SCREENING OF PHYTASE PRODUCING FUNGI ISOLATED FROM SYRIAN SOIL

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ABSTRACT

Due to the importance of phytase enzyme industrial applications,in this study, 301 local fungal isolates have been screened and evaluated for their phytase production. New fungal isolates have been selected for phytase production. The best phytase producing fungi isolates were FSS223 and FSS296. The identification for the two selected isolates have done on the basis of analyses of 5,8S gene sequencing. The closest phylogenetic neighbors according to 5,8S gene sequence data for the two isolates were *Aspergillus tubingensis* and *Aspergillus niger* with 99% homology, respectively. The results obtained for the two isolatesundersolid state fermentation showed that better carbon sources for phytase production were wheat bran and soya bean powder, the incubation times were 5th and 7th day, the initial moisture66.6 and 75%, and initial pH, 7 and 5, respectively. The experiments showed that best phytase activity was at 65°C and pH 2.5 for *A. tubingensis*, and at 60°C and pH 2.5 for *A.niger*.

Key words: Fungi, Phytase, Solid state fermentation, 5,8S gene.

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INTRODUCTION

The major portion of animal feed cereal bran, corn meal, oil seed cakes and soybean meal etc. contain (0.83-9.15%) of phosphorus as the phytate P. Phytic acid is considered anti-nutritional, due to its ability to chelate with divalent cations such as calcium, magnesium, iron and zinc. It forms under gastro-intestinal pH insoluble phytate metal complexes and inhibits the action of amylase, cellulose and phosphatase and reduces the protein, starch and lipid digestibility Vohra *et al.,* (2011).The unabsorbed inorganic phosphorus in monogastric derived faeces in are as of intensive animal production, have the potential to cause phosphorus pollution, leading to eutrophication Ullah *et al.,* (2003).

Phytase (myo-inositol-hexa kis phosphatephos phohydrolase EC 3.1.3.8) are the enzymes that can cleave phosphor-mono ester bonds in phytic acid, liberating sequentially a lower phosphate esters of myo-inositol and ortho-phosphates Mullaney & Ullah (2003).

Phytase enzyme can be found in animals, plants, bacteria, yeast and filamentous fungi. However, among microorganisms, phytase activity has been frequently detected in fungi, particularly in *Aspergillus* species Jafari-Tapeh *et al.,* (2012); Akturk *et al.,* (2013); Bhavsar *et al.,* (2013).

The use of filamentous fungi for the phytase production through under solid state fermentation (SSF) using agro-industrial wastes has gained many interests for research in the last years Pandey *et al.,* (2001). SSF system has generated much interest because it offers several economical and practical advantages including high products concentration, improved products recovery, simple cultivation equipment's and lower plant operational costs.

This study will be describes the screening of local fungal isolates for phytase production, and optimal condition to produce phytase enzyme by selected isolates.

MATERIALS AND METHODS

Screening of phytase isolated fungus

Three hundred and one pure fungi isolates culture were evaluated for phytase enzyme production on modified phytase screening medium containing (g/L):15 glucose, 1 Na-phytate, 2 NH4NO3, 0.5 KCl, 0.5 MgSO4.7H2O, 0.3 MnSO4, 0.3 FeSO4.7H2O,15 agar and pH was adjusted to 7.5 Mittal *et al.,* (2011). The isolates with higher clear zone diameter were selected for next experiments.

Innoculum preparation

Stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C. 7 days old spores were harvested using sterile saline solution containing: (3 g/LPepton, 5 g/L NaCl, 1mL/LTriton x100). Inoculum suspension adjusted at10^7 spore/mL.

Solid state fermentation and extraction of crude enzyme

Medium for SSF was prepared in 250 ml Erlenmeyer flasks using 5 g substrate with 10

ml of nutrient solution containing (g/L): 15 glucose, 5 $(NH4)_2SO_4$, 0.5 KCl, 0.1 NaCl, 2.5 $MgSO_4$.7H2O, 0.1 $CaCL_2$.2H2O, 0.01 $FeSO_4$.7H2O 0.01 $MnSO_4$. Different agro-industrial by-products including wheat bran, wheat straw, tomato pomace, olive pomace, corn cobs hulls, pea peel, orange peel, cotton seed cake, beet pomace, soya bean powder were tested for the phytase production by the selected isolates. pH was adjusted at 6.5 before sterilization. Flasks were sterilized at 121°C for 15 min, and then inoculated with 1 mL spore suspension and incubated for 5 day in 30°C. Crude enzyme extraction was carried out by addition 30 ml distilled water containing 1% Triton x100. The flasks were put on rotary shaker at 150 rpm in room temperature for 90 min. the flasks contents were centrifuged at 10000 rpm for 10 min and the supernatant used as enzyme source.

