimum for phytase production by *A. niger* NCIM563. However, *Pencillium purpurogenum* GE1 produced maximum phytase at 27ºC [18]. Probably the zone of comfort for the fungal growth required for a particular species plays an important role for the maximum production of phytase as the same physical condition is found optimal for their growth as well.

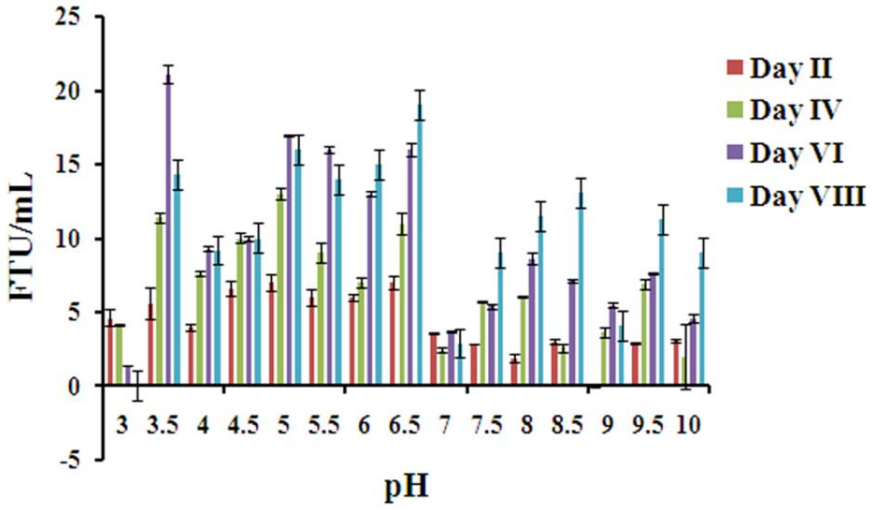

Fig. 1. Effect of physical parameter- pH on phytase production by *Aspergillus foetidus* MTTC 11682

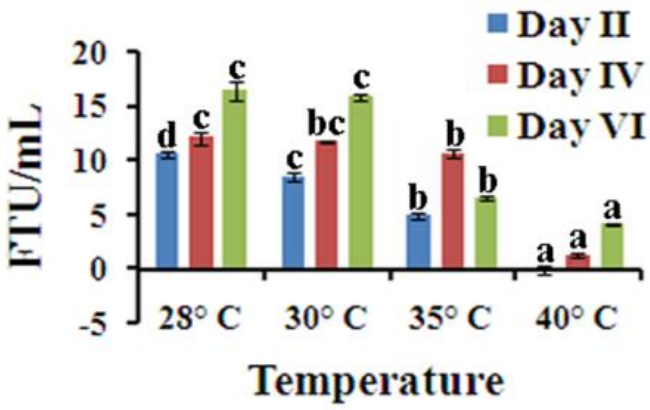

Fig. 2. Effect of physical parameter- temperature on phytase production by *Aspergillus foetidus* MTTC 11682

The bars (Mean±SEM) labeled with different letters indicate significant differences at P≤0.05

3.1.3 Biomass and phytase production

The fungal biomass increased with the increase in days of culture up to day 6 and declined thereafter (Fig. 3). The increase and decrease of fungal biomass coincided with the production of phytase indicating a proportional relationship between these two. At day 6, a biomass of 0.69 g/dw was obtained with maximum phytase production of 14.4 FTU/mL. The biomass and phytase production was found to be significantly higher on 6th day ($P<0.001$) as compared to other days. Similar proportional relationship of phytase production with the increase in biomass has been reported in the literature. Sabu et al. [19] stated that phytase yield and biomass formation in *Rhizopus oligosporus* are strongly correlated with the inoculum age, indicating strong growth associated phytase production. The low initial biomass of 0.471 g/dw in the present study might be due to the time required for the spore germination into vegetative hyphae. A dip in biomass after 6th day might indicate a lack of nutritive support to the proliferative vegetative hyphae due to the depletion of nutrients in the media. Awad et al. [20] have observed maximum growth of 135.7 mg/ds after 5 days of incubation in *Penicillium funiculosum* NRC467 by employing SSF.

