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Isolation, purification and characterization of phytase from *Bacillus subtilis* MJA

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In this study, three strains of bacteria were isolated from soil. Among the three isolated strains, one was identified morphologically and confirmed by the molecular techniques as *Bacillus subtilis* MJA with high phytase activity. The phytase-producing bacteria were isolated using phytate screening agar media (PSM) with only 1.5% glucose and 0.5% sodium phytate as only source for carbon. In order to optimize the phytase production by *B. subtilis* MJA, different factors were studied. A combination of 0.5% glucose and 0.5% sucrose showed to be the best carbon source. Also, malt extract used as a source of nitrogen gave the highest phytase production. Also, the maximum phytase production was detected after incubation for four days (720 U/ml) at an optimum pH value of 7. The produced phytase was purified through various chromatographic techniques. The estimated enzyme molecular mass was about 38 kDa and the phytase had an optimal temperature and pH of 37°C and 5 to 6, respectively. On the other hand, studying the enzyme stability showed that enzyme was stable at low temperature, and had good pH stability by retaining 80% of its initial activity over a wide range of pH from 2 to 8. Kinetic values of V_{max} and K_m for the purified enzyme were 510 U/mg and 0.485 mM, respectively. The phytase activity was affected by different divalent metal ions. Cations such as Cu^{2+} or Fe^{2+} showed an inhibition effect on the phytase activity and the effect was in a dose dependent manner while, cations such as Mg^{2+} or Ca^{2+} showed an increase in the phytase activity. On the other hand, among different matrices used to immobilize the cells for phytase production, agar-agar matrix indicated a promising immobilization matrix used for phytase production by *B. subtilis* MJA.

Key words: Phytase, microbial sources, optimization, purification, characterization, immobilization.

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 phosphomonoesterases (*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8 and *myo*-inositol hexakisphosphate 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) hexakisphosphate], which is considered the major storage form of phosphate in plant seeds and pollen (Blaabjerg et al., 2011).

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 (Mitchell et al., 1997). 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

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of phosphate from phytate (Cho et al., 2006; Guggenbuhl et al., 2012; Létourneau-Montminy et al., 2010). 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 by binding to proteins and by chelating minerals, a biotechnological application of phytase in the food area was taken into consideration (Rousseau et al., 2012). 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 (Tran, 2010). Since certain *myo*-inositol phosphates have been proposed to have novel metabolic effects (Vucenik and Shamsuddin, 2003), phytases may also find application in food processing to produce functional foods.

The action of phytase also contributes towards reducing the pollution in surface- and ground water caused by the phytate and phosphorus run-off from manure in intensive livestock regions. All the commercially available phytases are histidine acid phytases with optimum activities at low pH and with low thermostabilities. Alkaline phytases, known as β-propeller phytases, are active at neutral or slightly alkaline conditions, calcium-dependent, and are quite thermostable so as to withstand the high temperatures during the pelleting of animal feeds. They can hence exhibit activities in the small intestine of animals as well as during storage of feeds.

In the present study, the main objective was to screen for novel bacterial phytase. Then, we studied the optimization of physiological parameters for phytase production. After medium optimization, the enzyme purification steps were done with the aid of various chromatographic techniques using an automated ÄKTA Prime Plus system. Some previous studies on phytase used ion-exchange chromatographic methods as purification methods (Greiner et al., 1997, 1998, 2000, 2009), while other investigators used gel filtration as a purification method for phytase from different sources (El-Toukhy, 2001; Greiner et al., 1998 and 2000).

The properties of the pure enzyme were characterized and the kinetic parameters according to Lineweaver-Burk plot were calculated. Also, the effect of immobilized bacterial cells on the production of phytase was evaluated.

MATERIALS AND METHODS

Microorganism

Different microbial cultures were isolated from soil samples collected from different places in New Borg El-Arab city, Alexandria, Egypt.

