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Optimization of Culture Conditions to Produce Phytase from *Aspergillus tubingensis SKA*

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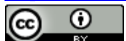

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Abstract

The effects of nutrients and physical conditions on phytase production were investigated with a recently isolated strain of *Aspergillus tubingensis SKA* under solid state fermentation on wheat bran. The nutrient factors investigated included carbon source, nitrogen source, phosphate source and concentration, metal ions (salts) and the physical parameters investigated included inoculum size, pH, temperature and fermentation duration. Our investigations revealed that optimal productivity of phytase was achieved using wheat bran supplemented with: 1.5% glucose. 0.5% $(NH_4)_2SO_4$, 0.1% sodium phytate. Additionally, optimal physical conditions were 1×10^5 spore/g substrate, initial pH of 5.0, temperature of fermentation 30˚C and fermentation duration of 96 h. Overall, a 34% improvement in phytase activity was achieved by using the optimal conditions.

Keywords

Phytase, *Aspergillus tubingensis SKA*, Solid State Fermentation, Optimization

1. Introduction

Phytic acid ($C_6H_{18}O_{24}P_6$) (*myo*-inositol hexakisphosphate (IP6), or *myo*-inositol polyphosphate), also known as phytate when in salt form, is the principle storage form of phosphorus in most plant tissues, and has been found to be a nearly ubiquitous component in cereals, legumes and oilseeds crops, constituting 1% - 5% of their weight [1] [2]. These crops are good sources of essential nutrients; however they contain high levels of phytic acid which may be considered an antinutritional factor. Phytic acid binds cations (Ca^{2+}, Fe^{2+}, Zn^{2+}) forming insoluble complexes in the intestinal tract which leads to mineral malabsorption. Moreover, phytic acid forms complexes with proteins causing a decrease in their solu-

bility and digestibility, therefore reducing their nutritive value. In addition phytic acid chelates vitamin B3 causing Pellagra, a vitamin deficiency disease [3].

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are a group of phosphatases which catalyze the hydrolysis of phytate to less phosphorylated myo-inositol derivatives and in some cases to free myo-inositol and inorganic phosphate (Pi) [4]. Phytases have a potential role in the food industry by reducing the phytic acid content leading to free phosphate. Phytases are widespread in nature and they are found in plants, animals and microorganisms. There has been an increase in research related to the production of industrial enzymes from filamentous fungi. Over 200 fungal isolates belonging to the genera *Mucor, Penicillium, Rhizopus* and *Aspergillus* have been identified as active producers of phytases, and the most remarkable one and a commercial source for phytase is *Aspergillus niger* [5]. Fungi are known for their ability to produce extracellular enzymes compared to intracellular enzymes produced by yeast and bacteria, and this makes fungi attractive for large scale production of enzymes [6].

Production of enzymes including phytase by solid-state fermentation (SSF) has advanced, due to the advantages of this method of fermentation with respect to economic and practical perspectives. These included: better product recovery, low-technology cultivation equipment, high product concentration and lower plant operation cost [7] [8] [9] [10]. SSFs take place in the absence or near absence of free water through employing natural substrates as solid supports [11]. They are not modern techniques, since they have been used for thousands of years and were used by ancient Egyptians for bread making and for the processing of Asian products such as soy sauce and koji [2].

Improving the production of microbial phytases is achieved either by optimizing nutritional and environmental conditions, or by genetic improvements of the producing isolate [12] [13] [14] [15]. The objective of this study was to optimize the culture conditions of *Aspergillus tubingensis SKA*, a new isolate of *Aspergillus*, for the production of phytase via SSF on wheat bran.

2. Materials and Methods

2.1. Instruments and Chemicals

Equipment sources were: pH meter (Sartorius, USA), water bath and spectrophotometer (Thermo Fisher Scientific, USA), incubator (Memmert, Germany), and shaking incubator (Eppendorf, Germany). Materials sources were: wheat bran (Bob's Red Mill, USA), beef extract, yeast extract, and Whatman No. 4 filter paper (Fisher Scientific), carbohydrates (Sigma-Aldrich, USA), cornstarch (purchases locally), all other materials and salts were purchased from Sigma-Aldrich (USA), Fisher Scientific (USA) and Bio-Rad (USA).

