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Der Pharmacia Lettre, 2015, 7 (12):148-153
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Production and optimization of phytase by *Aspergillus niger*

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ABSTRACT

The present article implies an extracellular phytase production by Aspergillus niger under submerged and solid fermentation. Physical and Chemical conditions tested for optimal production of phytase using the single variable mode optimization technique. A considerable higher phytase production was obtained using rice bran as solid substrate (17.8 U/ml & 28.6 U/ml) at pH 5, on 4th day of incubation at 30^{0}C. It has been inferred from the results that glucose as best carbon source for phytase production with 29.5 U/ml (SMF) and 42.4U/ml (SSF) activity and ammonium nitrate as good nitrogen source enhanced production of phytase from Aspergillus niger upto 31.8 U/ml in SMF and 45.2 U/ml in SSF.

Keywords: *Aspergillus niger*, Submerged and solid state fermentations, phytase optimization.

INTRODUCTION

Phosphorus is one of the essential micronutrients for the growth and development of living organisms since it plays a vital role in virtually every plant process that involves energy transfer. Most of the organic phosphorous is in the form of phytic acid (myo-inositol 1,2,3,4,5, 6-hexakisphosphate) which has a strong binding affinity to important minerals, such as calcium, magnesium [1], iron [2] and zinc [3], resulting in the formation of insoluble precipitates, which are in turn non-absorbable in the intestine of monogastric animals and also causes environmental pollution. Phytase is an enzyme that free the inorganic phosphorous from the phytate complex. It addresses both the anti-nutritional and eutrophication problem and hence finds an important role in the feed industry [4].This results in an increased availability of minerals, trace elements and amino acids as well as phosphate. Phytases have wide application, but commercial usage is hindered due to high production cost attributed to a high substrate cost and downstream processes [5]. The high production cost can be overcome with the usage of cheap substrates like agricultural residues. Phytase production using solid state fermentation with fungi would yield a high phytase titer and ease downstream processes. The culture conditions, type of strain, nature of substrate and availability of nutrients have to be taken into consideration for selecting a particular production technique, as they are critical factors affecting the yield. The metabolic differences between SSF and SMF have a direct impact on the productivity of the fungus. Various researches have shown the effect of range of parameters; including the fermentation technique adopted, culture conditions, different substrates, and media nutrients on the production of phytase [6, 7, 8, 9].

Several enzymes of industrial importance have been extracted from the fungi belonging to the genus *Aspergillus*. The importance of this genus is so much, that it has been studied as a model organism for fungal enzyme production [10]. *A.niger* is by far the single largest fungal source of enzymes. In view of industrial importance of phytase, the

present work was carried out to optimize the culture conditions for enhancing phytase production by the locally isolated fungus *Aspergillus niger*. In a preliminary study, this fungal isolate demonstrated the capacity for producing significant phytase activity.

MATERIALS AND METHODS

Optimization of culture conditions:
Various physical and nutritional parameters were optimized by changing one parameter at a time to get maximal production of phytase from *Aspergillus niger* in submerged and solid state fermentation. All the media ingradients and reagent chemicals were of analytical grade procured from E-Merck and Hi-Media, India.

Isolation and screening of phytase producing fungi
A phytase producing fungal strain was isolated from rhizosphere soil using serial dilution technique. The culture was screened for phytase enzyme production on phytase screening medium containing 0.5% sodium phytate as a substrate, further the potent fungal culture was identified as *Aspergillus niger* , based on molecular characterization of 18s RNA sequence [11]. The *A.niger* was inoculated on PDA agar slants and incubated for 3-4 days active culture.

Fungal inoculam preparation for phytase production
The *Aspergillus niger* inoculam was prepared by adding 1ml of inoculums containing $2x10^6$ spores to 100ml of medium in a 250ml flask.

Solid state fermentation:
A.niger was grown on solid medium was used for SSF. For this, 5gram of finely ground substrate in 250ml Erlenmeyer flask supplemented with 5ml of nutrient solution (0.2%glucose, 1%trptone, 0.5%Nacl, 0.1% Kcl) and sterilized at 121^0C for 20 minutes. The moisture content was adjusted by adding of sterile water prior to inoculation to 60%. One ml of inoculum containing 2×10^6 spores was used as inocula for all solid state fermentations. The flasks were kept in a rotatory shaker for 20 minutes at 200 rpm. The solids were separated by filtering through clean muslin cloth. Then it was centrifuged at 8944g for 10-15 minutes and assayed for phytase activity [12].