Polymerase Chain Reaction (PCR) Amplification and 5.8S **rDNA Sequencing**

The primers used for PCR and DNA sequencing are forward primer ITS1 (5'-TCC GTA GGT GAA CCTGCG G-3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TATGC-3') according to Bakri *et al.,* (2010).The amplification products were purified using Microcon Y-100 filters (Millipore) and sequenced using ABI Prism ® Big Dye ® terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacture instructions.DNA sequencing on both strands was directly performed on an ABI 310 sequencer machine (Department of Molecular Biology and Biotechnology, AECS) and subjected to aBLAST search against the full EMBL / GenBank database available at NCBI public database (http://www.ncbi.nlm.-nih.gov).

Phytase assay

Phytase enzyme activity was determined as described by Heinonen *et al.,* (1981), by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of 0.5 ml of glycin-HCl buffer (0.2 M, pH 2.5) containing 44.1mM phytic acid, 0.25 ml of the enzyme solution and 0.25 ml of distilled water. After incubation for 30 min at 37°C, the reaction was stopped by adding a freshly prepared solution of acetone: 5N H2SO4: 5% ammonium molybdate (2:1:1 v/v/v). One unit (U) of phytase is defined as the amount of enzyme releasing 1 µmol of inorganic phosphorus per ml per minute under the described assay conditions.

RESULT AND DISCUSSION

Screening of fungi isolates for phytase activity

Screening is often the first step for select the microorganism which has the required characteristics for industrial applications Maciel *et al.,* (2013). Three hundred and one isolates were tested on agar phytase screening medium. The best 10 fungal isolates which have clearing zone more than 29 mm were selectedand cultivated in submerged and solid state culture and the results shown in table 1. The table 1 demonstrates that the FSS223 and FSS296 isolates were the best for phytase production in solid fermentation and were selected for the following experiments.

Identification of the phytase producing fungal isolates FSS223 and FSS296

DNA sequence analysis methods are rapid means of identification, therefore, many researchers have been widely used, Bakri *et al.*, (2010). Identification of the FSS223 and FSS296 isolates was done using 5,8S DNA gene sequences.The phylogenetic analysis is based on the sequence gene 5,8S DNA was carried out by alignment of sequences consensus of genes 5,8S DNA collected in the Gene Bank database. The nucleotide BLAST similarity search analysis, revealedthat FSS223 and FSS296 were closely related to *Aspergillus tubingensis* and *Aspergillus niger*, respectively, with 99% of homology.

Effect of agro-industrial by-products on phytase production

The production cost of phytase is one of the major obstructions in wide spreading its application. The utilization of low cost agro-residual waste as a substrate was targeted to obtain high enzyme titre by many researchers, kumar & Sushma (2012) and Sandhya *et al.,* (2015). The production of phytase by FSS223 and FSS296 on different agro-industrial substrates was assayed to select a suitable carbon source. All the substrates used demonstrated phytase activity in varying levels as shown in figure 1 and 2. For FSS223 isolate, maximum phytase production was achieved with wheat bran, followed by soya bean powder. While,for FSS296 isolate, the highest phytase production record was with soya bean powder followed by pea peel. Javed *et al.,* (2010) and kumar & Sushma (2012) obtained the best phytase production from *Sporotrichum thermophile* and *Aspergillus niger* with wheat bran as carbon source compared to other carbon sources tested, respectively. On other hand, Sandhya *et al.,* (2015) obtained the best phytase production from *Aspergillus niger* using rice bran.