2.2. Microorganism

Isolate *Aspergillus tubingensis SKA* was isolated according to Qasim *et al.*, 2016. Thereafter, a phenotypic/morphologic and molecular characterization of the isolate was conducted by (Fungus Testing Laboratory/University of Texas-

Health Science Center, USA) to accurately classify this organism as a new strain of *Aspergillus tubingensis*.

2.3. Media and Growth Conditions

A. tubingensis SKA was cultivated in malt extract media slants for 5 days at 30˚C and all the slants were stored at 4˚C for further use. For SSF, *A. tubingensis SKA* was cultivated in 250 ml Erlenmeyer flasks containing 10 g of wheat bran as the fermentation substrate (support). After the flasks were autoclaved at 121˚C for 15 min, they were cooled before supplementation with (1:1) (w/v) sterilized modified phytase screening broth (PSB) containing 15.0 g/L D-glucose, 3.0 g/L sodium phytate, 5.0 g/L NH_4NO_3, 0.5 g/L $MgSO_4·7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4·7H_2O$, 0.01 g/L $MnSO_4·4H_2O$ and adjusted to pH 5.5. Flasks were inoculated with the pre-prepared fungal spores at a density of 1×10^6 spore/g and then incubated at 30˚C for 5 days (120 hrs). In order to reach the optimal conditions for the production of the extracellular phytase from the isolate *A. tubingensis SKA*, the variables that affect the production/growth; carbon, nitrogen, phosphate and metal sources were studied. The physical conditions including pH, temperature, size of inoculum and duration of fermentations were also investigated. In all the conducted experiments, conditions of fermentation were retained as they mentioned above (growth in PSB) at 30˚C for 5 days, except for the studied factor, which varied, according to requirements of the experiment. All experiments were replicated 3 times and analyzed in triplicate. T-tests were performed to test for statistical significance (a = 0.05) using SAS 9.4.

2.4. Effect of Nutrients

Fermentation was carried out with different carbon sources including, sucrose, maltose, fructose, galactose, cornstarch and lactose at a concentration of 1.5%, replacing the glucose in the PSB. Fermentation was carried out with different nitrogen sources including, $(NH_4)_2SO_4$, (AmSu) $NaNO_3$, NH_4Cl, peptone (Pep), yeast extract (YE) and beef extract (BE) at concentrations of 0.5% replacing the NH_4NO_3, (AmNi) in the PSB. Fermentation was carried out with different phosphate sources including, KH_2PO_4, Na_2HPO_4 replacing the sodium phytate in the PSB. All these sources were used at concentrations of 0.3%, and a control sample with the absence of any source of phosphate was conducted. Fermentation was carried out with different concentrations of sodium phytate (0.05, 0.1, 0.3, 0.5 and 1.0 %). In order to study the effect of metal salts one of these salts ($MgSO_4·7H_2O$, KCl, $FeSO_4·7H_2O$ or $MnSO_4·4H_2O$) was eliminated at time while the others were used at the concentrations stated above in PSB. The control was all 4 salts as stated in the PSB.

2.5. Effect of Physical Conditions

Fermentation was carried out with different sizes of inoculum, (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 2×10^6) spore/g substrate with 1×10^6 as the control. Fermentation was carried out at different periods of incubation, (48, 72, 96,

120, 144, 168, and 192 h) with 120 hrs as the control. PSB was prepared at different pH values (2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0), using HCl or NaOH with pH 5.5 as the control. Fermentation was carried out at different temperatures, (25˚C, 30˚C, 35˚C, 40˚C, 45˚C) with 30˚C as the control.