Submerged fermentation:
Submerged fermentation was carried out in 250ml Erlenmeyer flask using 100 ml potato dextrose broth containing substrate sodium phytate (0.5%) as the cultivation medium and autoclaved at 121^0C for 20 minutes. After cooling, various chemical components were added separately and then 1ml of inoculum containing 2×10^6 spores was added and incubated in an orbital shaker at 200 rpm and 30^0C for 7 days. After incubation the medium was filtered through whatmann No.1 filter paper, centrifuged at 8944g for 10-15 minutes and assayed for phytase activity [12].

Effect of agro-residued substrates on phytase production
Various agro-residual substrates, wheat bran (WB), rice bran (RB), corn cobs (CB), Mango seeds (MS) citrus peel (CP), groundnut oil cake (GOC) and sugarcane bagasse (SCB) were obtained from a local market and used as solid substrates for phytase production. The residues were prepared by exhaustive washing with distilled water, dried at 80ºC for 24-48h and milled (35 mesh). All these substrates were used in SSF and SMF for phytase production by *Aspergillus niger*. The best solid substrate was selected phytase production in subsequent studies.

Optimal conditions:
Effect of temperature:
Effect of temperature on phytase production in *A. niger* was studied. The fungus was incubated at temperatures varying from 25 to 45^0C with 5^0C interval. The optimum temperature of the solid substrate was studied.

Optimization of pH: The effect of medium pH on phytase production from fungal strain was studied by adjusting the media pH between 3 and 7.

Optimization of incubation time on phytase production
The effect of fermentation time on phytase production was studied by incubating *A.niger* in submerged and solid state cultivation medium for 7 days and periodically testing the enzyme activity.

Effect of carbon and nitrogen sources

Various carbon sources were supplemented to the medium for phytase production with glucose, fructose, maltose, sucrose, galactose, as carbon sources (1% W/W), and peptone, Yeast extract, ammonium sulfate, ammonium chloride, ammonium nitrate, and sodium nitrate used as nitrogen sources (0.5% w/w).

Phytase activity assay

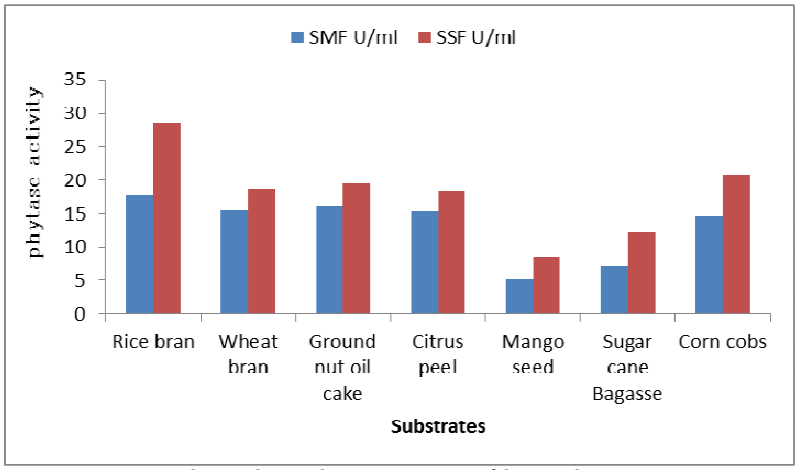
The phytase activity of fungal culture in filtrate was assayed. The enzyme activity was determined by release of phosphorous from sodium phytate substrate by the method Engelen *et al* [13]. Phytase activity was measured as the amount of enzyme required that liberated 1 µmol phosphorous per minute under the reaction conditions (37 °C, 10 min, and pH 5.5). The phytase activity was expressed as Units per milliliter (U/ml) in submerged fermentation as well as solid state fermentation.

RESULTS AND DISCUSSION

Effect of substrates

The potent phytase producing fungal strain *Aspergillus niger* used in this study was isolated from agricultural soils [11], and used for optimal production of phytase on medium supplemented with suitable physical parameters and chemical nutrients. In this study, various agro-residues, rice bran (RB), wheat bran (WB), groundnut oil cake (GOC), citrus peel (CP), mango seeds (MS) and sugar cane bagasse (SB) were used in the growth medium for phytase production by *Aspergillus niger*. All substrates supported microbial growth and phytase production (Fig.1). Among those, rice showed higher phytase yield (28.6U/ml in SSF &17.8 U/ml in SMF) followed by ground nut oil cake with 20.9 U/ml in SSF &16.2U/ml in SMF (table 1) than the other substrates..Similarly, Wien Kusharyoto [14] reported rice bran as the best substrate for phytase production by *Aspergillus ficcum*.Similarly , Subramaniyam Suresh *et al* [15] Gunashree and Venkateswaran [12] also reported rice bran as good substrates for phytase production by *Rhizopus oligosporus*, *Aspergillus ficcum* and *Aspergillus* niger. Based on this the rice bran has been used as an alternative substrate for phytase production in subsequent studies.