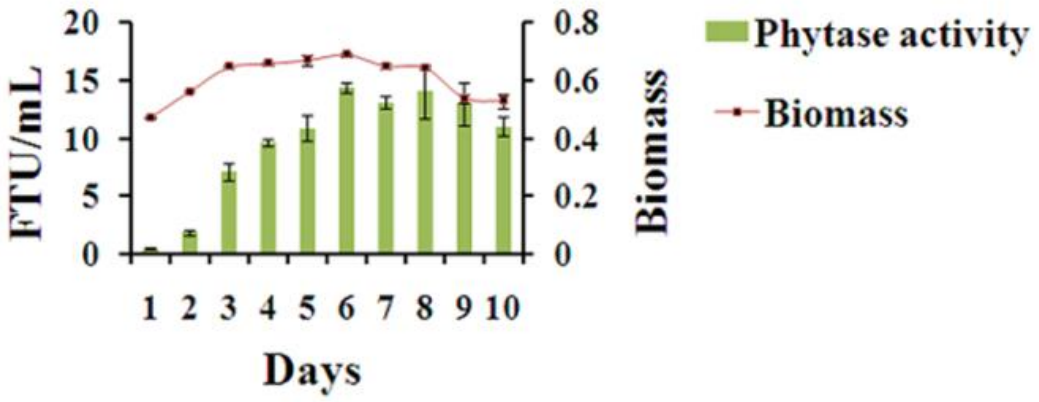

Fig. 3. Effect of physical parameter- biomass vs phytase production by *Aspergillus foetidus* MTTC 11682

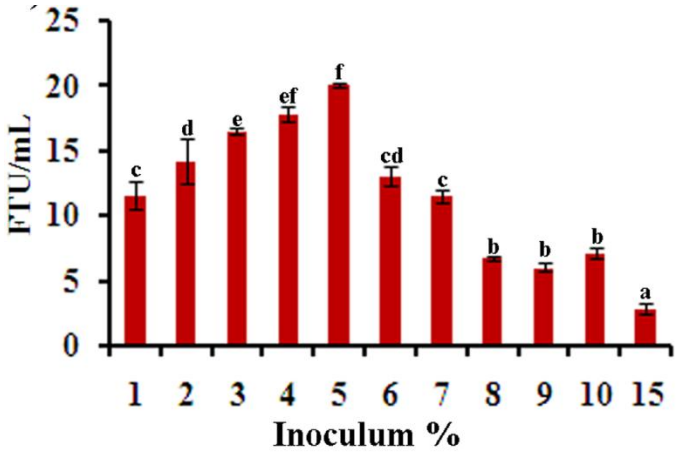

Fig. 4. Effect of physical parameter- inoculum concentration on phytase production by *Aspergillus foetidus* MTTC 11682

The bars (Mean±SEM) labeled with different letters indicate significant differences at P≤0.05

3.1.4 Phytase production in different inoculum sizes

The present investigation showed a gradual increase in enzyme production up to 5% of inoculum size, followed by a drop on 6th day of phytase production. A maximum yield of 20 FTU/mL ($P<0.001$) was obtained at 5% w/v inoculum concentration as compared to 3 FTU/mL at the highest inoculum concentration of 15 % w/v (Fig. 4). Optimum phytase production at 2-4 % inoculum concentration in *Bacillus* sp EBD 9-1 was reported by Elif et al. [21]. Reports of an inoculum concentration as low as 2% to be the optimum for phytase production in *Mucor racemosus* and *P. funiculosum* NRC467 are also available in the literature [22,20]. Decreased phytase production with the increase in inoculum size might be due to the adopted survival strategies of the increased biomass under limited available nutrients in the culture media.

3.2 Optimization of Nutritional Parameters for Phytase Production

3.2.1 Optimum carbon source

The lactose was found to be the preferred carbon source for the fungal strain employed in the present study (Fig. 5A). Lactose at 5% level of supplementation resulted in an activity of 19 FTU/mL. Although this organism can grow and produce reasonably good quantity of phytase in the presence of other sugars like glycerol,

mannitol, galactose and glucose with an activity of 17.9, 15.9, 15.2 and 12.9 FTU/mL, respectively in comparison to the control (8.9 FTU/mL). Sucrose was found to suppress phytase production in this fungal species. On the contrary, *Bacillus* sp. DSII and *B. amyloliquefaciens* FZB45 have the best preference for sucrose as compared to other sugars for phytase production [23,24]. *A. niger* CFR 335 [25] and *Mucor racemosus* [22] showed an inclination towards glucose as the carbon source for phytase production in SSF. Unlike the commonly preferred monosaccharide glucose, this organism preferred disaccharide lactose. This preferred utilisation of carbon source developed in an organism could be due to shifts in their metabolic pathways. A detailed study on carbohydrate metabolism of these fungi at different levels of supplementation of different sugar and studying the molecular pathways could throw light on these differences. However, one probable reason that could justify this finding might be the level (5%) of metabolisable substrates used for the culture. It has been opined that readily metabolised carbon source like glucose at higher concentration might cause catabolic repression in some organism [26]. In immobilised *Rhizopus arrhizus* maximum lipase production was noticed at 1 to 2.5 g/L of glucose concentration but further increment of it caused depletion of the enzyme [27].