Effect of incubation time on phytase production from FSS223 and FSS296 isolates

The time for maximum phytase production by FSS223 and FSS296 was investigated (Figure 3). Phytase production by FSS223 isolate using wheat bran as carbon source reached at maximumafter 120 h of incubation. While, the phytase production by FSS296 using soya been powder reach to maximum at 168 hours of incubation. The phytase activity was decreased on prolonged cultivation time which could be due to the reduction in the nutrient level of medium or may be related to the autolysis of fungal mycelium according to Santos *et al.,* (2004). Sandhya *et al.,* (2015) and Qasim *et al.*, (2017) reported that highest phytase production by *Aspergillus niger* and *Aspergillus tubingensis* were after 4 daysusing rice bran and wheat bran, respectively.

Effect of initial moisture on phytase production

To achieve the best conditions to use in SSF for phytase production, the effect of initial moisture on phytase production by FSS223 and FSS296 was investigated The effect was studied by changing the substrate to mineral solution ratios. It was taken into consideration that the soluble medium ingredients concentration was not

changed. The results obtained in figure 4 showing the best phytase production by FSS223 was at 66.6% initial moisture. The best initial moisture for phytase production byFSS296 was at 75% as shown in figure 4. A moisture level is a critical factor affecting on growth of microorganism and their product biosynthesis, Gaind & Singh (2015).At higher moisture contentthan optimum might resulted in decreased porosity, lowered oxygen transferredand enhanced aerial mycelium formation. However, at lower moisture level than optimum causes higher water tension and reduced nutrients solubility, Ramchandran *et al.,* (2007). Gaind & Singh (2015) found that thebest initial moisture content at 58.0%for phytase production by *Aspergillus flavus* ITCC6720 using mustard cake after 4 days of incubation. Tian & Yuan (2016) observed the highest enzyme production by *Aspergillus ficum* ATCC66876 using potato wastes was at 77% initial moisture content.

Effect of initial medium pH on phytase production

The production of phytase by FSS223 and FSS296was studied at different pH values ranging from 4.0 to 10.0 (Figure5). The results showed that the best initial medium pH for phytase productions by FSS223 and for FSS296 were at 7 and 5 respectively. The pH of the growth medium has a direct impact oninducing enzyme production and metabolism changes in the microbes, Moreira *et al.,* (2014). Muniz-Marquez *et al.,* (2016) reported that the *Aspergillus* sp. showed the highest metabolic activities when the pH was around 5. Sapna & Singh (2014) and Qasim *et al.,* (2017) obtained the best phytase production by *Aspergillus oryzae* and *A. tubingensis* SKA using wheat bran at pH 5 after4 days of incubation.While Hassouni *et al.,* (2006) reported that the optimum pH for phytase production by *Myceliophthorathermophilia* in SSF was at 5.5 using sugar cane bagasse. Different microorganisms have different pH optima and increaseor decrease in pH value results in poorof microbial growth and reduction in enzyme production.

Effect of temperature on activity of phytase produced by FSS223 and FSS296 isolates

The effect of temperature on phytase activity of the crude enzyme showed that the optimal activities of phytase produced from FSS223 and FSS296 were at 65 and 60°C, respectively as shown in figures 6 and 7. Similar results were reported by Moreira *et al.,* (2014) and Casey and Walsh (2003), the optimum temperatures were at60 and 65°C for maximum activity of phytase produced by *Aspergillus japonicus* Saito URM 5633 and *Aspergillus niger* ATCC 9142, respectively. In other studies, the temperatures for optimal activity of phytase produced from *Aspergillus* species were in the range of 45–55°C, Vohar *et al.,* (2003), Vats and Banerjee (2004), Singh & Satyanarayana (2011), Singh *et al.,* (2011), singh & Satyanarayana (2014).

Table 1. Screening of fungi isolate for phytase activity

Fungal Isolates	Phytase clear zone (mm)	Submerged fermentation Phytase (IU/mL)	Solid fermentation Phytase (IU/g)
FSS28	29	0.41	5.44
FSS36	32	0.65	2.48
FSS50	30	0.44	2.08
FSS66	34	0.59	1.92
FSS69	32	0	2.32
FSS110	36	0.21	1.68
FSS114	77	0.29	9.12
FSS155	29	0.57	1.68
FSS223	81	0.54	20.56
FSS296	79	0.26	19.2