2.6. Production of Phytase under Optimum Conditions

Phytase was produced under the optimum conditions: 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% sodium phytate, 0.5% $MgSO_4 \cdot 7H_2O$, 0.5% KCl, 0.1% $MnSO_4 \cdot 4H_2O$, with an incubation temperature of 30˚C, at pH 5.0, with an inoculum size of 1×10^5 spore/g substrate and an incubation time of 96 h (4 days). These conditions were compared with the fermentation conditions using PSB as described above for 120 hrs (5 days) at pH 5.5, at 30˚C with 1×10^6 spore/g substrate.

2.7. Phytase Assay

Crude enzyme (phytase) was collected at the end of incubation period by the addition of 50 ml acetate buffer (0.2 M, pH 5.5) to the samples in the 250 ml Erlenmeyer flasks. The flasks were shaken in a rotary shaker at 200 rpm for 1 h at room temperature. The mixture was separated from solid biomass by filtration through filter paper (Whatman No. 4), then the filtrate was centrifuged at 10,000 rpm for 10 min at 4˚C. The supernatant was used as the source of crude enzyme for phytase assay.

Phytase activity was assayed by determining by measuring the amount of liberated inorganic phosphate as described by Awad *et al.* [13]. The enzyme assay contained 0.9 ml of acetate buffer (0.2 M, pH 5.5), 1 mM phytate and 0.1 ml of the crude enzyme supernatant. After a 30 min incubation at 37˚C, 1 ml of 10% trichloroacetic acid was added to stop the reaction. The liberated inorganic phosphate was determined according to the method of Fiske and Subbarow [16]. One unit of phytase is defined as the amount of the enzyme that releases one µg inorganic phosphate per ml per min under the assay conditions.

3. Results and Discussion

3.1. Effect of Nutrients

A. tubingensis SKA was isolated as described by Qasim *et al.* [17]. Results of the phenotypical and morphological identification of the isolate, which was conducted at the Fungus Testing Laboratory, University of Texas- Health Science Center (San Ontonio, TX, USA) confirmed that this isolate belongs to genus and species of *Aspergillus tubingensis* (**Figure 1**) and labeled as *SKA*.

A series of experiments were conducted to examine the best nutrient sources for the production of phytase from *A. tubingensis SKA* under SSF. Wheat bran was chosen as a substrate for the production since it has a high nutritional value (a rich source of carbon, protein and other nutrients) and low cost. For such characteristics, wheat bran has aroused the attention of researchers over years as

Figure 1. Isolate *Aspergillus tubingensis SKA.*

an acceptable nutrient source for use in the production of microbial enzymes via SSF [18] [19] [20].

Optimum conditions for the production of phytase from *A. tubingensis SKA* were studied and for the effect of carbon sources, six carbon sources were added at concentrations of 1.5% to the fermentation medium (**Figure 2(a)**). Among the six carbon sources investigated, the highest phytase yield (42.00 unit/ml) from isolate *A. tubingensis SKA* was gained by addition of glucose to the fermentation medium which was significantly higher than the other 5 carbon sources. The use of sucrose resulted in 29.23 unit/ml which was significantly higher than the use of cornstarch, galactose, fructose and lactose. There were no significant differences in phytase yield with the use of cornstarch, galactose or fructose with lactose yielding significantly less phytase.

Glucose is considered a simple carbon source commonly utilized by microorganisms, leading to an increase in the fungal biomass with a high yield of phytase [14]. This observation comes in agreement with Awad *et al.* [13] whose findings showed that glucose was a favorable source of carbon for phytase production by *Penicillium purpurogenum*. Regarding the increase in phytase production when cornstarch is used in SSF medium, Roopesh *et al.* [21] obtained high yields of phytase from the fungus *Mucor racemosus*; such finding is related to the ability of some genera of fungi e.g. *Aspergillus, Mucor* and *Rhizopus sp.* to produce accessory enzymes as well as phytase in SSF. The accessory enzymes may have resulted in hydrolysis of starch, which improved phytase production via an increase in glucose and maltose which are simple carbohydrate nutrient sources.