3.2.2 Optimum nitrogen source

Phytase production by the organism was found to increase ($P<0.001$) at the supplemented level of $NaNO_3$ as compared to control (23 FTU/mL vs 0.6 FTU/mL). All other nitrogen sources showed little effect on the phytase production by this novel strain (Fig. 5B). Nampoothiri et al. found the $NaNO_3$ as the best nitrogen source for *Thermoascus aurantiacus* with an activity of 247.2 U/mL [28]. *A. niger* NCIM 563 with an optimum yield of 41.47 and 10.71 IU/mL at pH 2.5 and 4.0 was reported by Soni and Khire [29]. On the contrary, $NaNO_3$ was also reported to be severely inhibitory for the phytase production for *Bacillus* sp T4 [30]. Elif et al. [21] recommend organic nitrogen sources, however $NaNO_3$ was the best among the available inorganic sources for phytase production by *Bacillus* sp EBD 9-1. Choi et al. [31] showed beef extract and peptone as the best nitrogen source for phytase production for *B. subtilis* KHU-10. In *B. laevolacticus* [17] beef extract, peptone and tryptone had not shown any effect on phytase production. These findings indicate that there are organism specific differences for the preferences of nitrogen sources for the production of this enzyme.

3.2.3 Metal ions on phytase production

Results from the present study indicated that incorporation of Mg^{++}, K^{+}, Fe^{++} and Mn^{++} ions increased ($P<0.001$) the phytase activity when compared to the control (2.8 FTU/mL). Mg^{++} and K^{+} showed maximum phytase activity at 20.4 and 18 FTU/mL respectively (Fig. 5C). The role of Fe++ and Mn^{++} were also substantial with an activity of 13 FTU/mL. The results clearly categorized the phytase supporting metal ions into bulk and trace metals. Mg^{++} and K^{+} were assigned as the bulk metals incorporated at 0.05% w/v and Mn^{++} and Fe^{++}as the trace metals assimilated at 0.001% w/v. Pb^{++} and Hg^{++} had an inhibitory effect on phytase synthesis. Similarly Fe^{++}, K^{+} and Mg^{++} were studied to enhance phytase production by *A. niger* NCIM612 with activities of 128.79, 126.92 and 125.05 U/gds respectively [32]. Zn^{++}, Ni^{++}, Co^{++}, Hg^{++} and Pb^{++} were reported as the strong inhibitory metals causing repression of phytase production by *A.niger* NCIN563 [29]. Some cations such as Fe^{++}, Zn^{++} and Cu^{++} caused 50% subjugation of enzyme activity [33]. The current study also agreed to the inhibitory effects of Zn^{++}, though Fe^{++} had a positive response to phytase production. The trivial change in the effect of metal ions could be due to the variance of strain and cultural conditions.

3.2.4 Surfactants on phytase release

Surfactants are known to increase the cell wall/ membrane permeability through the change in lipid layer leading to increased secretion of extracellular enzyme during fermentation [34]. The tritonX 100 and tween 80 was found to significantly increase the phytase activity of 40.7 and 32.5 FTU/mL as compared to the control of 19.4 FTU/mL. Mercaptoethanol and SDS acted as suppressor for phytase production (Fig. 5D). An enhanced production of phytase from 154 U/g ds to 188 U/g ds by *P. purpurogenum* GE1 was obtained employing triton X100 [18]. Mandiwala and Khire [35] reported that among the various surfactants added to SSF, triton X 100(0.5%) exhibited 30% increase in *A. niger* phytase production. Likewise, the phytase production of *A. niger* NCIM563 was reported to increase when supplemented with tween 80 and triton X 100

