https://doi.org/10.46344/JBINO.2023.v13i02(a).07

PRODUCTION AND OPTIMIZATION OF PHYTASE BY *BACILLUS SUBTILIS*

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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 *Bacillus subtilis* (se1) 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 (162.45 U/ml) at pH 6.5, on 2th day of incubation at 35^{0}C and Agitation 200rpm. Glucose is considered as suitable carbon source whereas pepton is for nitrogen. The phytases produced can be used further for various applications.

Keywords: *Bacillus subtilis*, Submerged fermentations, phytase optimization.

of the total grain phosphorus **Ajith *et al.*,(2019).**When phytate is hydrolyzed, it provides inorganic phosphorus and minerals for absorption into the human stomach. Phytate forms complexes with divalent and trivalent cations, starch, proteins and lipids, making phosphorus unavailable for utilization; therefore, phytic acid is known as an anti-nutritional factor **Kumar *et al.*, (2010).** Phytases (myo-inositol hexakisphosphate phosphohydrolases) are a class of phosphatases that catalyze the hydrolysis of phytates to myo-inositol, inositol phosphate, and inorganic phosphates. Phytase enzymes are reported in plants, animals, bacteria and fungi. Humans and Monogastric Animals like poultry, pig and fish cannot hydrolyze phytate as they do not have gastrointestinal phytase **Sardar *et al.*, (2022).**

INTRODUCTION

Antinutrients and mineral absorption inhibitors are chemical compounds found naturally in some plant products, and they are organic substances that have the ability to chelate binding to metal electrolytes leading to a decrease in the vital benefit of essential nutrient mineral elements, as well as trace elements in food, the most important of which are phytic **Mihrete (2019),** Phytate, also known as phytic acid (myo-inositol (1,2,3,4,5,6) hexakisphosphate), internationally coded IP6, $C_6H_{18}O_{24}P_6$, 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) %

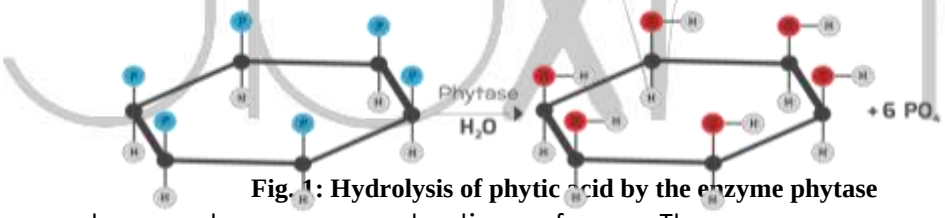

Fig. 1: Hydrolysis of phytic acid by the enzyme phytase

In the most recent years, production of phytase by Submerged fermentation (SMF) has grown up, due to 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 biotechnological applications, interest in the isolation of new bacterial isolates, producing novel and efficient phytases, is increasing **Mehak *et al.*, (2021)** & **Kumar *et al.*, (2021).** There are only few reports of phytase producing bacteria in Syria,

The enzyme phyterase 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. Bacteria are among the most productive sources of phytease, including species of the genus *Bacillus* sp. **Abdolshahi *et al.*, (2021)** & **More *et al.*, (2014).**

The isolate (Se1) was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology. The strain was initially examined for cell morphologies and cell arrangement by gram staining, presence or absence of spores and capsules and motility using microscopy and biochemical characteristics (Catalse test, Oxidase test, Urease test) following Bergey's manual of Determinative Bacteriology, 9th edition **Holt *et al.*, (1994).** The various biochemical tests carried out were also performed by API 50 CH system. API kit was used according to manufacturer's instructions.

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).** To optimize physical factors, the isolate (Se1) was grown in PSM broth with incubated at different temperatures (28°C, 32°C, 35°C, 37°C, 40°C, 42°C, 47°C, 50°C, and 55°C) and different pH (3.5, 4.5, 5.5, 6.5, 7, 7.5, and 8.5), Incubation time (18h, 24h, 30h, 48h, 72h, and 96h) and different agitation (50 rpm, 100 rpm, 150 rpm, 200 rpm, and 250 rpm). For chemical optimization, the isolate (Se1) was grown in PSM broth modified with different carbon sources (glucose, lactose and maltose) at different concentrations (0.1%, 0.3%. 0.5%), and different nitrogen sources (peptone, tryptone and urea) at different concentrations (0.07%, 0.1%, and 0.3%). After 48 hours incubation, culture filtrates were measured for phytase activity.

therefore present study was designed to determine the ability of the isolation of the bacterial type *Bacillus subtilis* isolated from whole sesame seeds to produce phytase and to determine some optimal physical and chemical conditions for its production (temperature, pH, incubation time, agitation, carbon and nitrogen sources).