For the optimum source of nitrogen, 0.5% of various sources of nitrogen were used in the fermentation medium (**Figure 2(b)**). The highest phytase yield (55.26 unit/ml) was achieved using $(NH_4)_2SO_4$ (ammonium sulfate). The use of NH_4NO_3 (ammonium nitrate) beef extract and $NaNO_3$ (sodium nitrate) resulted in the second highest phytase activity. The use of peptone and yeast extract

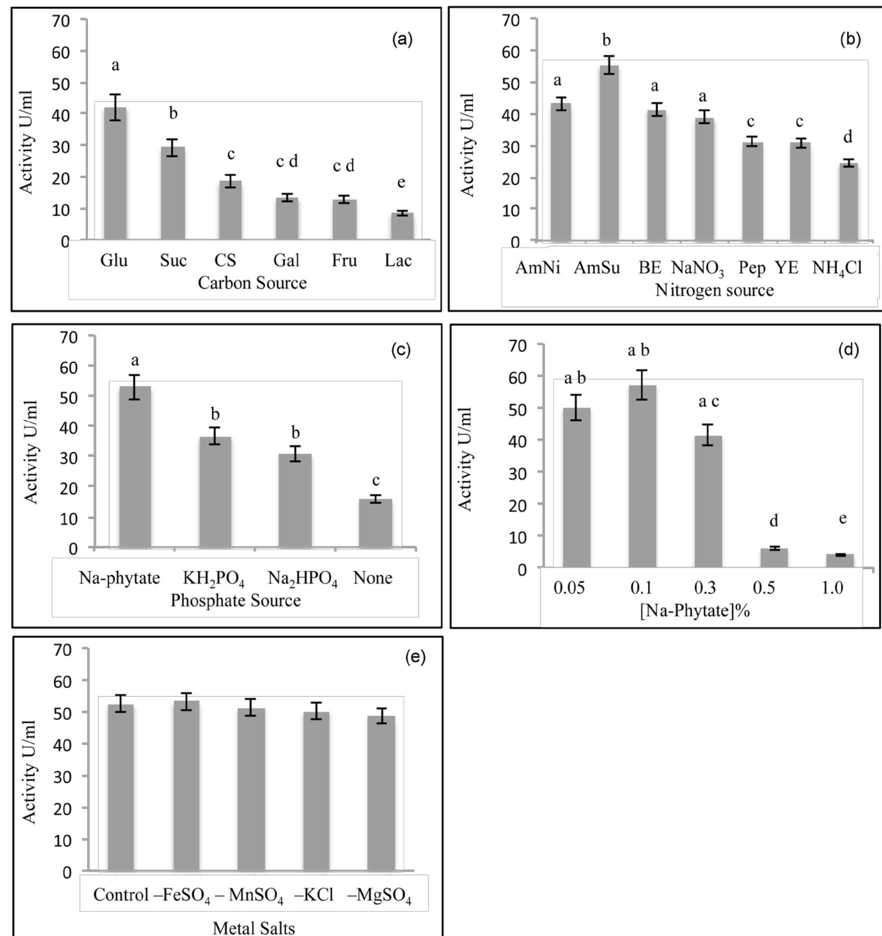

Figure 2. Effect of nutrients on the production of phytase from *Aspergillus tubingensis SKA*. (A) Carbon sources: glucose, sucrose, cornstarch, galactose, fructose, lactose; (b) Nitrogen sources: Ammonium nitrate (AmNi), Ammonium sulfate (AmSu), beef extract (BE), sodium nitrate ($NaNO_3$), peptone (Pep), yeast extract (YE), ammonium chloride (NH_4Cl); (c) Phosphate source: sodium phytate (Na-phytate), monopotassium phosphate (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), no phosphate added; (d) sodium phytate concentration; (e) Metal salts: Control (all metal salts), minus $FeSO_4$ (ferrous sulfate), minus $MnSO_4$ (manganese sulfate), minus potassium chloride (KCl), minus $MgSO_4$ (magnesium sulfate). All experiments were conducted in PSB, pH 55 at 30˚C for 5 days (120 hrs) with 1×10^6 spores/g. Error bars indicate standard deviation and values with no common letter are significantly different at $a = 0.05$.