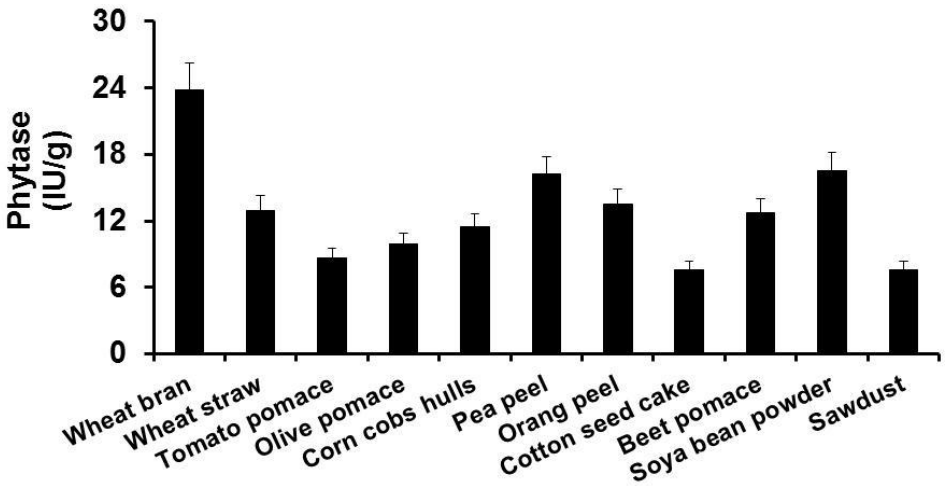

Figure 1. Phytase production form FSS223 using agro-industrial by-product

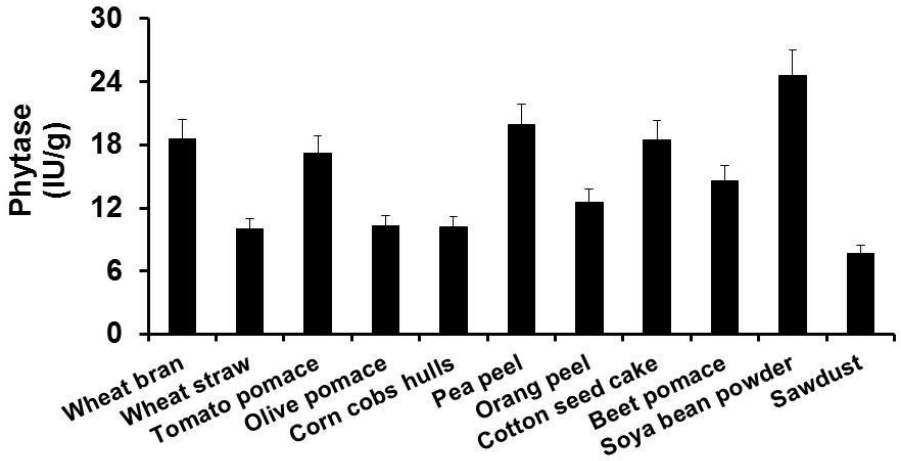

Figure 2. Phytase production form FSS296 using agro-industrial by-product

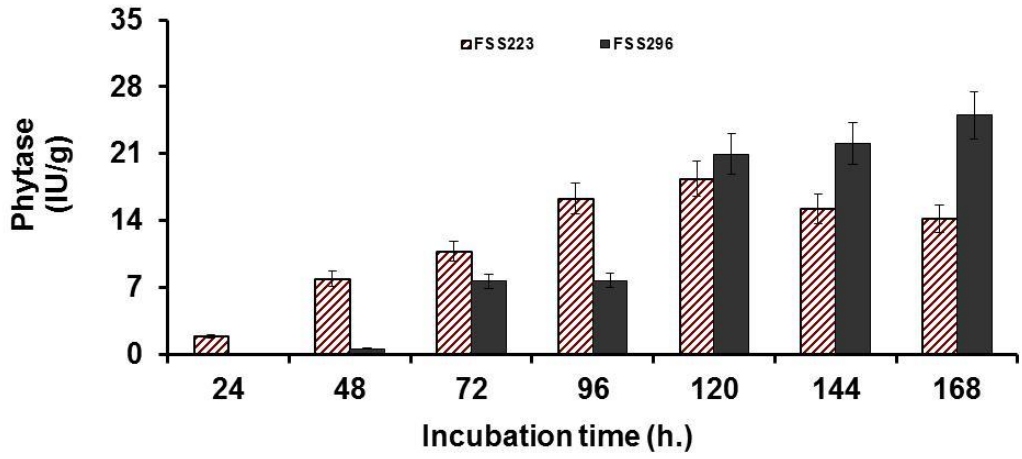

Figure 3. Effect of incubation time on phytase production by FFSS223 and FSS296 isolates

