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PRODUCTION AND OPTIMIZATION OF PHYTASE BY *ASPERGILLUS NIGER* USING SUBMERGED FERMENTATION

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ABSTRACT

Phytases are the enzymes hydrolyzing phytic acid to less phosphorylated myo-inositol derivatives, releasing inorganic phosphate. Phytase has become an important industrial enzyme and is the object of extensive research. The present article implies an extracellular phytase production by *Aspergillus niger*(b2) under submerged 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 phytate sodium substrate (102.46 U/ml) at pH 5, on 5^{th} day of incubation at 30^{0}C and Agitation 200 rpm. Glucose is considered as suitable carbon source whereas pepton is for nitrogen. The phytases produced can be used further for various applications.

Keywords: *Aspergillus niger*, Submerged fermentations, phytase optimization.

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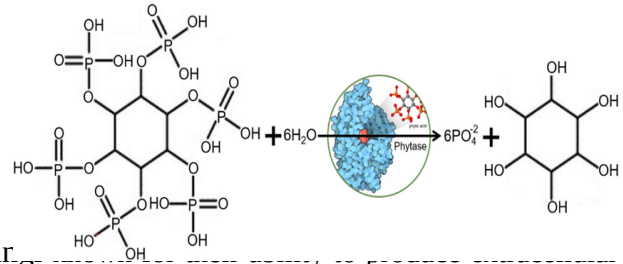
INTRODUCTION

Cereals, Oilseeds and legumes crops are the main ingredients of the food basket of the population especially in developing countries **Katina *et al.*, (2005);** they represent good sources for the essential nutrients especially for the proteins and minerals. In addition whole grain products which are recommended for consumption for their therapeutic role as anti-cardiovascular diseases, diabetes and certain type of cancers **Kumar *et al.*, (2010).**However all these products contain phytic acic (phytate). also known as(myo-inositol (1,2,3,4,5,6) hexakisphosphate), internationally coded IP6, C6H18O24P6, molecular weight 660,035 g/mol is an organic acid found in different proportions in most plant foods **Khan (2018),** especially in food grains (wheat, barley, corn, rice), as it is concentrated in large proportions in the outer grain layers (bran) **Bohn et al.,(2008)** &**Vats et al.,(2009),** and it is a major store of phosphorus, which contains (90-60) % of the total grain phosphorus **Ajith et al.,(2019).** Phytic acid is an indigestible constituent for human and nonruminant animals and it has a unique structure with six phosphate groups that makes it highly charged and acts as a strong chelator of cations causing inhibition for the absorption on minerals, as well as its ability to form complexes with proteins reducing their solubility and digestibility. therefore, phytic acid is known as an anti-nutritional factor **Kumar et al., (2010).** When phytate is hydrolyzed, it provides inorganic phosphorus and minerals for absorption into the human stomach. The enzyme phytase was first discovered in 1907 by Suzuki and his colleagues **Irshad *et al.*, (2017)**. then researchers went to produce it commercially and mainly through the use of microorganisms secreting this enzyme, and stimulating them to produce it because of its economic feasibility, and importance in the field of agriculture and food industries. Phytases (myo-inositol hexakisphosphate phosphohydrolases), have the ability to catalyze the hydrolysis of phytic acid to lower inositol polyphosphates or in some cases free inositol and release inorganic phosphate (Pi), **Qasim*et al.*, (2016)**Phytate degrading enzymes (phytases) have potential application in food industries and that is due to their role in the reduction of phyic acid content in food **Singh & Satyanarayana (2012).**Phytases are present in many plants, animal tissues and microbial sources (bacteria, yeasts and molds), **Gaind& Singh (2015).**In the case of fungi various species were described as a good producers of phytases specially *Apergillus***Sariyska*et al.*, (2005)**&**Antia*et al.*, (2012).**



Fur[illegible] enzymes compared to intracellular enzymes produced by yeast and bacteria, and that was reflected positively on the operation cost, especially for the industrial scale **Tariq *etal.*, (2017),**Production of phytase under submerged fermentation (SmF) was promoted as the best technique.In the most recent years, production of phytase by Submerged fermentation (SMF) has grown up, dueto the advantages of method, both in economic and practical perspectives as better product recovery, low technology cultivation equipment, higher product concentration, and lower plant operation cost. Because of its potential biotechnologicalapplications**El-batal& Abdel karem (2001).**There are only fewreports of phytase producing fungi in Syria,therefore present study was designed to determine the ability of the isolation of the fungi type *Aspergillus niger* isolated from barley grains to produce phytase and to determine some optimal physical and chemical conditions for its production (temperature, pH, incubation time, agitation, carbon and nitrogen sources) using submerged fermentation technology (SmF).