yielded significantly higher phytase activity than the use of NH_4Cl (ammonium chloride). The phytase activity with $(NH_4)_2SO_4$ is similar to the findings of Santos [2] who found that the addition of 1.8% of $(NH_4)_2SO_4$ led to a 7% increase in the yield of phytase produced by *Aspergillus niger* under SSF. Kumari *et al.* [22] also found that $(NH_4)_2SO_4$ was the optimal source of nitrogen for the production of fungal phytase by SSF. Bala *et al.* [23] demonstrated that $(NH_4)_2SO_4$ was the best among various nitrogen sources in the production of phytase form the fungus *Humicola nigerescens* with SSF. A simple inorganic nitrogen source may be more convenient for the production of extracellular enzymes compared to organic sources, which have a role in stimulating growth. Organic nitrogen sources

in the fermentation medium may stimulate the production of proteases which have a negative effect on the overall yield of enzymes [13].

Fungi need a phosphorus source for their growth and production of metabolites. Inorganic sources include phosphate salts while an organic source is phytate. Among the various phosphate sources used in this study (**Figure 2(c)**), highest phytase activity (52.96 unit/ml) was gained by adding sodium phytate to the medium. There were no significant differences in phytase activity using either KH_2PO_4 or N_2HPO_4, while the lease phytase activity was observed with no phosphorus addition.

Phosphorus acts as a regulator for phytase production, therefore the type and concentration of the phosphate in the medium is one of the important factors in the production of microbial phytases [24]. In this regard, Lata *et al.* [12] found that calcium phytate was the best phosphate source for the production of phytase from *Aspergillus heteromorphus* MTCC 10685, and similar result were found by Das and Ghosh [14] but with the use of *Aspergillus niger* NCIM 612 for phytase production. While Selvamohan *et al.* [14] showed that the optimal source of phosphorus was tricalcium phosphate in the production of phytase from *Pseudomonas* sp., they added that inorganic phosphate is an essential mineral in phytase production medium, since it plays an important role in the regulation of the enzyme production. Since sodium phytate showed significantly higher phytase production than the other sources, we explored the use of different phytate concentrations on phytase activity (**Figure 2(d)**). Results showed that concentrations of 0.05 to 0.1% of sodium phytate resulted in significantly higher phytase activity compared with higher concentrations, which showed adverse effect on phytase activity.

The above results are similar to those of Lata *et al.* [12] who found that the maximum phytase activity produced from *Aspergillus heteromorphus* MTCC was achieved when 0.1% of calcium phytate was used, but sharp decreases in the activity were recorded at higher concentrations, especially that of 0.6%. Tungala *et al.* [26] found that high concentrations of potassium phosphate (KH_2PO_4) in a medium containing wheat bran caused a decrease in bacterial phytase activity. This may be referred to the phenomenon of phosphate repression expressed on phytase production, which was observed clearly in fungal phytase as well as phytase produced from yeasts [27] [28]. As stated by Selvamohan *et al.* [25], phosphates can act as both repressors and inducers of phytase production in different microorganisms. Here we showed that at phytate concentrations greater than 0.3%, phytate elicits a repressor activity in *A. tubingensis SKA*. Das and Gosh [14] observed a sharp decrease in phytase production from *Aspergillus niger* NCIM 612 associated with high concentrations of inorganic phosphates in growth medium. High concentrations of phosphate in fermentation medium may lead to the formation of non-reversible bonds with surface proteins of the microorganism and impair microbial growth [29].

For the effect of metal salts, four metal salts were added to the fermentation medium and given as the control in **Figure 2(e)**. For the treatments, only three

of the salts were included while the fourth one was eliminated from the medium. Results show that there were no significant differences in phytase activity among the treatments and control. Results reported by Soni *et al.* [28] found that presence of Fe^{2+} in the fermentation medium of *Aspergillus niger* NCIM 563 lead to a decreased phytase activity. In addition, Sreedevi and Reddy [30] found that the presence of Mg^{+2}, Mn^{+2} and K^{+1} in bacterial phytase production medium increased enzyme activity significantly. Minerals are generally included in microbial growth medium since they can be included in the composition of enzymes and aid in maintaining ionic balance. The activation or inhibition of phytase activity by metal ions is attributed to their effect on the growth of fungi itself [30] [31].