Preliminary screening of phytate-degrading bacteria

1 g of soil samples were suspended in 0.9% saline. The suspension was filtered by Whatman filter paper and 100 µl of the filtered sample was spread onto the phytate screening agar media (PSM), and incubated at 30°C for 1 to 2 days. The bacterial colonies, which were capable of hydrolyzing sodium phytate were recognized by their surrounding clear zone. The isolate with the highest clear zone was re-plated and used for further studies with phytase producing media (Chunshan et al., 2001). A single colony was streaked on LB agar-filled plates, and incubated overnight at 30°C. A drop of the bacteria was transferred into 100 ml conical flask containing phytase production medium (10 g sodium phytate, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.H_2O$ and 1 g D-mannose per liter and adjusted to pH 7), and the culture was incubated at 30°C with 200 rpm shaking for 6 days. The culture was centrifuged and the supernatant was used as the enzyme source in phytase activity assay (Rodriguez and Fraga, 1999).

Molecular identification and confirmation of the selected bacterial strain

The bacterium was characterized and identified by 16S rRNA gene sequencing using synthesized universal primers (ETABION, Germany). The forward and reverse primers were of the following sequences 5`-AACTGGAGGAAGGTGGGGAT-3` and 5`-AGGAGGTGATCCAACCGCA-3`, respectively. The PCR was carried out for amplifying the 16S rRNA. The Thermocycler (MJ Research, model PTC-200 Peltier, USA) was used for performing PCR amplification with modified method according to Innis et al. (1990).

After completion, a fraction of the PCR mixture was examined using 2% agarose gel electrophoresis according to Sambrook et al. (1989). The amplified PCR product of 16S rRNA gene was visualized on Ultra-Violet Transilluminator (M-20, Upland, USA) and photographed using Gel Documentation System (Alpha-ChemImager, USA). The remnant mixture was purified using QIA quick PCR purification kit manufacturer instructions (Qiagen) according to Vogelstein and Gillespie (1979).

The 16S rRNA gene fragment (1200 bp length) was sequenced in both direction and Blast program (www.ncbi.nlm.nih.gov/blast) using the data submitted into GenBank database to assess the DNA similarities (Altschul et al., 1990). Alignment of 16S rRNA sequence was conducted using CLUSTAL W (1.83) multiple sequence alignment software (Blackshields et al., 2007; Higgins et al., 1994). Phylogenetic tree and molecular evolutionary analyses were conducted according to NCBI nucleotide BLAST, GenBank database and generated by CLC Genomics workbench-3. Also, the sequence has been deposited in the GenBank under accession no. JQ045381.

Enzyme production conditions

The phytase specific medium was prepared with the following composition: 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$ and 0.5% sodium phytate (Sigma-Aldrich, USA) according to Kerovuo et al. (1998). Luria-Bertani broth (LB), consisting of 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter and adjusted to pH 7, was used for culturing bacterial isolates. The phytase production medium (PPM) consisted of 10 g sodium phytate, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.H_2O$ and 1 g D-mannose per liter and adjusted to pH 7 and sterilized by low binding protein filtration system.

For determination of bacterial growth in LB media, the incubation process was carried out at 30°C under shaking conditions (200 rpm) overnight. A single bacterial colony was transferred into 5 ml of LB as pre-culture and 1 ml of the culture was transferred into 500 ml flask containing 100 ml LB broth media and the inoculated broth was incubated under the same condition of the pre-culture. 1 ml of

the cell culture was taken out at time intervals of every 2 h and centrifuged at 10,000 rpm for 10 min then the turbidity was measured at OD_{600} nm with a spectrophotometer (BECKMAN DU530, USA) according to Zwietering et al. (1990).

Determination of phytase enzyme activity

Enzyme activity assays were performed as described by Heinonen and Lahti (1981) with some modifications. Enzyme activity was measured at 37°C. Prior to determination of enzyme activity, the enzymatic reactions were started by adding 10 µl enzyme to the assay mixtures. The incubation mixture of phytase determination consisted of 350 µl of 0.1 M sodium acetate buffer pH 5 containing 500 nmol sodium phytate. After an incubation period of 30 min, the liberated orthophosphate was measured by a modification of the ammonium molybdate method (Heinonen and Lahti, 1981). 1½ ml of freshly prepared stop solution (acetone:5 N H_2SO_4:10 mM ammonium molybdate (2:1:1 v/v) and 100 µl of 1.0 citric acid) was added to the assay mixture. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. Blank was run without degradation of the substrate by adding the stop solution prior to the addition of the enzyme to the assay mixture. In order to calculate the enzyme activity, a calibration curve was drawn over the range 100-1500 µg/mL orthophosphate. Enzyme activity was expressed in international units (U). One unit of the enzyme activity was defined as the amount of the enzyme able to hydrolyze phytate resulting in liberation of 1 µmol of inorganic phosphorus per minute under the assay condition.