MATERIALS AND METHODS

Isolation and screening of phytase producing Fungi (Phytate hydrolyzing Fungi):

Phytase producing Fungi were isolated from different sources samples (n=9) from various reginos in Aleppo and its Countryside, Syria and were transported to the laboratory. One gram of each sample was suspended in (10) ml of sterile distilled water and was serially diluted and the best dilution of each sample was spread onto PSM (Phytase Screening Medium) agar plates composedof 20g glucose; 4g sodium phytate; 2g $CaCl_2$; 5g NH_4NO_3; 0.5g KCl; 0.5g $MgSO_4.7H_2O$; 0.01g $MnSO_4.H_2O$; 0.01g $FeSO_4.7H_2O$ and 20g agar preparedin 1 literofdistilled water **at pH 5.5.** The inoculated plates were incubated at

temperatures **of (30)** °C **for (5)** days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production. To indicate the phytaseactivity of the fungal isolates, diameters of clear zone around colonies on PSM agar weremeasured. the fungal isolate the highest phytase activity was selected for the next studies **Howson & Davis (1983).**

Identification Morphological of the fungal isolates producing the phytase enzyme:

Fungal isolates that formed transparent clear zone around their colonies were classified to the genus and species level based on the taxonomic keys of the genus *Aspergillus***Raja *et al*., (2017)**. Culture characteristics were studied in terms of the speed of growth of colonies on PDA culture medium, the nature of the colonies formed, their color, the opposite color, the shape of the vesicle, and the presence or absence of mitula, as well as phialides and spores **Pitt & Hocking (2009).**

Fungal inoculam preparation for phytase production:

The *Aspergillus niger*(b2)inoculam was prepared by adding 1ml of inoculums containing $2x10^6$ spores to 100ml of medium in a 250ml flask.

Submerged fermentation:

Submerged fermentation was carried out in 250ml Erlenmeyer flask using 100 ml PSM(Phytase Screening Medium)as described previously **Gunashree&Venkateswaran (2008).**containing substrate sodium phytate (0.4%) 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 $x10^6$ 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 3500rpm for 10-15 minutes and assayed for phytase activity **Gunashree&Venkateswaran (2008**).

Physico-chemical optimization of enzyme production:

physical (temperature, pH, Incubation time, agitation) and chemical conditions (carbon and nitrogen source) for optimum enzyme production were determined by using PSM broth as described previously **Gunashree&Venkateswaran (2008).**

Effect of temperature:

Effect of temperature on phytase production in *A. niger*(b2)was studied. The fungus was incubated at temperatures varying from 28 to 42^0C.

Effect of pH:

The effect of medium pH on phytase production from *A. niger*(b2) was studied by adjusting the media pH between 4.5 and 6.

Effect of incubation time on phytase production

The effect of fermentation time on phytase production was studied by incubating *A.niger*(b2) in submerged cultivation medium for 5 days and periodically testing the enzyme activity.

Effect of Agitation:

The effect of agitation on phytase production by *A.niger* (b2) was studied. The fungus was incubated at different agitation varying from (50 rpm, 100 rpm, 150 rpm, 200 rpm, and 250 rpm).

Effect of carbon and nitrogen sources:

For chemical optimization, the isolate *A.niger*(b2) was grown in PSMbroth modified withdifferent carbon sources (glucose, lactose and maltose) atdifferent concentrations (0.1%, 0.3%. 0.5%),anddifferent nitrogen sources (peptone, tryptone and urea) atdifferent concentrations (0.07%, 0.1%, and 0.3%).After 5 day incubation,culture filtrates were measured for phytase activity.

Phytase activity assay:

The enzyme activity was determined by release of phosphorous from sodium phytate substrate by the method **Engelen*et al*., (1994).** Phytase activity was measured as the amount of enzyme required that liberated 1 µmol phosphorous per minute under the reaction conditions). The phytase activity was expressed as Units per milliliter (U/ml) in submerged fermentation.