3.2. Effects of Physical Conditions

Physical parameters for the production of phytase were investigated. For the effect of inoculum size (**Figure 3(a)**), there was significantly higher phytase production with 1×10^5 and 1×10^6 spores/g. Significantly lower phytase activity was observed with an inoculum of 2×10^6 spores/g with the lowest phytase activity observed with 1×10^2 spores/g. There was a gradual increase in phytase activity with an increase in the inoculum size until 1×10^5. Increasing the size of inoculum greater than 1×10^6 caused an obvious decrease in phytase activity. This is similar to other studies that reported a 1×10^5 spores/g inoculum was the optimum for the production of phytase from *Aspergillus niveus* and thermophilic fungi [18] [32]. The interpretation of these results is due the inhibitory effect of higher levels of inoculum; where at a higher level of inoculum, enzyme

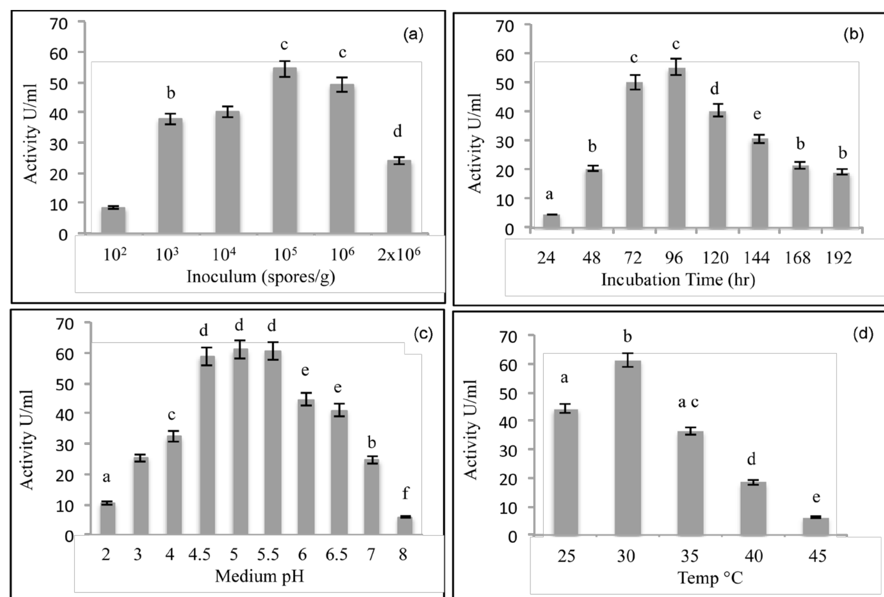

Figure 3. Effect of physical conditions on phytase from *Aspergillus tubingensis SKA*. A. Inoculum size. B. Fermentation time. C. Medium pH. D. Fermentation temperature. All experiments were conducted in PSB, pH 55 at 30˚C for 5 days (120 hrs) with 1×10^6 spores/g unless denoted on the x-axis. Error bars indicate standard deviation and values with no common letter are significantly different at a = 0.05.

production is reduced due to the competition among the fungal population for the nutrients in the growth medium, such as carbon and nitrogen sources, which lead to exhaustion of nutrients resulting in a decrease in enzyme activity. On the other hand, low levels of the inoculum resulted in low phytase activity due to the small number of fungal cells [33].