Optimization of phytase production from *B. subtilis* MJA

Effect of carbon source

The effect of carbon source was studied in order to optimize the production of the phytase enzyme from the isolate *B. subtilis* MJA. Different carbon sources such as 1% glucose, 1% maltose, 0.5% glucose plus 0.5% maltose, 0.5% sucrose, and 0.5% glucose plus 0.5% sucrose, were used instead of the original carbon source (0.01% D-mannose) in the phytase production medium and the effect on the phytase production was observed and compared with the phytase production medium as control.

Effect of nitrogen source

Different nitrogen sources, such as 0.1% glycine, 0.1% malt extract and 0.1% yeast extract, were used instead of the original source of nitrogen [$(NH_4)_2SO_4$] in the phytase production medium in order to optimize the production of the phytase enzyme from *B. subtilis* MJA. The production of the phytase enzyme was observed and compared with the phytase production medium as control.

Effect of pH value

Phytase production media with different pH values of 4, 7 and 9 were prepared in order to optimize the production of phytase enzyme produced from *B.subtilis* MJA. The results obtained were compared with the phytase production medium (pH 7) as control.

Optimization of the growth curve

A single bacterial colony was inoculated into 5 ml LB broth and incubated in PPM at 30°C under shaking conditions (200 rpm) overnight. 2 ml of the cell culture was centrifuged at 10,000 rpm and the cell pellet was transferred into 500 ml flask containing 100 ml phytase production media and incubated further at 30°C with shaking to induce the cells to produce phytase. Samples of 1 ml of the cell culture were taken out for turbidity measurement at OD_{600} and for Pi measurement at every 2 h. For determination of optimum time for phytase production, 1 ml of the culture was sampled every 24 h and the supernatant from centrifugation was used for enzyme activity assay (Wang et al., 2004).

Determination of protein concentration

In the process of enzyme purification, concentrations of total proteins in all steps were determined by using Bradford method (Bradford, 1976), and the absorbance was measured at 595 nm with a spectrophotometer. The protein concentration was calculated based on a convenient standard curve using bovine serum albumin (BSA).

Purification of the phytase enzyme

The cell-free culture supernatant was dialyzed and concentrated using the Labscale TFF filtration system (Millipore, Bedford). The dialyzed and concentrated culture was then applied to an anion-exchange chromatographic column (diethylaminoethyl, HiPrep 16/10 DEAE-Sepharose FF, Pharmacia, Sweden) equilibrated with 20 mM Tris-HCL buffer pH 8. After washing the column, the bound enzyme was eluted at flow rate of 1 ml/min by using a linear gradient from 0 to100% of 1 M NaCl in 20 mM Tris-HCL buffer pH 8. Fractions were collected in volumes of 5 ml. Fractions containing phytase activity were pooled together, dialyzed and concentrated using the Labscale TFF filtration system (Millipore, Bedford). The concentrated enzyme was applied to gel filtration column (HiPrep 16/60 Sephacryl S-100 HR, Pharmacia, Sweden) pre-equilibrated with 50 mM phosphate buffer and 200 mM NaCl at pH 8, and eluted by using the same buffer at flow rate 1 ml/min. Fractions were collected every 5 min and that of high phytase enzymatic activity were collected and pooled together. The pooled fractions were subjected to dialysis procedure using the ultrafiltration tubes at a speed of 5000 rpm for 20 min at 4°C in Centricon 10 (Amicon, USA) ultrafiltration concentrators (membrane cut off of 10 kDa). During all the purification procedures, all collected and pooled fractions were tested for absorption (wavelength 280 nm), total protein (wavelength 595 nm), and phytase activity (wavelength 355 nm).

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% running gel as described by Laemmli (1970) and the resolved protein visualized by Commassie Brilliant Blue staining. The process of the electrophoresis was conducted using mini-Protein II Slab cell (Bio-Rad Company). An unstained broad range protein standard ranging from 14 to 116 kDa (Jena Bioscience, Germany) was used in order to detect the molecular mass of our purified phytase enzyme. Finally the gel was photographed by using gel documentation system (Syngene, G: Box, USA).

