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Production of extracellular phytase from *Bacillus subtilis* isolated from Syrian soil

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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 objective of the present study was to isolate a potential phytase producing bacterial strains from soil samples of Damascus countryside, Syria. The phytase producing bacteria were screened using PSM plates, containing selectable media. The best phytase producing strain was preliminary identified by microscopic and biochemical tests as *Bacillus sp.* Further, the identification of the strain was confirmed by subjecting it to 16S rDNA sequencing followed by BLAST analysis.From the (15) bacterial isolates, The isolate C4 with high potential for phytase production was selected and identified as *Bacillus subtilis* strain X3. The isolateC4 produces significant amount of phytase during (72th) h of incubation at (37) °C with the pH of (7). The phytases produced can be used further for various applications.
Keywords: Phytase –Sodium Phytate – *Bacillus Subtilis.*

Introduction

During the last (20) years, phytases attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. These enzymes belong to a special class of phosphor-mono-esterases (*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8 and *myo*-inositol hexakis-phosphate 6-phosphorylase, EC 3.1.3.26), and are capable of initiating the stepwise release of phosphate from phytate *myo*-inositol (1, 2, 3, 4, 5, 6) hexakis-phosphate[1], which is considered the major storage form of phosphate in plant seeds and pollen[2].

Phytases were originally proposed as an animal feed additive to enhance the nutritional quality of plant material in feed for simple-stomached animals by liberating phosphate[3]. More recently, addition of phytase has been seen as a way to reduce the level of phosphate pollution in areas of intensive animal production. Several studies have shown the effectiveness of supplemental microbial phytases in improving utilization of phosphate from phytate[4]. Therefore, inorganic phosphate supplementation in the diets for simple-stomached animals can be obviated by including adequate amounts of phytase and as a result, the fecal phosphate excretion of these animals may be reduced by up to (50) %. Because of the action of phytate as an anti-nutrient[5] by binding to proteins[6]and by chelating minerals[7], a biotechnological application of phytase in the food area was taken into consideration. On the other hand, phytase can improve the nutritional value of plant based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach or during food processing[8]. Since certain *myo*-inositol phosphates have been proposed to have novel metabolic effects[9], phytases may also find application in food processing to produce functional foods[10]. A thermo stable

phytase could have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid would not produce carcinogenic and highly toxic by-products[11]. Therefore, application of phytases in the pulp and paper process could be environmentally friendly and would assist in the development of cleaner technologies.

Phytases are widespread in nature and can be derived from a host of sources including plants, animals and microorganisms. Microbial sources are more promising for the production of phytases on a commercial scale[12]. Some of the phytase producing microorganisms include bacteria such as *Bacillus subtilis, Escherichia coli*, fungi such as *Aspergillus niger, A. oryzae, A. flavus and Penicillium sp.*, and yeasts such as *Saccharomyces cerevisiae, Schwannoiomyces castelii*[13]. Due to several biological characteristics, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases have considerable potential in commercial applications. The increasing potential of phytase application prompts screening for newer phytase producing microorganisms, which can meet the conditions favorable to the industrial production. Bacteria are though ubiquitous in their occurrence, the most common sources for their isolation are soils[14].

The present study report the isolation of phytase producing bacteria from soil samples of Damascus countryside, and focus on the production of extracellular phytase by *Bacillus subtilis* strain X3.

Materials and Methods

Isolation and screening of phytase producing bacteria

Total of (30) rhizosphere soil samples of Legumes, cattle shed soil samples and poultry farm soil samples were collected from various regions in Damascus countryside, Syria.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) plates[8-15]. The agar media composed of Glucose (1.5) %, (NH4)2SO4 (0.5) %, MgSO4.7H2O (0.01)%, KCL(0.05)%, NaCl(0.01)%, CaCl2.2H2O(0.01)%, FeSO4(0.001) %, MnSO4(0.001) %, Sodium Phytate (0.5) %. The pH was adjusted to (7)and (1.5) % agar was added before autoclaving at (121) °C for (15) minutes. 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. Each such colony was picked up and maintained till further use[16].