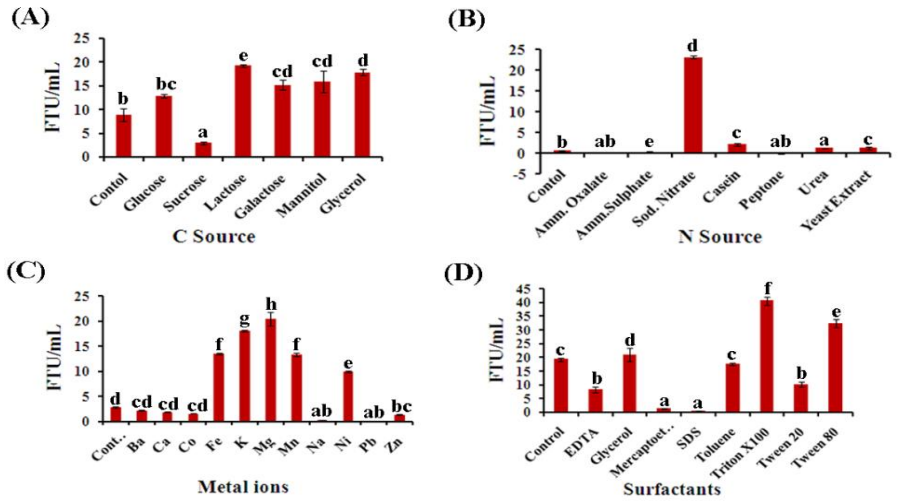

Fig. 5. Effect of nutritional parameters on phytase production by *A. foetidus* MTCC 11682

Effect of nutritional parameters on phytase productivity by Aspergillus foetidus MTTC 11682 against a control on 6th day of fermentation (A) carbon source at 5% w/v (B) organic and inorganic nitrogen sources at 0.86% w/v(C) metal ions at 0.05% w/v (D) surfactants at 0.1%w/v. The bars (Mean±SEM) labeled with different letters indicate significant differences at P≤0.05 as compared to control

[29]. On the contrary, the studies of Lan et al. [36] reported that tween 80 (0.5 % and 1%) and Triton X 100 (1%) did not affect the phytase production.

Optimisation studies resulted in a two fold increase in the total yield of phytase. A phytase activity of 20 FTU/mL was obtained for basal media, whereas, the optimised media resulted in an activity of 41 FTU/mL on the 6th day of fermentation. An increase in phytase activity with 1.8 fold for *Mucor racemosus* and 1.7 fold for *A. ficuum* were studied on statistical optimisation [37]. An improved phytase activity of 1.75 times for yeast *Pichia anomala* in synthetic medium was observed by Vohra and Satyanarayana [16].

3.3 Phytase Production by Immobilised Fungal

Immobilisation of *Aspergillus foetidus* was found to enhance phytase production and changes its physio-chemical properties. Intense amount of hyphal aggregation on all the carrier matrices were observed.

3.3.1 Phytase production by immobilised fungus employing entrapment

The entrapment technique employing calcium alginate and carrageenan were effective in confining the fungal spores. However, on the 6th day a sumptuous fungal growth was observed resulting in rupture of beads and clogging of media with vegetative cells. This indicated low mechanical strength, regeneration ability and hindrance in downstream processing. The phytase activity obtained by employing calcium alginate beads and carrageenan cubes were 4 and 10 FTU/mL, respectively (Fig. 6). A similar finding stressing on issues of mechanical strength and lack of open spaces to accommodate active cell growth resulting in their rupture and cell release into the growth medium was reported by Barbotin and Nava Saucedo [38]. To counter this problem one can use the strengthening solution. However, use of higher concentration of strengthening solution might damage to the viability/activity of the cells [39]. Several other reports indicated the fragility of natural polymers in comparison to synthetic polymers [40,7]. Inefficiency of the matrix coupled with low phytase activity obliterated their use as an ideal immobilising matrix in this study.

3.3.2 Phytase production by immobilised fungus employing adsorption

Immobilisation of fungus by adsorption proved to be more effective in our studies as good phytase activity was observed when fungus were grown in wood shavings and PUF cubes. Areca sheath didn't support phytase production, though good adsorption and sporulation of fungi was observed. Wood shavings recorded the maximum phytase activity of 34 FTU/mL;