The effect of temperature on the phytase enzyme activity

Phytase enzyme was incubated with the substrate in a concentration of 10 and 100 mM sodium acetate buffer at pH 5 over a temperature range of 30 to 60°C for 30 min to find out the optimum temperature for the enzyme activity. On the other hand, temperature stability was assayed by incubating the enzyme with 100 mM sodium acetate buffer pH 5 at temperature range 30-70^{o}C for different incubation periods (0, 1, 4, 8, and 24 h). After that, the 10 mM substrate concentration was added at pH 6 and the enzyme activity assay was carried out at 37°C for 30 min (El-Toukhy, 2001).

The effect of pH value on the phytase enzyme activity

In order to determine the optimum pH value, phytase activity was measured by incubating with the mixture of 10 mM substrate concentration and 100 mM of different buffers in a pH-value ranged between 2 to 8 and the incubation was done at 37°C for 30 min. The pH stability was determined by mixing the purified phytase with 100 mM of different buffers in different pH values that ranged between 2 to 8 and then the mixture were incubated for 0, 1, 4, 8 and 24 h. The pH stability was determined at room temperature (25°C). The phytase activity at each pH-value was calculated as a percentage of the phytase activity of the certain pH-value at the initial time (El-Toukhy, 2001).

Determination the denaturation rate of the phytase enzyme activity

Phytase enzyme was heated in 100 mM sodium acetate buffer pH 5 at 50, 60, 70, 80 and 90°C for 0 to 100 min, cooled and then sodium phytate was added to a final concentration of 10 mM (El-Toukhy, 2001).

The effect of substrate and enzyme concentrations on the phytase enzyme activity

Different substrate concentrations that ranged from 100 up to 1100 nmole were used to determine the kinetics data. According to the method of Lineweaver and Burk (1934), values of maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) were determined for the substrate. To study the effect of different enzyme concentrations on the activity, the same enzyme assay was used except for enzyme concentration. Enzyme concentrations used were 0.01, 0.02, 0.1, 0.2 and 0.3 µg/ml and 10 µl of such enzyme concentrations were tested separately.

The effect of different divalent metal ions on the phytase enzyme activity

The effects of metal cations, in the form of metal salts, on phytase enzyme activity were tested. These metal salts were $CaCl_2$, $MgSO_4$, $CuSO_4$, $FeSO_4$. 10 µl of each metal salt was added at concentration of 0.5, 1, 1.5, 2, 5 and 10 mM to the mixture of the purified phytase. The phytase activity value for each metal salt at different concentrations was taken as a percentage of the phytase activity without adding the metal salt (El-Toukhy, 2001).

Immobilization of *B. subtilis* cells

3 ml of sterile distilled water was added to a 24 h old slant of *B. subtilis*. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension at 10% level was transferred-aseptically into 250-mL conical flasks containing 45 mL of sterile inoculum medium (PPM). The flask was kept in a shaker incubator at 200 rpm at 30°C. The contents of the flasks were centrifuged at 3000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0 g/L potassium chloride solution, followed by sodium chloride solution and sterile distilled water subsequently. Finally, the cell mass was suspended in sterile sodium chloride solution (0.9%). This cell suspension was used as inoculum for immobilization (Adinarayana et al., 2005). Immobilization by calcium alginate was performed according to the method of Johnsen and Flink (1986), while immobilization by agar-agar was done according to the method described by Veelken and Pape (1982), and immobilization by polyacrylamide was done according to the method described by Kumar and Vats (2010).

RESULTS AND DISCUSSION

Preliminary screening of phytate-degrading bacteria

In the preliminary screening program for isolation of phytase-producing bacteria, many isolates were collected from soil of New Borg El-Arab city, Alexandria, Egypt. One *Pseudomonas* isolate and three *Bacillus* isolates were identified morphologically. Qualitative determination of phytase enzyme showed that the *Pseudomonas* isolate did not produce a detectable clear zone, therefore, it was excluded from further work. The three species of *Bacillus* isolate, namely S1, S2 and S3, showed clear zones with different sizes on PSM.