Fig: 1 Effect of agro-residues on phytase production by *A.niger*



Values in the graph represents mean of three replicates.

Effect of Temperature

The effect of incubation temperature on phytase production was studied in the temperature range of 25^0-45^0C under submerged and solid state fermentation (Table.1). The optimum temperature for growth and phytase production was found to be 30^0C (Fig 3). Further rise in temperature, decreased the production of phytase. The fermentation temperature for optimum production of phytase is mostly reported as 30^0C by many researchers. For instance Tahir *et al* [16] reported maximum phyatse production from *Aspergillus niger* at 30^0C. Similarly, Suman latha *et al* [17] found that *Aspergillus heteromorphus* secreted maximum phytase at 30^0C. Similar reports made Soni *et al* [18] and Vats and Benerjee *et al* [19].

Table 1: Effect of temperature on phytase production

Temperature ^{0}C	SMF U/ml	SSF U/ml
20	12.2	24.6
25	15.5	31.9
30	23.8	34.5
35	18.6	30.7
40	14.7	22.5
45	10.9	18.4

Each value represents mean of three replicates.

Effect of pH

The phytase production from *Aspergillus niger* was increased from pH 3 to 5 in both submerged and solid state fermentations. Further change in pH declined the enzyme production drastically (Table.2). Similar results were reported by Singh and Sathyanarayana [20] who stated that production of phytase from *Sporotrichum thermophile* was also maximum at pH 5. Phytase production was mostly reported that acidic to neutral pH range. *Aspergillus niger* strain 89 produced phytase optimally at pH 3.0 was reported by Volfova [21] while Sano et al [22] found that *Arxula adeninivorans* showed maximum activity at pH 5.5. *Aspergillus. oryzae* produced highest phytase titers at pH 6.5 [23,24].

Table 2: Effect of pH on phytase on SMF and SSF by *A.niger*

pH	SMF U/ml	SSF U/ml
3	2.8	12.6
4	14.5	18.8
5	21.9	32.4
6	18.6	30
7	17.8	28.6

Each value represents mean of three replicates.

Effect of incubation time

The effect of incubation time on phytase production by *A.niger* was studied (Table.3). Maximum growth as well as phytase production was measured at 4th day of incubation (25.6 U/ml in SMF & 38.5U/ml in SSF). Thereafter, the enzyme productivity slightly decreased. Shimizu [18] observed maximum phytase activity (0.4 U/ml) at 4-5 days of fermentation by *Aspergillus oryzae* K1. Singh and Satyanarayana found maximum phytase production at 5 days of incubation by thermophilic mould *Sporotrichum thermophile* BJTLR50 (20). However, Howson and Davis [25] reported highest extracellular phytase activity from *Aspergillus ficuum* NRRL 3135 after 10 days of incubation**.** Similarly, maximum phytase activity of 68 U/ml by *Aspergillus niger* NCIM 563 on the 11th day of fermentation was reported by Bhavsar [26].

Table 3: Effect of incubation time on phytase production

Number of days	SMF U/ml	SSF U/ml
1	10.8	12.6
2	14.5	26.5
3	20.2	33.3
4	25.6	38.5
5	22.7	30.2
6	18.4	24.8
7	12.9	16.5

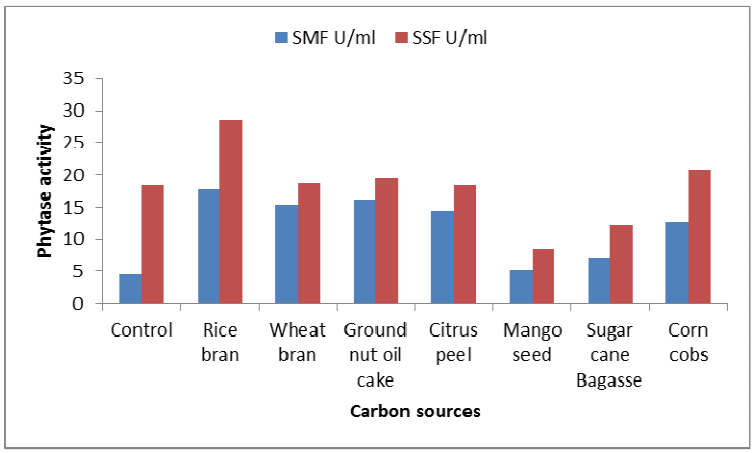
Each value represents mean of three replicates.