Statistical Analysis: The results were analyzed statistically using the Genestate12 program by Anova test of variance, according to the one-way analysis, determining the least significant difference (LSD), and comparing the mean enzyme activity (unit/ml) of the *A. niger* (b2) isolate using the Duncan distribution at the 1% level of significance.

RESULTS AND DISCUSSION

Isolation and screening of phytase producing fungi:

In the present study, phytase producing fungi were isolated from soil, wheat grain and Barley grain collected from various regions of Aleppo and its countryside.The results indicate the ability of (9) fungal isolates to form a transparent clear zone around their colonies as an indicator of the production of the phytase enzyme. The average

diameter of the transparent clear zone formed around their colonies ranged between (63-77 mm)(Table.1). The isolate *A.niger*(b2), isolated from barley grain, showed the highest enzyme productivity among the tested isolates with an average diameter of the transparent clear zone (77.33)and was selected for further studies.

Table 1: Hydrolysis efficiency of isolates

Isolate. no	Source of isolation	Clear zone diameter (mm)
S1	Soil	64.36
S2	Soil	63.12
S3	Soil	66.47
S4	Soil	71.44
B1	Barlygrains	72.34
B2	Barlygrains	77.33
B3	Barlygrains	69.78
W1	Wheat grains	65.33
W2	Wheat grains	68.99

Identification of the isolate (b2):

These isolates showed typical cultural and microscopic characteristics. A. niger colonies were rapidly growing at a temperature of 25°C on the surface of the PDA culture medium. They were white in color at first, then turned black when conidial spores began to form, and a paleyellow opposite color appeared after... Duration of grooves on the upper surface of the colony. This fungus forms an undivided, double-walled, long, colorless spore that darkens towards the spherical-shaped vesicle, and on it is placed the mitula layer bearing two rows of phialides, on which are placed the black, or blackish-brown, spherical conidia spores **Moslem *et al*., (2010).**

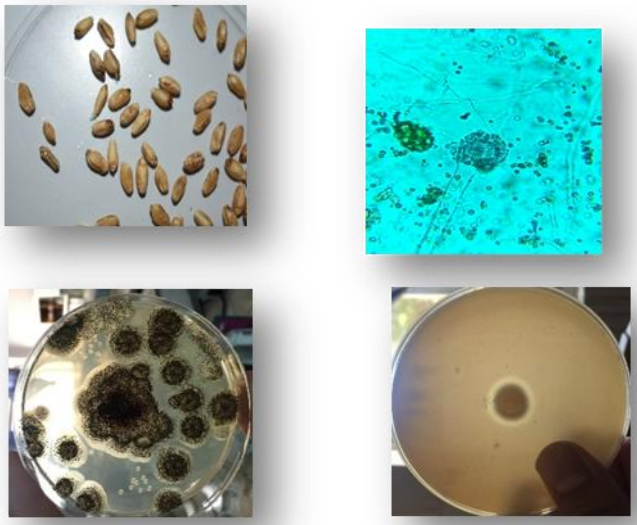


Fig. 2: clear zone (halo) formation of the isolate *A.niger (b2)*

Physico-chemical optimization of enzyme production:

Effect of Temperature:

The effect of incubation temperature on phytase production was studied in the temperature range of (28-42)^{0}Cunder submerged fermentation. The optimum temperature for growth and phytase production from *A.niger*(b2) was found to be 30^{0}C (Fig 3). Further rise in temperature, decreased the production of phytase. The fermentation temperature for optimum production of phytase is mostly reported as 30^{0}C by many researchers. For instance**, Sandhya *et al*., (2015).** reported maximum phyatse production from *Aspergillus niger* at 30^{0}C. Similarly, **Tahir *et al*., (2010).** found that *A.niger* secreted maximum phytase at 30^{0}C. Similar reports made **Nacimento*et al*., (2012), Antia*et al*., (2012), Griener*et al*., (2009)** and **Xiong*et al*., (2009)**.while Tariq *et al*., (2017) found that *Aspergillus niger* showed maximum activity at 25^{0}C.