In order to determine the optimum incubation period for the maximal phytase production by SSF, isolate *A. tubingensis SKA* was incubated at various times (**Figure 3(b)**). Results showed that times of 3 and 4 days (72 and 96 hrs) resulted in significantly high phytase activity. Phytase activity was initially detected at 24 h and progressively increased with incubation time until 4 days. However, phytase production dropped significantly at 5 days and longer. Such observations are similar to other studies, which reported that the optimal period for the production of phytase from *Mucor racemosus* [24], *Rhizopus oryzae* [34], *Aspergillus sp.* [35], *Aspergillus heteromorphus* MTCC 10685 [12] and *Aspergillus oryzae* [36], was after incubation for 96 h. The pyhtase activity was decreased on prolonged cultivation which could be due to the reduction in the nutrient level of medium after 120 h of incubation, or may be related to the autolysis of fungal mycelium which is accompanied by the release of intracellular enzymes that have a dramatic impact in reducing phytase production [2].

The effect of different pH values of the SSF on phytase activity is shown (**Figure 3(c)**). Significantly high values of phytase activity were obtained in SSF within a pH range of 4.5 - 5.5. The maximum activity 60.89 unit/ml was reached at a pH of 5.0, while the minimum production of phytase, 5.81 unit/ml, was at pH 8.0. Earlier related studies reported that pH values ranging from 4.5 - 6.0 were optimal for production of phytase by filamentous fungi [37] [38]. Singh [36] found that the optimal pH of fermentation medium for *Aspergillus oryzae* was at pH 5.0 for phytase activity. While Hassouni *et al.* [39] reported that the optimum pH for phytase activity from the fungus *Myceliophthora thermophilia* in SSF was 5.5. The results of this study showed that the production of phytase was achieved at acidic conditions, and this is consistent with the fact that acidic environment allows germination of fungi spores, in general, and even promotes their growth [40]. The pH has a direct impact on the activity of extracellular enzymes and metabolism of the microorganisms. It affects the ionization of the materials involved the growth medium of the microorganism and influence enzyme production [41].

A specific trend can be seen for the influence of incubation temperature on phytase activity (**Figure 3(d)**). Incubation of fungal isolate *A. tubingensis SKA* at 30˚C resulted in significantly higher phytase activity (61.31 unit/ml) than at lower or higher temperatures. Lata *et al.* [12] found that the optimal temperature for phytase production from *Aspergillus heteromorphus* MTCC 10685 was 30˚C, higher temperatures reduced the enzyme production, and the maximum reduction occurred at 40˚C. Shivanna and Venkateswaran [42] also found that maximum production of phytase by *Aspergillus ficuum* SGA 01 was at 30˚C. Each type of fungi has an optimal temperature for growth, which varies. For

example, the optimal growth for most species belonging to the genus *Aspergillus* is at 30˚C, for that, this genus is widely distributed in moderate environments. Furthermore, enzymes are produced during the exponential growth phase which is why temperatures outside the optimal can lead to deficient growth and consequently reduced enzyme production [34].

We then compared phytase activity from *A. tubingensis SKA* under optimal conditions. The changes to the PSB buffer were the use of ammonium sulfate in place of ammonium nitrate and the use of sodium phytate at 0.3%. The physical parameter changes included using 1×10^5 spores/gram substrate and an incubation time of 4 days (96 hrs). Overall, a 34% improvement in phytase activity was achieved by using the optimal conditions, with an enzymatic activity value that reached 60.42 unit/ml, compared to 45.02 unit/ml activity under original conditions.

Optimizing the conditions which affected phytase production has been the goal of many studies. Rani *et al.* [43] found that optimizing parameters affecting phytase production from *Rhizopus oryzae* resulted in increasing enzymatic activity of about 7.95 fold compared to enzymatic activity prior optimization. Thyagarajan *et al.* [44] was able to increase phytase activity produced from the fungus *Hypocrea lixii* SURT01 to 17.5-fold compared to phytase activity under non optimized production.

The ultimate aim for optimization is to create an idealistic condition for the production of an enzyme by using straightforward procedures and cost-effective materials that would be feasible to implement on a large scale. To improve any bioprocess it is very important to optimize all the parameters that affect the production of any bio products. Here we showed that we could increase the phytase activity from *A. tubingensis SKA* by 34% by determining the optimum nutrient and physical conditions.

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