Effect of carbon sources

The effect of carbon on phytase production was studied and results were shown in Fig (2). Among the five carbon sources tested in this study, glucose showed considerable higher titre of phytase (29.5 U/ml & 42.4 U/ml) when compared to fructose, sucrose, galactose and maltose used in this study. Das and Ghosh 2014 [27] also studied the effect of various carbon sources, Among the carbon sources tested, glucose supported highest phytase production from *Aspergillus niger* NCIM 612 as compared to other carbon sources. Suman latha *et al* [17] found glucose as the

best carbon source for phytase production from *Aspergillus heteromorphus* MTCC 10685. Similar results were supported by some other researchers [16,21].

Fig 2: Effect of carbon sources on phytase production by *A.niger*



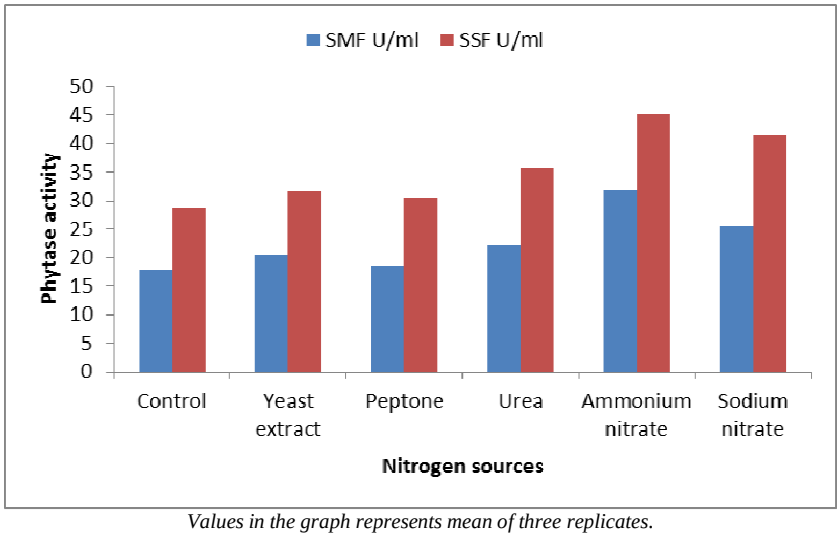
Values in the graph represents mean of three replicates.

Effect of nitrogen sources

The effect of nitrogen source on phytase was studied by supplementing the production medium with various inorganic and organic nitrogen sources (Fig 3). Among different nitrogen sources studied, ammonium nitrate, exhibited maximum phytase activity (31.8U/ml & 45.2U/ml) than control (17.8 U/ml & 28.6 U/ml). Similarly, phytase production from *Bacillus* sp. DS11, *Aspergillus niger* St-6 and *Aspergillus niger* van Teighem was found maximum with ammonium nitrate as nitrogen source [16,19,23].

Compared to the liquid medium higher titre of phytase was exerted in solid medium. For instance, the phytase activity at pH 5 in liquid medium was 17.8 U/ml where as in solid medium 28.6 U/ml. Nearly 1.6% increase in phytase activity in SSF was observed when compared to SMF. The same trend of phytase activity was observed even with chemical parameters. For instance, the phytase activity in SMF was 29.5 U/ml and 42.4U/ml in SSF when glucose was used as carbon source.

Fig 3: Effect of various nitrogen sources on phytase production by *A.niger*



Values in the graph represents mean of three replicates.

CONCLUSION

In this study optimization of phytase production was carried out by potent fungal strain *Aspergillus niger* through submerged and solid state fermentation. The present study reports production of phytase by utilization of less expensive rice bran as substrate. Enrichment of rice bran with glucose, ammonium nitrate as carbon and nitrogen sources at pH 5.0, temperature 30^0C resulted in better yield of enzyme. Compare to submerged fermentation, solid state fermention proved a good method for enhanced production of phytase by *A.niger*. The results indicate that the *A.niger* could be a potential candidate for phytase production and hence can be used as feed additive. Phytase supplementation to animal feed is proved as an effective way to increase the digestion in mono gastric animals and to reduce manure-born phosphorous pollution.

Acknowledgements
The author Sandhya is gratefully acknowledges the University Grants Commission (UGC), India for the financial support to carry out this work under UGC-PDF project.

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