Evaluation of the higher phytase-producing isolate

The three colonies, which showed clear zone, were cultivated separately on PSM media. The highest Phytase producing isolate, namely isolate S3 was picked up based, on quantitative estimation of its phytase production, and sub-cultured in PPM. The bacterium was induced to produce extracellular phytase when grown in minimum medium containing phytate as the sole source of phosphorus. The induce enzyme production was induced in parallel to the amount of phosphorus released into the broth and reached a plateau around days 4 to 5 (data not showed). Thus, the amount of phosphorus released reflects the amount of enzyme produced (Wang et al., 2004; Xueying, 2003).

Molecular identification of the isolate

Bacillus isolate (S3) was subjected to molecular identification using 16S rRNA. The 16S rRNA gene was amplified by PCR using 16S rRNA. The PCR product, with reference to the marker used, was estimated to be approximately 1200 bp. Partial nucleotide sequence of 16S rRNA gene was done as shown in Figure 1, and deposited into GeneBank with the accession number (ac: JQ045381). The sequence analysis revealed a close relation to *B. subtilis* MJA; Class: Bacilli, Order: Bacillales, Family: Bacillaceae. By conducting the phylogenetic tree of *B. subtilis* MJA with reference to 16S rRNA, the gene sequence indicated the degree of similarity between our isolate and other species.

Optimization of growth parameters of phytase-producing *Bacillus subtilis* MJA

The combination of 0.5% glucose plus 0.5% sucrose was the best carbon source when compared with original PPM medium after incubation for 2 days (Figure 2). Also, the medium containing malt extract, as a nitrogen source,

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   1 ggcgcattag ctagttggtg aggtaatggc tcaccaaggc aacgactggc atttgcatgc
  61 actgcattgc atgcgtagcc gacctgagag ggtgatcggc cacactggga ctgagacacg
 121 gcccagactc ctacgggagg cagcagtagg gaatcttccg caatggacga aagtctgacg
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 241 acaagtaccg ttcgaatagg gcggtacctt gacggtacct aaccagaaag ccacggctaa
 301 ctacgtgcca gcagccgcgg taatacgtag gtggcaagcg ttgtccggaa ttattgggcg
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 481 gtgaaatgcg tagagatgtg gaggaacacc agtggcgaag gcgactctct ggtctgtaac
 541 tgacgctgag gagcgaaagc gtggggagcg aacaggatta gataccctgg tagtccacgc
 601 cgtaaacgat gagtgctaag tgttaggggg tttccgcccc ttagtgctgc agctaacgca
 661 ttaagcactc cgcctgggga gtacggtcgc aagactgaaa ctcaaaggaa ttgacggggg
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 781 cttgacatcc tctgacaatc ctagagatag gacgtcccct tcgggggcag agtgacaggt
 841 ggtgcatggt tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc
 901 aacccttgat cttagttgcc agcattcagt tgggcactct aaggtgactg ccggtgacaa
 961 accggaggaa ggtggggatg acgtcaaatc atcatgcccc ttatgacctg ggctacacac
1021 gtgctacaat ggacagaaca aagggcagcg aaaccgcgag gttaagccaa tcccacaaat
1081 ctgttctcag ttcggatcgc agtctgcaac tcgactgcgt gaagctggaa tcgctagtaa
1141 tcgcggatca gcatgccgcg gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca
1201 ccacgagagt ttgtaacacc cgaagtcggt gaggtaacct tttaggagcc agccgccacg
1261 atgctacgta cgt
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Figure 1. *Bacillus subtilis* MJA 16S ribosomal RNA gene, partial sequence.