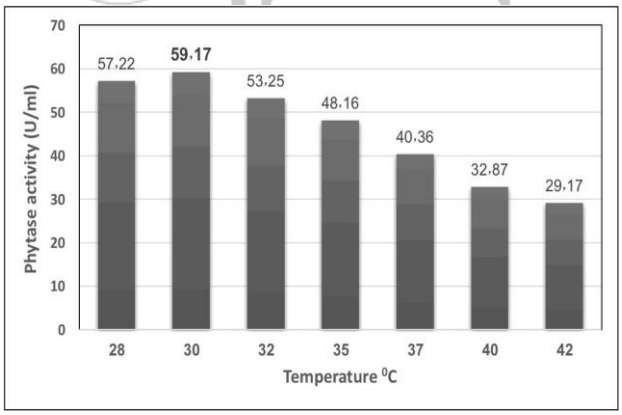


Fig. 3: Effect of temperature on Phytase productionby the isolate *A.niger* (b2)

Effect of pH:

The phytase production from ***A.niger (b2)*** was increased from pH 4.5 to 6 in submerged fermentation. Further change in pH declined the enzyme production drastically. The optimum pH for growth and phytase production from (b2) was found to be pH 5 (Fig 4). Similar results were reported by **Sandhya *et al*., (2015) and Gargova*et al*., (2003)**who stated that production of phytase from *Aspergillus niger*was also

maximum at pH 5. while **Tariq *etal*., (2017),Soni*et al*., (2007) and Antia*et al*., (2012)** found that *Aspergillus niger*showed maximum activity at pH 5.5.**Nacimento*et al*., (2012)** found maximum phytase production at pH 4 by *Aspergillus niger*.

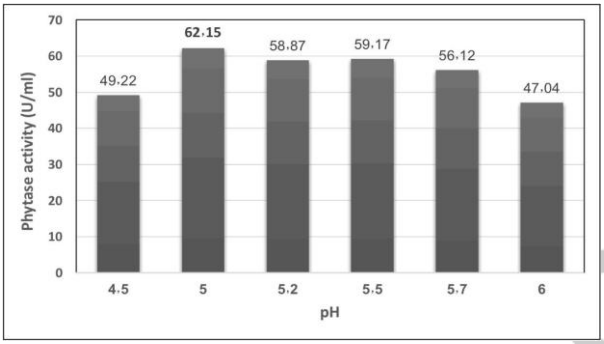
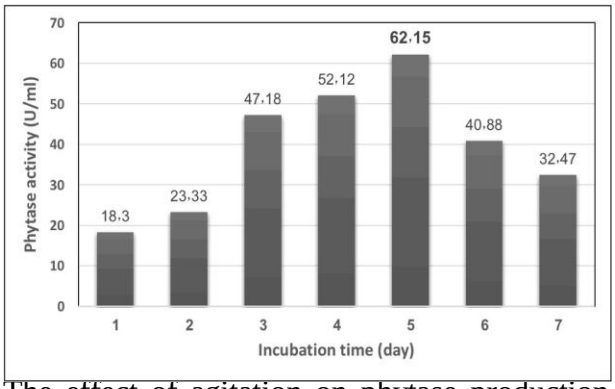


Fig. 4: Effect of pH on Phytase productionby the isolate *A.niger* (b2)

Effect of incubation time:

The effect of incubation time on phytase production by *A.niger*(b2) was studied (Fig 5). The optimum incubation time for growth and phytase production from *A.niger*(b2) was found to be 5day. Thereafter, the enzyme productivity slightly decreased. **Tariq *et al*., (2017)** and **Betancur*et al*., (2012) and Qasim*et al*., (2016)** observed maximum phytase activity at 5 day of fermentation by *Aspergillus niger*. **Sandhya*et al*., (2015)** found maximum phytase production at 4 day of incubation by *A.niger*.



The effect of agitation on phytase production by *A.niger*(b2) was studied. The optimum agitation for growth and phytase production from *A.niger*(b2) was found to be 200rpm.Thereafter, the enzyme productivity slightly decreased (Fig 6). **Sandhya *et al*., (2015),** and **Vats &Banerjee *et al*., (2002).** observed maximum phytase activity at 200rpm of fermentation by *Aspergillus niger*. **Papagianni*et al*., (2001), Qasim*et al*., (2016)** and **Betancur*et al*., (2012)**found maximum phytase production at 150 rpm of incubation by *Aspergillus niger*.