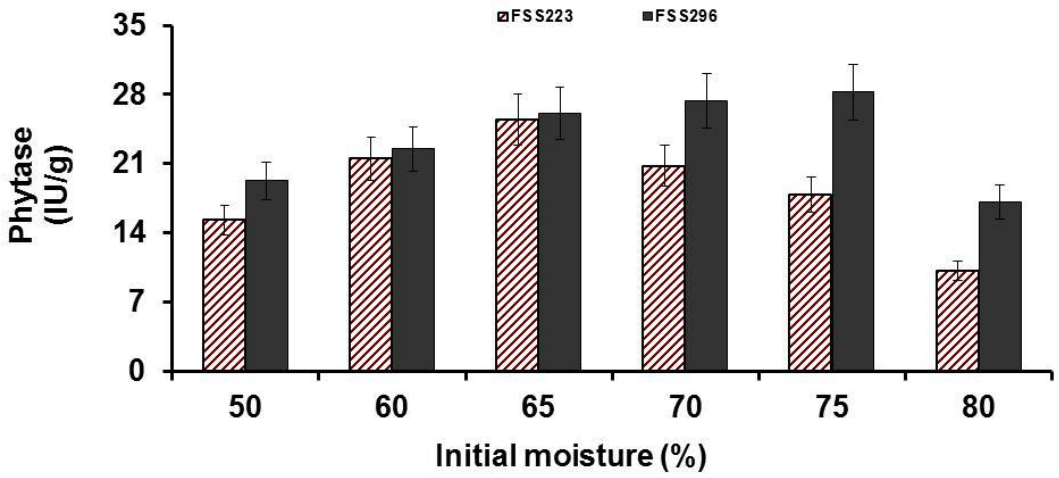

Figure 4. Effect of initial moisture on phytase production by FFSS223 and FSS296 isolates

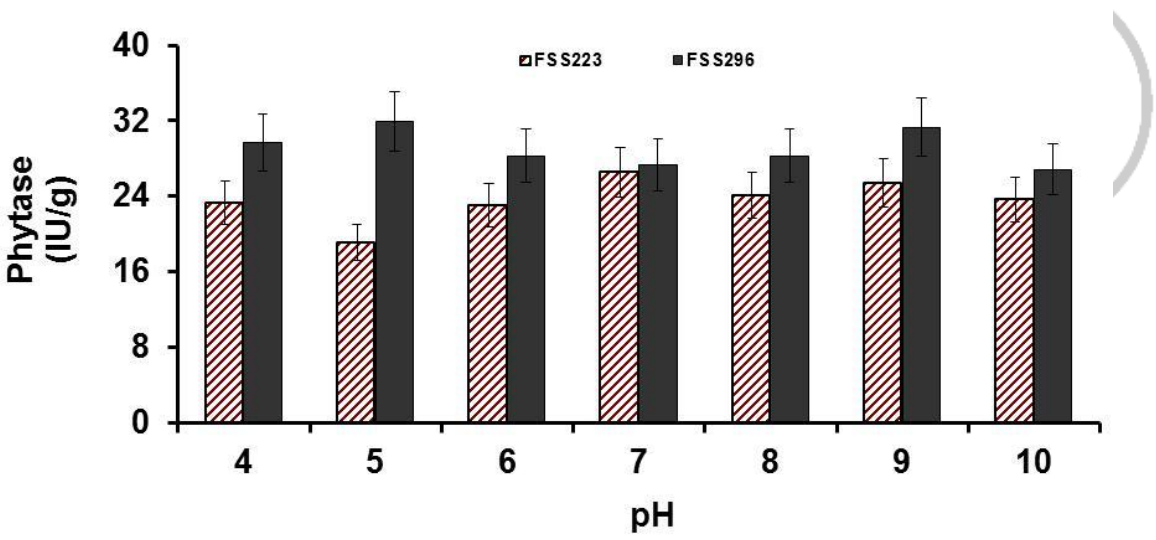

Figure 5. Effect of initial pH on phytase production by FFSS223 and FSS296 isolates