Screening for best phytase producing strain microbial colonies capable of hydrolyzing phytate which can be recognized by their surrounding clear halo were obtained by re-plating single colonies. The halo (Z) and colony (C) diameters were measured after (3) days of incubation at (37) °C. Hydrolysis efficiency of all the isolates was determined by the formula Z-C/ C[17-18]. The isolate (C4) with(50) % efficiency was selected and stored at (4) °C until use.

Identification of the selected phytase producing bacterial isolate

Morphological and Biochemical tests

The isolate (C4) was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology[19]. 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. The various biochemical tests carried out was performed by API 50 CH system. API kit was used according to manufacturer's instructions.

16S rRNA gene sequence Analysis

For the sequence analysis, bacterial genomic DNA was extracted and purified using CTAB method[20]. Two primers annealing at the 5´ and 3´ end of the 16S rDNA were Forward:5´- AGAGTTTGA TCCTGGCTCAG-3´, Reverse:5´-TACCTTGTTACGACTT-3´[21]. PCR amplification was performed in a final reaction volume of (100)µl. The PCR reaction was run for (35) cycles in a DNA thermal cycler. The amplified PCR products were then analyzed in a (1.0) % (w/v) agarose gel, excised from the gel, and purified. The amplified DNA sequence was then sequenced. The 16S rRNA gene sequence of the isolates was aligned with reference 16S rDNA sequences of the GenBank using the BLAST algorithm available in NCBI.

Phytase production and activity assay

The production of enzyme was carried out in the production medium without addition of agarusing Shaken flask fermentation method. The inoculum of the selected strain was produced during (24) husing LB Broth. (0.3) % of inoculum was inoculated on (30) ml of production medium[11-22]taken in (100) ml conical flask. The flask was then incubated at (37) °C for (4) days at shaken condition at (200) rpm for better aeration and growth of organism. The fermented broth from the flask was transferred every day into centrifuge tubes and centrifuged at (4000) rpm for (15) minutes at (4) °C.The supernatant was then transferred into clean test tube which was used as crude enzyme solution[23].Phytase activity was determined by quantification of the phosphate released from phytate during the enzymatic reaction.

The enzymatic activity was measured by a modification of the Heinonen-Lahti method using sodium phytate as substrate[24].

Results and Discussion

Isolation and screening of phytase producing bacteria

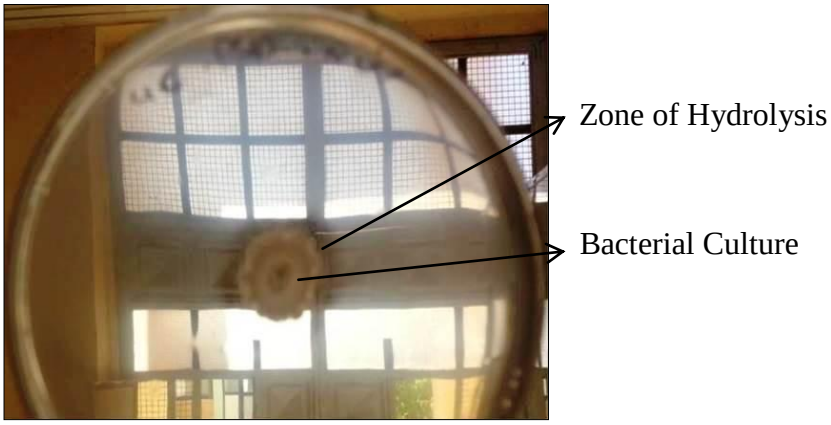

Fig. 1: Halo formation of the isolate C4

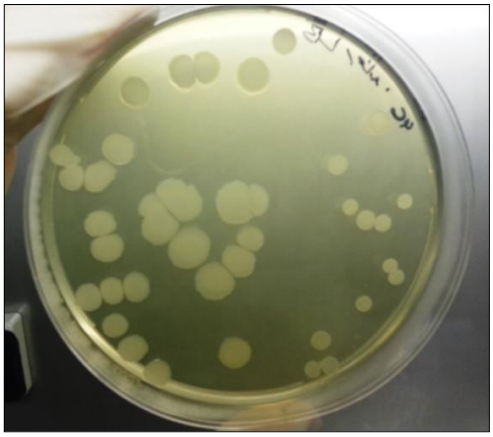

Fig. 2:Colony formation of the isolateC4after incubation on PSM agar at 37°C-72h after incubation on LB agar at 37°C-24h