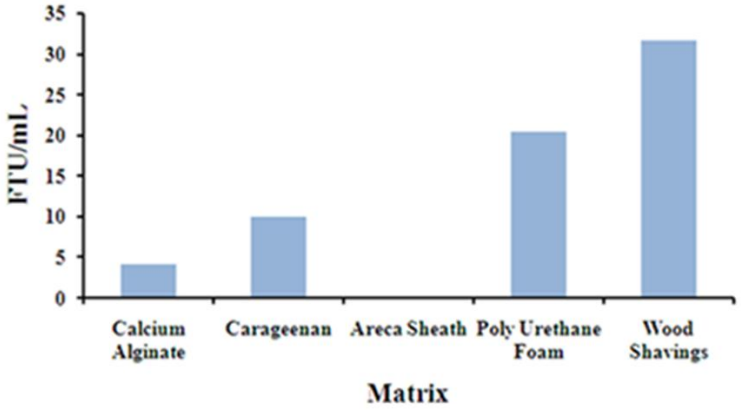

Fig. 6. Immobilised fungal phytase production on 6th day employing adsorption and entrapment technique

however, it was subjected to microbial decomposition easily and not durable for a long term production. Permanent loss of enzymes from fungal extracellular matrix due to degradation and loss of supporting material concerned with waste water treatment has been reported by Bohmer et al. [41]. Results from the present study indicated that Poly Urethane Foam was a better immobilisation matrix for proliferation of fungus. The activity of 20 FTU/mL was obtained in the spent medium after 6 days of culturing. An ideal immobilisation matrix should exhibit high chemical and biological stability, mechanical resistance to abrasion, appropriate permeability to reagents and large surface, capacity and porosity [7]. Synthetic polymeric carrier, polyurethane foam circumvents all the problems associated with culturing such organism as being inert [42]. Enhanced secondary metabolite (gluconic acid, vinegar and liognolytic enzymes) production using PUF was reported by Nakamura et al. [43]; Ory et al. [44]; Mukhopadhyaya et al. [45]; Krishnaprasad et al. [10].

Phytase production by PUF immobilised *A. foetidus* was low (20 FTU/mL) as compared to the free cells on the 6th day of culture (40 FTU/mL). This could be due to constraint of nutrients and oxygen diffusion during the pre-culture period leading to a languid mycelial growth in immobilised condition. Similar findings were reported on phosphatase production by immobilised *Humicola lutea* that yielded 800 U/L in a 72 h period where as, the free cells reached the maximum of 2500 U/L [46]. However, on scale up fermentation and increased incubation period, immobilised fungus exhibited 103% higher phytase activity compared to the free cells. On 10th day of fermentation an activity of 52.7 FTU/mL was obtained while submerged fermentation by free cell yielded only 25.5 FTU/mL. Improved surface area of fungal mycelia immobilised on PUF cubes and reduced limitation of mass transfer, enabling easy interaction with substrate might be the cause of enhanced phytase production.

3.4 Phytase Production under Repeated Batch Process by PUF Immobilised Fungus

Under immobilised condition, the phytase activity was found to increase from 20 FTU/mL on the 6th day to 49 FTU/mL on 12th day of production after which no significant increase was observed. However, the same activity was retained till 25th day with little decrease in phytase yield (Fig. 7).

As the maximum phytase activity was recorded on 10th day, the time was designated as one cycle for the corresponding batch culture. Periodic replenishment of the media on every cycle enabled the durability of the immobilised cells in repeated use. Until 10 fermentation cycles, the activity was retained but declined thereafter (Table 1). The phytase activity was found to stabilize after a few cycles of batch fermentation. The retained activity after the 15th cycle was 55% of the initial value. Though the phytase expression was suppressed after 10th cycle, the mycelial aggregation was not inhibited. Similar to this observation, phytase production up to 10th cycle in repeated batch process was also reported by Papagianni [46] in *A. niger* by SSF. Mycelial aggregation gives the advantage of better availability of oxygen and substrates due to shoot differentiation resulting in improved cell

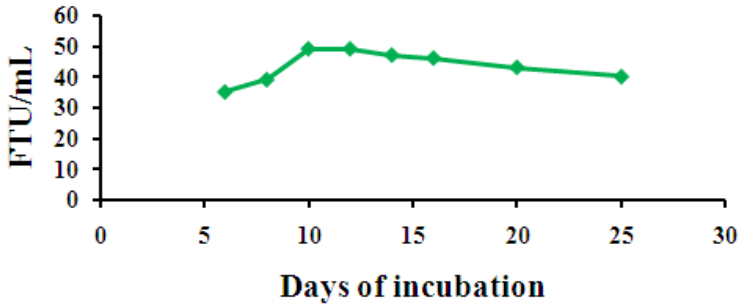

Fig. 7. Immobilised fungal phytase production using PUF cubes employing optimised media and the retention power of phytase activity in each batch of fermentation