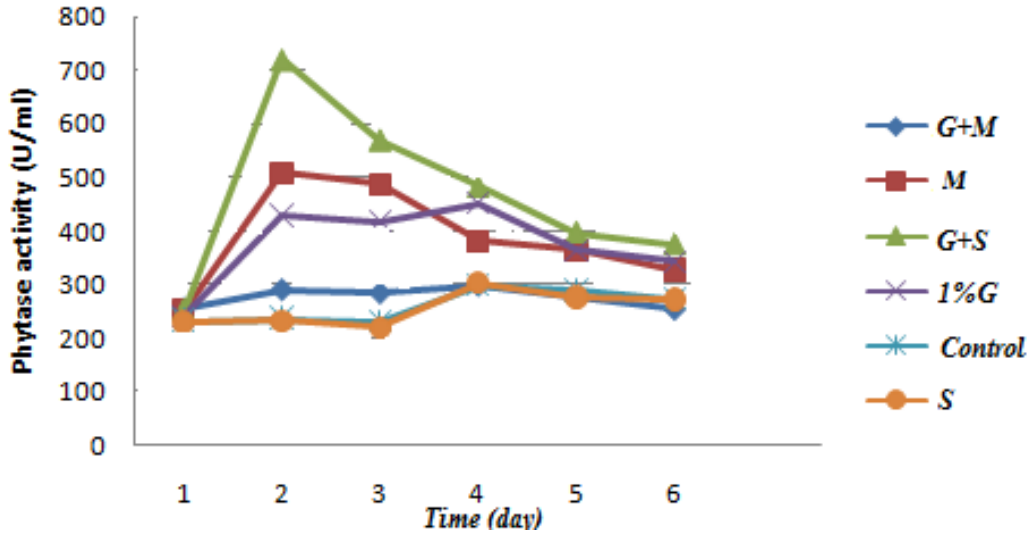

Figure 2. Optimization of culture condition (carbon source) of the isolated *Bacillus subtilis* MJA. G, glucose; S, sucrose; G+S glucose + sucrose; G+M, glucose+maltose; M, maltose. Result shows that the best carbon source was G+S which produced 720 U/ml phytase after 2 days.

gave the highest phytase production (716 U/ml) after incubation for 4 days as compared with the original PPM medium (Figure 3).

Regarding the optimization of phytase production using the pH values, Figure 4 shows that the highest phytase production (720 U/ml after 4 days incubation) was obtained by using the original PPM medium pH 7. Also, in the present study, growth curve for *B. subtilis* MJA was drawn in the optimized media for phytase production. The maximum production rate of phytase enzyme was after about 36 to 60 h. The overall results for optimizing our strain *B. subtilis* MJA towards phytase production are in agreement with those of previous studies (Gulati et al., 2007; Hosseinkhani et al., 2009; Kammoun et al., 2011).

Purification of the phytase enzyme

Table 1 shows that during the purification steps, the total phytase activity was reduced to about 416 U (about

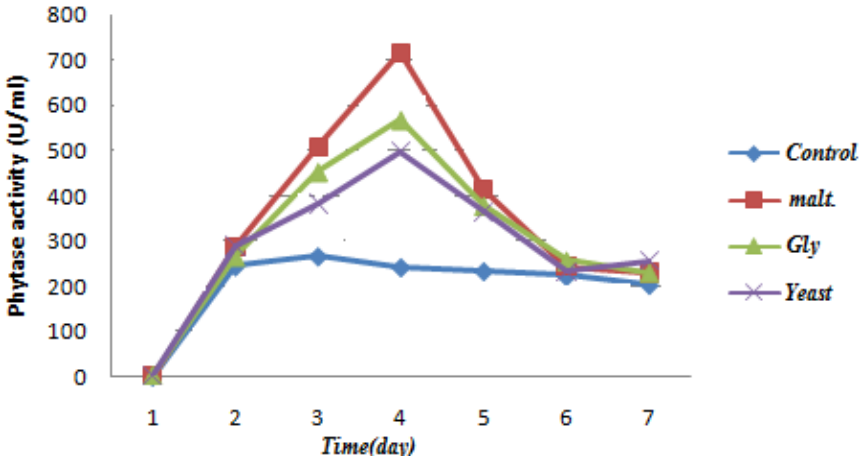

Figure 3. Optimization of culture condition (nitrogen source) of the isolated *Bacillus subtilis.* MJA. Malt, malt extract; Yeast, yeast extract; Gly, glycine. Result show that in medium with malt extract as nitrogen source, phytase production was in maximum (value of 716 U/ml recorded after 4 days incubation).