MATERIALS AND METHODS

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

Phytase producing bacteria were isolated from different sources samples (n=45) from various reginos in Aleppo Countryside, Syria and transported to bacteriology. 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 composed of 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 prepared in 1 liter of distilled water at pH 7. The inoculated plates were incubated at temperatures of (37) °C for (1-3) days and observed for the clear zones of hydrolysis around the colonies which gave an indication of extracellular phytase production. To indicate the phytase activity of the bacterial isolates, diameters of clear zone around colonies on PSM agar were measured. A bacterial isolate with the highest phytase activity was selected for the next studies **Kumar *et al.*, (2011).**

Identification of the bacterial isolate with highest phytase activity:

Morphological and Biochemical tests

screening of phytase producing bacteria

In the present study, phytase producing bacteria were isolated from sesame, whipped chickpeas, soil, fermented bran, bran bread, flour, sourdough and bran collected from various regions of Aleppo and its countryside. The results indicate the ability of (15) bacterial 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 (6.83-26.5) (Table.1). The isolate (se1), isolated from sesame seeds, showed the highest enzyme productivity among the tested isolates with an average diameter of the transparent clear zone (26.5) and was selected for further studies.

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 *Bacillus subtilis* (Se1) isolate using the Duncan distribution at the 1% level of significance.

RESULTS AND DISCUSSION

Isolation and d

Isolate. no	Source of isolation	Clear zone diameter (mm)
Se1	Sesame seeds	26.5
Se3	Sesame seeds	24.83
S6	Soil	22.83
FB2	fermented bran	18.5
FB3	fermented bran	17.67
B1	Bran	16.83
S4	Soil	15.5
S3	Soil	13.83
B2	Bran	13.67
BB1	Bran Bread	12.67
BB3	Bran Bread	12.17
Sd1	sourdough	11.33
F1	Flour	9.17
gch2	ground chickpeas	8.33
gch3	ground chickpeas	6.83

Table 1: Hydrolysis efficiency of isolates

identification scheme in Bergey's Manual of Systematic Bacteriology **Krieg & Holt (1984).** (se1) was identified as *Bacillus* sp. Results of biochemical tests by API 50 CH indicate the isolate (se1) to be *Bacillus subtilis*.

Identification of the isolate (se1):

Identification of the isolate (se1) was carried out by Gram staining, microscopic examination and biochemical tests. The organism was seen as a Gram-positive *Bacilli*. Based on the

Fig. 2: clear zone (halo) formation of the isolate se1

optimum production of phytase is mostly reported as 35^{0}C by many researchers. For instance, **Demirkan *et al*., (2014).** reported maximum phyatse production from *Bacillus* sp. at 35^{0}C. Similarly, **Javaid *et al*., (2022).** found that *Bacillus subtilis* secreted maximum phytase at 35^{0}C. Similar reports made **Shamna *et al*., (2012), and Aziz *et al*., (2015).**

Physico-chemical optimization of enzyme production:

Effect of Temperature:

The effect of incubation temperature on phytase production was studied in the temperature range of (28-55) ^{0}C under submerged fermentation. The optimum temperature for growth and phytase production from (se1) was found to be 35^{0}C (Fig 3). Further rise in temperature, decreased the production of phytase. The fermentation temperature for

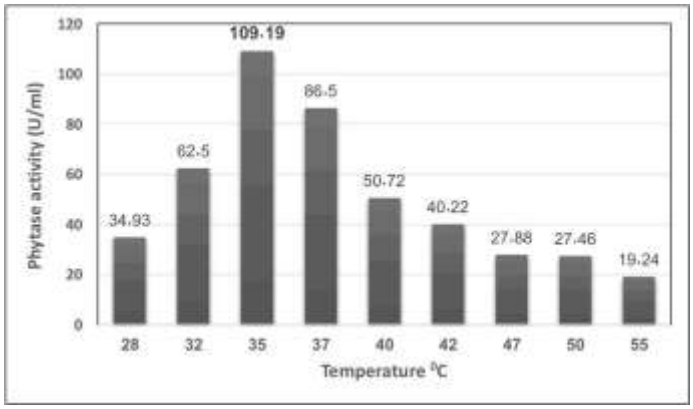

Fig. 3: Effect of temperature on Phytase production by the isolate Se1

Similar results were reported by **Shamna *et al.*, (2012).** who stated that production of phytase from *Bacillus subtilis* was also maximum at pH 6.5. Similar reports made **Aziz *et al.*, (2015).** and **More *et al.*, (2014).** while **Trivedi *et al.*, (2021).** and **Kammoun *et al.*, (2012)** found that *Bacillus subtilis* showed maximum activity at pH 6.