In the present study, phytase producing bacteria were isolated from rhizospheresoil; cattle shed soil and poultry farm soil collected from various regions of Damascus countryside. Total of (15) colonies showed positive for phytase production. Among these, (9) were from rhizosphere soil samples and were designated as R1 to R9and (4)were from cattle shed soil samples which were designated as C1 to C4and (2) were from poultry farm soil samples and designated as P1, P2[25-29]. All the (15) isolates were replated and their halo (Z) and colony (C) diameters were measured after (3) days of incubation at (37) °C (Table 1). Hydrolysis efficiency of all the isolates was calculated which ranged from (5) % to (50) %. The isolateC4 was found to produce phytase with significantly higher activity (Fig.1) and was selected for further studies[26-29].

Table 1: Hydrolysis efficiency of isolates

Isolate. no	Colony diameter, C (mm)	Halo diameter, Z (mm)	Hydrolysis Efficiency, Z-C/C (%)
R1	40	42	5
R2	23	27	17
R3	30	40	33
R4	30	33	10
R5	14	19	36
R6	26	34	31
R7	29	34	17

R8	22	27	23
R9	26	32	23
C1	31	33	6
C2	20	27	35
C3	18	23	28
C4	16	24	50
P1	80	90	13
P2	50	60	20

Table 2: Morphological identification of the isolate C4

Microscopic Characteristic	Result
a) Form	Bacilli
b) Gram Staining	Gram-positive
c) Motility	-
d) Spore	+
e) Capsule	+

Identificationof the isolate C4

Identification of the isolate C4 was carried out by Gram staining, microscopic examination and biochemical tests. The organism was seen as a Gram-positive Bacilli. Based on the identification scheme in Bergey's Manual of Systematic Bacteriology[27], C4 was identified as Bacillus sp. Results of biochemical tests by API 50 CH indicate the isolate C4 to be *Bacillus subtilis.*

Hence, the identification of the strain was further done by 16S rRNA gene sequencing analysis. Comparison of the 16S rRNA gene sequence of the isolate C4 with those in GenBank using BlastN showed (99) % sequence homology with *Bacillus subtilis* strain X3. The sequence is deposited under accession no. KM187652.1 in GenBank.