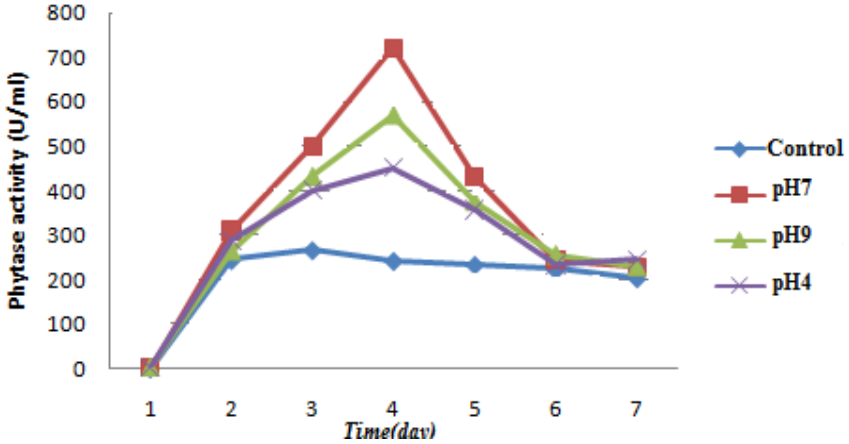

Figure 4. Optimization of culture condition (pH) of the isolated *Bacillus subtilis* MJA. Result shows that in medium adjusted at pH 7, phytase production was maximum (value 720 U/ml recorded after 4 days incubation).

Table 1. Purification scheme of phytase from *Bacillus subtilis MJA.*

Purification step	Phytase (U)	Total protein activity (mg)	Specific (U/mg)	Yield activity (%)	Purification (fold)
Crude enzyme	721	682	1.075	100	1
16/10DEAE-Sepharose FF	510	254	2.008	70.54	1.87
16/60 Sephacryl S-100 HR	416	98	4.244	57.70	3.95

57.7% of the whole phytase activity in the culture filtrate) in comparison with the initial phytase activity (about 721 U), and also the total protein content reduced to 98 mg in comparison with the initial protein content of 682 mg. However, the specific activity at the end of the purification steps was found to be almost 4.24 U/mg as compared to 1.08 U/mg at the culture filtrate. Also, phytase enzyme at the end of the extraction and purification steps was purified to almost 4-fold. Using ion-exchange chromate-graphy such as the anion DEAE-Sepharose technique formed the main purification part and recovered about 70.5% of the total phytase enzyme with almost 1.9-fold

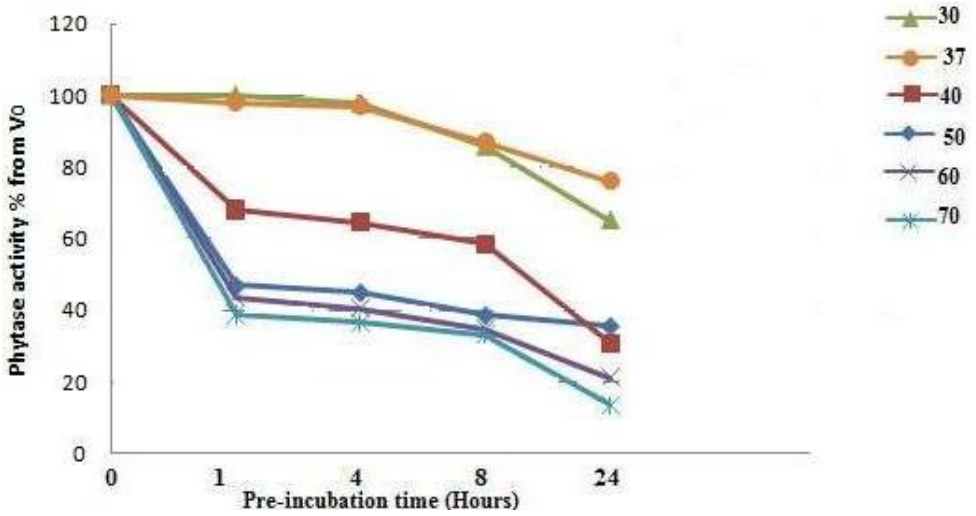

Figure 5. Study of the thermal stability for the purified phytase enzyme produced by *Bacillus subtilis* MJA.

of purification (Table 1). Previous studies demonstrated the use of ion-exchanger chromatographic methods for purification of phytase enzyme (Greiner et al., 1997, 1998, 2000 and 2009). Gel filtration technique, as a last purification step, was used in order to get a highly purified phytase enzyme which was found to be only 57.7% of the original enzyme and with a 4-fold purification (Table 1). Gel filtration was used as a purification method for phytase from different sources in previous studies (Greiner et al., 1998 and 2000).