Effect of pH:

The phytase production from *Bacillus subtilis* (se1) was increased from pH 3.5 to 8.5 in submerged fermentation. Further change in pH declined the enzyme production drastically. The optimum pH for growth and phytase production from (se1) was found to be pH 6.5 (Fig 4).

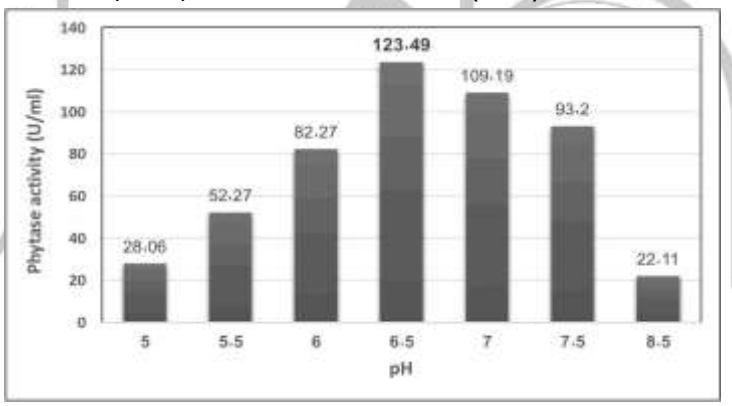

Fig. 4: Effect of pH on Phytase production by the isolate Se1

***et al.*, (2021)** and **Demirkan *et al.*, (2014).** observed maximum phytase activity (123.49 U/ml) at 48hr of fermentation by *Bacillus subtilis*. **Abdolshahi *et al.*, (2021)** and **El-toukhy *et al.*, (2013).** found maximum phytase production at 72 hr of incubation by *Bacillus* sp.

Effect of incubation time:

The effect of incubation time on phytase production by *B.subtilis* (se1) was studied (Fig 5). The optimum incubation time for growth and phytase production from (se1) was found to be 48hr. Thereafter, the enzyme productivity slightly decreased. **Shamna *et al.*, (2012), Trivedi**

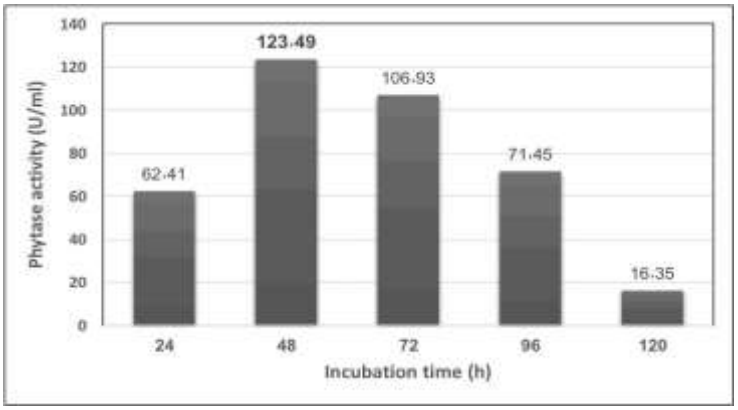

Fig. 5: Effect of Incubation time on Phytase production by the isolate Se1

activity at 200rpm of fermentation by *Bacillus subtilis*. **Abdolshahi *et al.*, (2021)** found maximum phytase production at 250rpm of incubation by *Bacillus* sp. **Trivedi *et al.*, (2021).** also studied the effect of Agitation on phytase production from *Bacillus subtilis* observed maximum phytase activity at 150rpm.