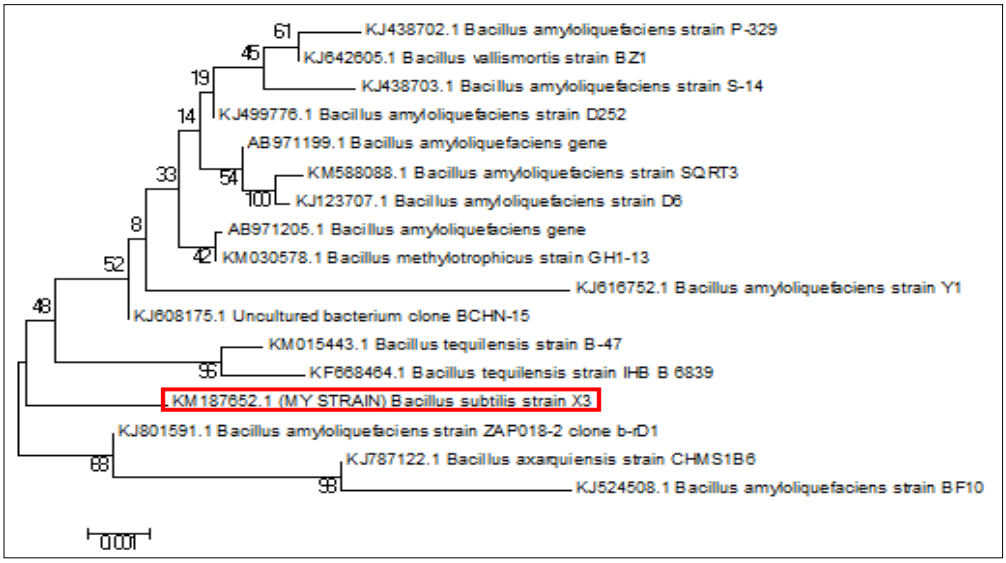

Fig. 3: Phylogenetic trees of bacterial strain C4 and its related species CLUSTAL X version 1.81 program

Effect ofincubation time on phytase production

Hence it is necessary to optimize the fermentation parameters for the maximum production of phytase with a view to develop economically feasible technologies[28]. Incubation time plays an important role in maximum enzyme production[29]. The present results showed that the significantly high level enzyme activity was obtained during (72th) h of incubation at (37) °C with the pH of (7)(Fig.4).Extending the fermentation resulted in a slight decrease in phytase activity, which might be due to proteolytic degradation of the enzyme[30].

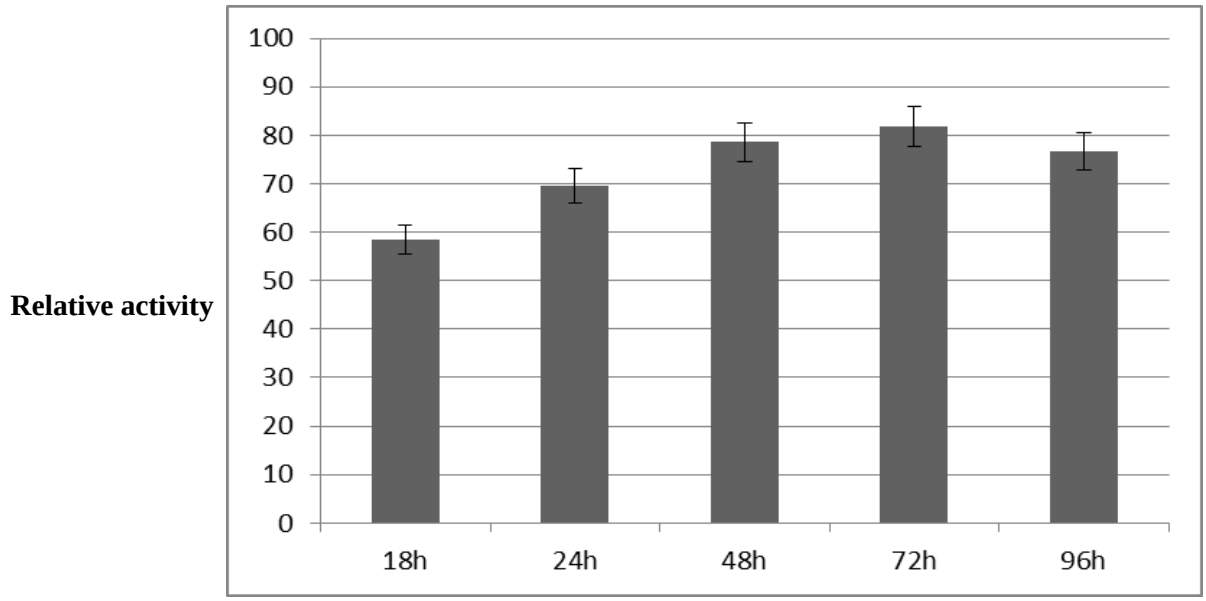

Fig. 4: Effect of Incubation time on phytase production by the isolate C4

Conclusion

Thus Phytase producing bacteria were isolated from various soil sources and the high yielding bacterial isolate C4 was selected for identification. Microscopic, Biochemical and 16S rRNA gene sequence studies revealed the isolated phytase producing bacterial isolate as *Bacillus Subtilis* strain X3. The target protein is secreted to the culture medium which makes its purification simpler and more economical. This strain will be further studied for characterization of purified phytase enzyme.

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