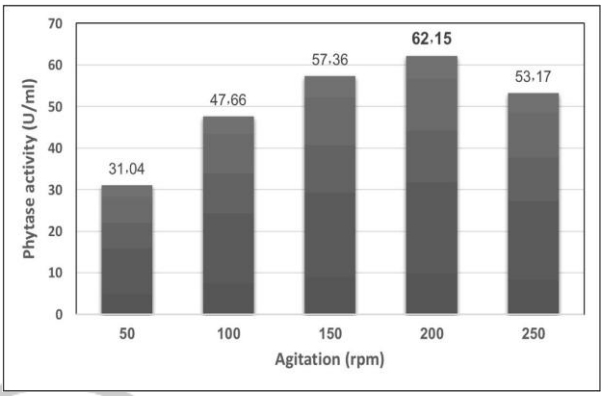


Fig. 6: Effect of Agitation on Phytase productionby the isolate *A.niger* (b2)

Effect of carbon sources:

The effect of carbon on phytase production was studied and results were shown in Fig (7). Among the three carbon sources tested in this study, glucose showed considerable higher titre of phytase (85.15 U/ml) when compared to lactose and maltose used in this study. **Sandhya *et al*., (2015),** and **Das & Ghosh*et al*., (2014)** observed maximum phytase activity at glucose showed considerable of by *A.niger*. also studied the effect of various carbon sources, Among the carbon sources tested, glucose supported highest phytase production from *A.niger* (b2) as compared to other carbon sources.

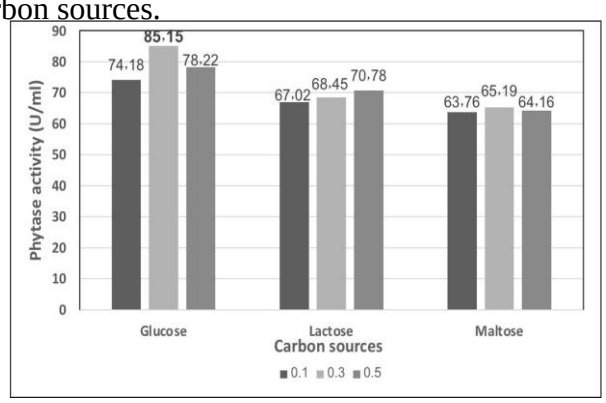


Fig. 7: Effect of Carbon sources on Phytase productionby the isolate *A.niger* (b2)

Effect of nitrogen sources:

The effect of nitrogen source on phytase was studied by supplementing the production medium with various nitrogen sources (Fig 8). Among different nitrogen sources studied, pepton, exhibited maximum phytase activity (102.46.45U/ml) when compared to trepton and urae used in this study. and **Tariq *et al.*, (2017) and Vats &Banerjee *et al.*, (2002).**also studied the effect of Nitrogen sources on phytase production from *A.niger* observed maximum phytase activity at ammonium nitrate.

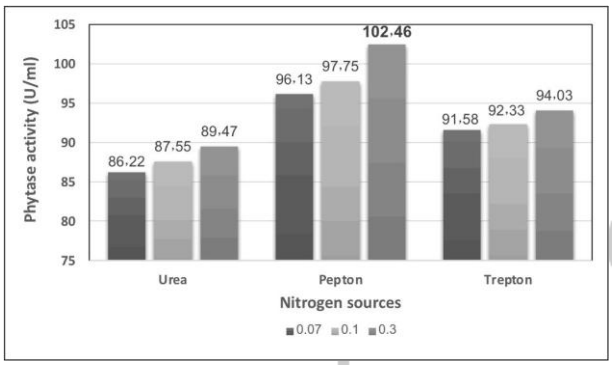


Fig. 8: Effect of Nitrogen sources on Phytase productionby the isolate *A.niger* (b2)

CONCLUSION

In this study optimization of phytase production was carried out by *Aspergillus niger* (b2) through submerged fermentation. The present study reports production of phytase by utilization of phytate sodium as substrate. Enrichment of phytate sodium with glucose, pepton as carbon and nitrogen sources at temperature 30^0C, pH 5, Incubation time 5 day, and Agitation (shaker speed) 200 rpm resulted in better yield of enzyme.

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