Table 1. Production of phytase by immobilised *A. foetidus* in repeated batch process

No. of cycle	Phytase activity (FTU/mL)	Relative activity (%)
1	49 (control)	100
2	49	100
3	53	108
4	50	102
5	49	100
6	48	98
7	50	102
8	48	98
9	46	94
10	42	86
11	40	82
12	36	73
13	35	71
14	32	65
15	27	55

Fifteen cycles of repeated batch fermentation with phytase activity and the relative activity.

to cell interaction and signaling that was divergent from that of free dispersed mycelia [47]. The eventual depletion in the phytase yield after 11th cycle might be due to the excessive agglomeration of mycelia in numerous stratums on the PUF cubes, exhibiting an adverse effect on the availability of substrate and oxygen. This constrains the contact between the mycelia and substrate to the peripheral zone ensuing reduced surface area, resulting in reduced phytase production and activity. Excessive agglomeration of mycelia after 10th cycle is reported by Heath [48] who described a phenomenon of tip extension of hyphae for cell extension that cause this process.

3.5 Effect of pH and Temperature on Immobilised Fungal Phytase

The PUF immobilised fungal phytase showed better activity over a wide range of pH and temperature. An activity of 47 FTU/mL was obtained at pH 5.5 in comparison to 24 FTU/mL by the free cells at same pH. Maximum phytase activity was exhibited at 37ºC by both immobilised and free cells of 50 and 23 FTU/mL, respectively. With the increase in temperature, both immobilised and free cells lost their capacity to produce the phytase. However, regarding the stability of enzymes, phytases produced by immobilised fungus was found to be more stable as compared to the free cells. Incubation at 70ºC retained 74% of the phytase activity for immobilised fungus as compared to 39% for the free cells (Fig. 8). This clearly indicated the supremacy of immobilised fungal phytase. Though the precise cause of enhanced stability is vague, mimicking the natural phenomenon of fungi to adhere firmly on surfaces through immobilisation on matrix might lead to a protective effect making it more resistant to changes of environmental parameters such as temperature, pH or inhibitory effect of different compounds. This in turn improves enzyme operational stability.

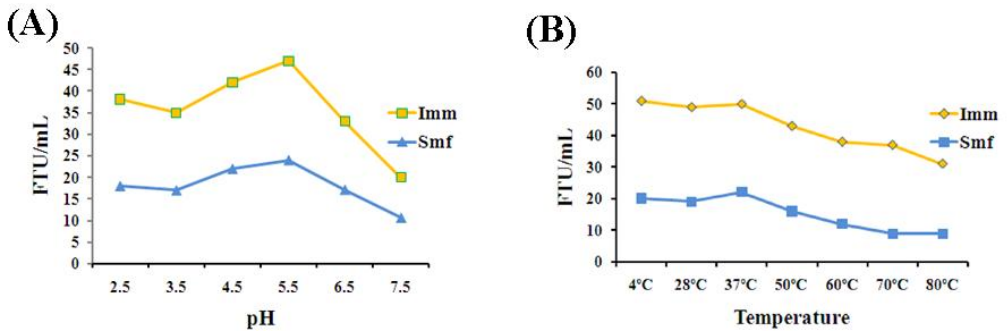

Fig. 8. Effect of pH (A) and temperature (B) on the activity of phytase produced by immobilised (Imm) and free (Smf) fungal cells

Table 2. Immobilised phytase and storage stability at -20ºC

S. no.	Time period	Phytase activity (FTU/mL)		
		Sample 1	Sample 2	Sample 3
1	Quarter 1	53	50	48
2	Quarter 2	52	51	48
3	Quarter 3	52	50	48
4	Quarter 4	52	50	48

3.6 Storage Capacity of Phytase Produced by Immobilised Fungus

The shelf life of immobilised fungal phytase was studied over a period of one year stored at -20ºC (Table 2). The quarterly examination showed stable retention of phytase activity over a period of one year.

4. CONCLUSION

The results indicated that immobilisation of *A. foetidus* MTCC 11682 on synthetic PUF adsorption can be a realistic alternative for the large-scale production of phytase. Optimisation of the nutritional and physical parameters of the production media augmented phytase production by two-fold when compared to production in basal media. An upscale fermentation on a bioreactor design is recommended for commercial exploitation. Characterization of this gene would give a better incite to the enzyme which would help to design an engineered construct for recombinant production of better phytase in the heterologous expression system.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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