Effect of Agitation:

The effect of agitation on phytase production by *B.subtilis* (se1) was studied. The optimum agitation for growth and phytase production from (se1) was found to be 200rpm.Thereafter, the enzyme productivity slightly decreased (Fig 6). **Shamna *et al.*, (2012),** and **Demirkan *et al.*, (2014).** observed maximum phytase

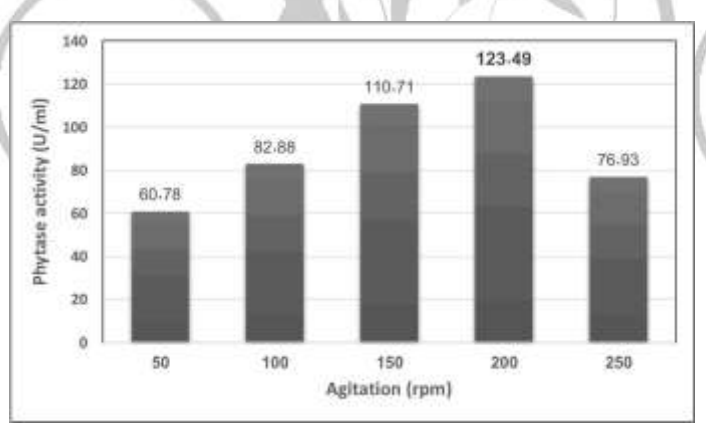

Fig. 6: Effect of Agitation on Phytase production by the isolate Se1

Shamna *et al.*, (2012), and **Aziz *et al.*, (2015).** also studied the effect of various carbon sources, Among the carbon sources tested, glucose supported highest phytase production from *Bacillus subtilis* (se1) as compared to other carbon sources.

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 (148.22 U/ml) when compared to lactose and maltose used in this study.

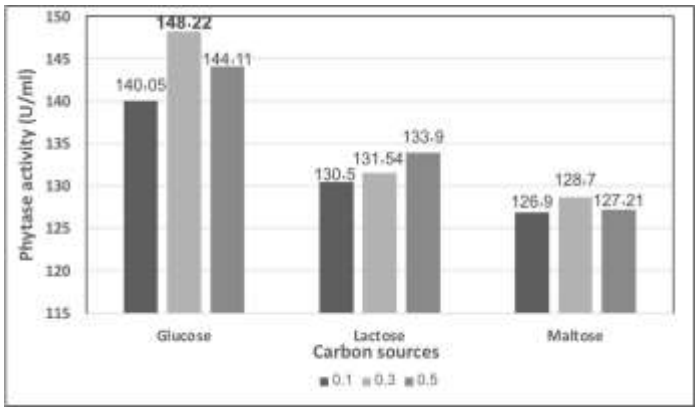

Fig. 7: Effect of Carbon sources on Phytase production by the isolate Se1

phytase production from *Bacillus subtilis* (se1) as compared to other Nitrogen sources. **Demirkan *et al*., (2014).** also studied the effect of Nitrogen sources on phytase production from *Bacillus subtilis* observed maximum phytase activity at meat extract. while **Shamna *et al*., (2012),** found that *Bacillus subtilis* showed maximum activity at yeast extract.

Effect of nitrogen sources:

The effect of nitrogen source on phytase was studied by supplementing the production medium with various nitrogen sources (Fig 6). Among different nitrogen sources studied, pepton, exhibited maximum phytase activity (162.45U/ml) when compared to trepton and urae used in this study. and **Aziz *et al*., (2015).** also studied the effect of various Nitrogen sources, Among the Nitrogen sources tested, peptone supported highest

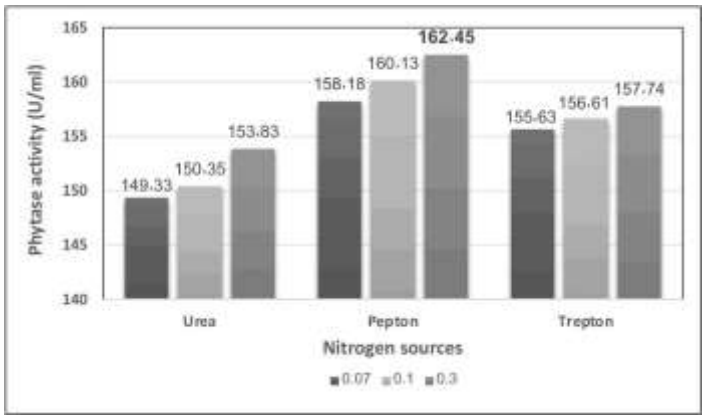

Fig. 8: Effect of Nitrogen sources on Phytase production by the isolate Se1

Bacillus subtilis (se1) through submerged fermentation. The present study reports production of phytase by utilization of

CONCLUSION

In this study optimization of phytase production was carried out by

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phytate sodium as substrate. Enrichment of phytate sodium with glucose, pepton as carbon and nitrogen sources at temperature 35^{0}C, pH 6.5, Incubation time 48h, and Agitation 200rpm resulted in better yield of enzyme.

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