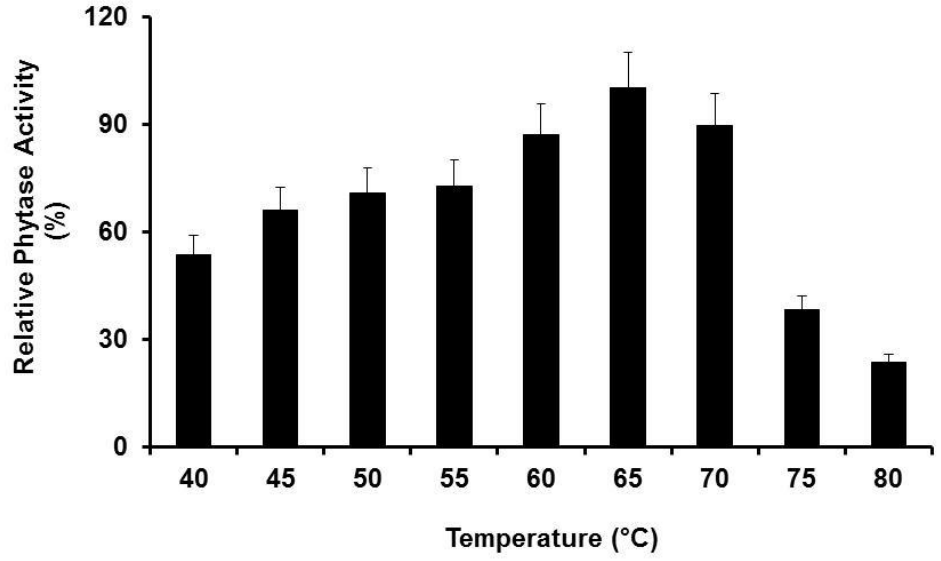

Figure 6. Effect of Temperature on activity of phytase produced by FSS223 isolate

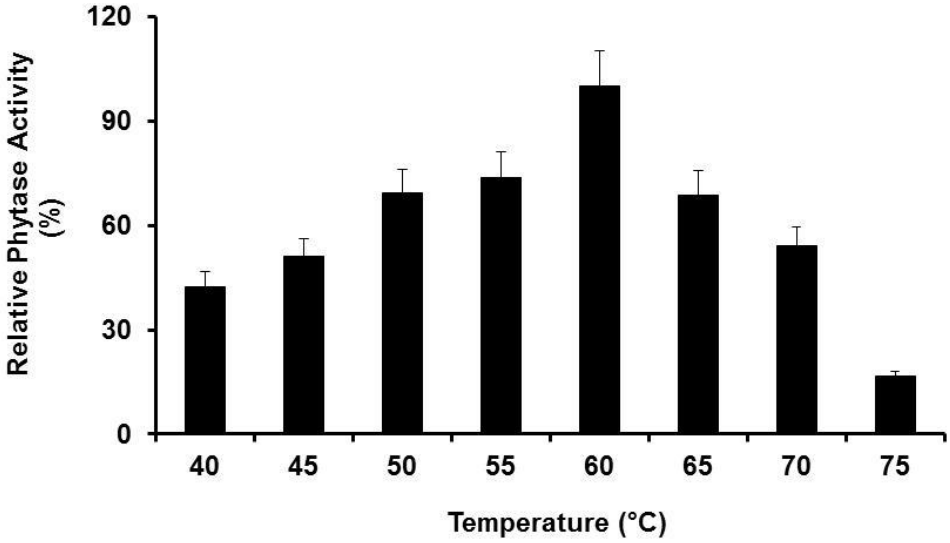

Figure 7. Effect of Temperature on activity of phytase produced by FSS296 isolate

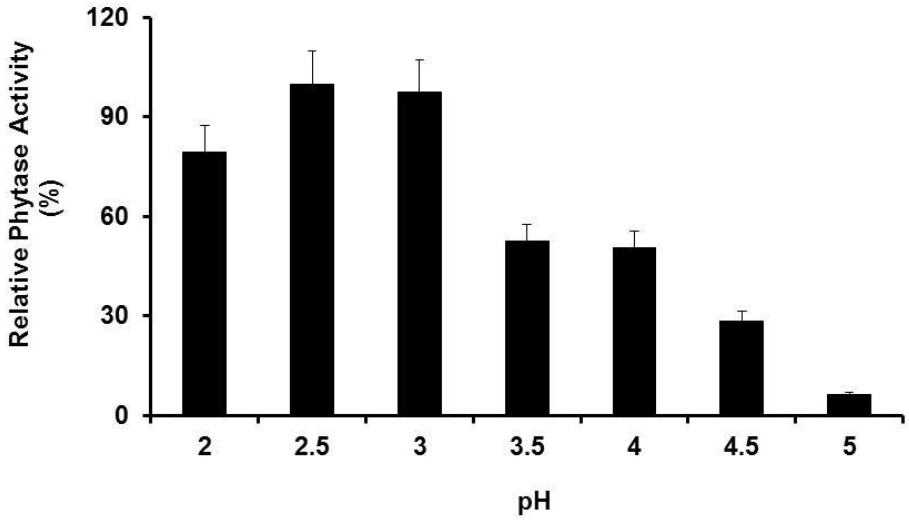

Figure 8. Effect of pH on activity of phytase produced by FSS223 isolate

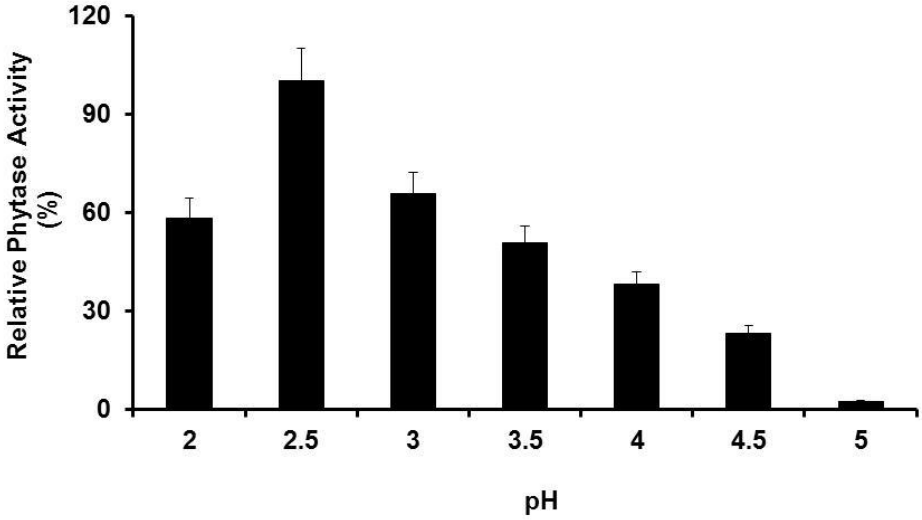

Figure 9. Effect of Temperature on activity of phytase produced by FSS296 isolate

Effect of pH on activity of phytase produced by FSS223 and FSS296 isolates

A pH range from 2 to 5 was used to study the effect of pH on the activities of phytase produced from FSS223 and FSS296 and the results are given in figures 8 and 9, respectively. The optimum pH for phytase activity ofFSS223 and FSS296wasat pH 2.5.Similar results obtained by Vats & Banerjee (2005) and Singh & Satyanarayana (2014) on phytase produced by *Aspergillus niger*. Moreira *et al.,* (2014) found the optimum activity for phytase from *Aspergillusjaponicus* Saito

URM 5633 occurred at pH 3.6.Rani &Ghosh (2011) working on phytase production with *Rhizopusoryzae*, found the optimal pH activity at 5.5. This wide range of differences in pH optimal for enzyme activity could be due to the variation in molecular conformation of the protein from different sources,Vats & Banerjee (2004).

CONCLUSIONS

This study has allowed the identification of two phytase producing microorganism,FSS223 and FSS296. The 5,8S gene sequence data for the 2 isolates were *Aspergillus tubingensis* and *Aspergillus niger*, respectively. Phytase production by the two isolates FSS223 and FSS296 was investigated by the optimization of different parameters. The optimization experiments showed that a maximum phytase production obtained by FSS223 and FSS296 using wheat bran and soya bean powder, incubation time 120 and 168 h, initial moisture content 67 and 75%, initial pH medium 7 and 5, respectively.The optimum activities of phytase produced from FSS223 and FSS296 were at pH 2.5 and at 65 and 60°C, respectively.

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