Molecular mass of the purified phytase enzyme

Determination of the purified phytase enzyme using SDS-PAGE electrophoresis with Commassie Brilliant Blue staining is considered for determining the molecular mass of the purified enzyme and indicating the enzyme purity. The molecular mass of the purified phytase enzyme was estimated to be about 38 kDa. Phytases with different molecular masses were obtained from various sources. Phytases in molecular mass range of 36 to 40 kDa were obtained bin previous studies (Greiner et al., 1997; Powar and Jagannathan, 1982; Valikhanov et al., 1981). In other investigations, phytases with molecular masses of 200 and 92 kDa were obtained from *Aspergillus niger* (Skowronski, 1978), and *Aspergillus fumigatus* (Rodriguez et al., 2000).

Effect of temperature on phytase enzyme activity and stability

Phytase activity was determined at different temperatures from 30 to 60°C. The optimum temperature for enzyme activity was found to be about 37°C. Some investigators showed results on optimal temperature from different microbial sources, which were in agreement with our results (Greiner et al., 1997 and 2009; Miao et al., 2013; Pavlova et al., 2008). On the other hand, studying the enzyme thermal stability showed that the enzyme was stable at low temperatures (Figure 5). For example, incubating the enzyme at 40°C for 8 h resulted in loss of about 35% in the activity in comparison with the initial phytase activity while, incubating the enzyme at the same temperature for 24 h inhibited the enzyme activity to 75% from the initial activity (Figure 5). Also, increasing the incubation temperature above 60°C resulted in inhibition of enzyme activity by about 80% from the initial incubation temperature after 24 h. Other studies (Greiner et al., 1997) showed that phytases obtained from different microorganisms had higher optimal temperature with more thermal stability than that obtained in our study.

Effect of pH on phytase enzyme activity and stability

The purified enzyme had a preference to work over a wide range of pH 3 to 7, where an optimum pH plateau was observed in range of pH from 5 to 6. The enzyme retained more than 80% of its activity over a wide pH range of 2 to 8 for 4 h (Figure 6). While, exposing the enzyme to pH values 2 or 8 for 24 h, resulted in an inhibition in the enzyme activity of about 50% of its initial activity. Our results are highly similar to the literature results for optimal pH (Menezes-Blackburn et al., 2011; Xueying, 2003) and for pH stability (Mittal et al., 2011).

Determination of the denaturation rate of the phytase enzyme

Phytase enzyme activity decreased with increasing pre-incubation time within the same pre-incubation temperature. Pre-incubating the phytase enzyme at temperature

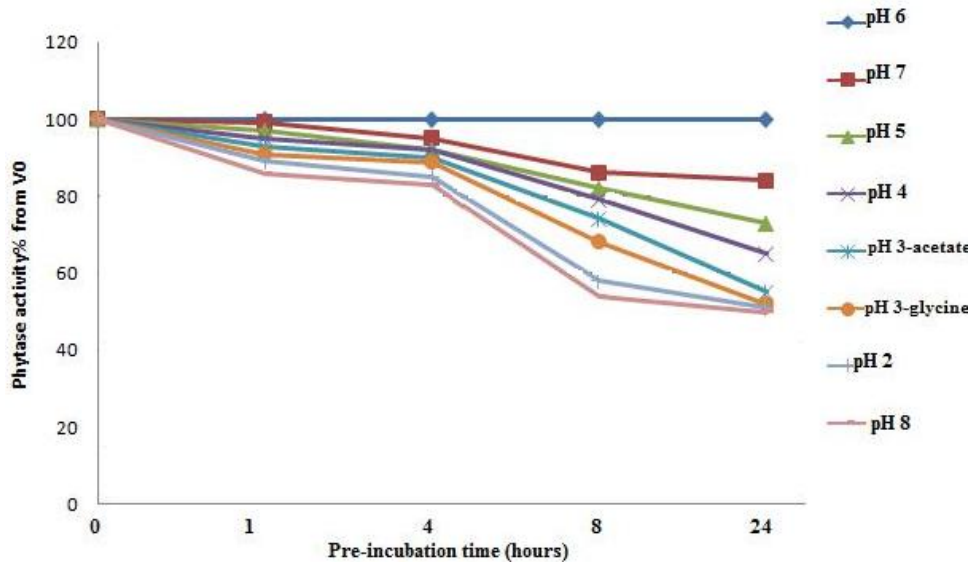

Figure 6. Study of pH stability for the purified phytase enzyme produced by *Bacillus subtilis* MJA.

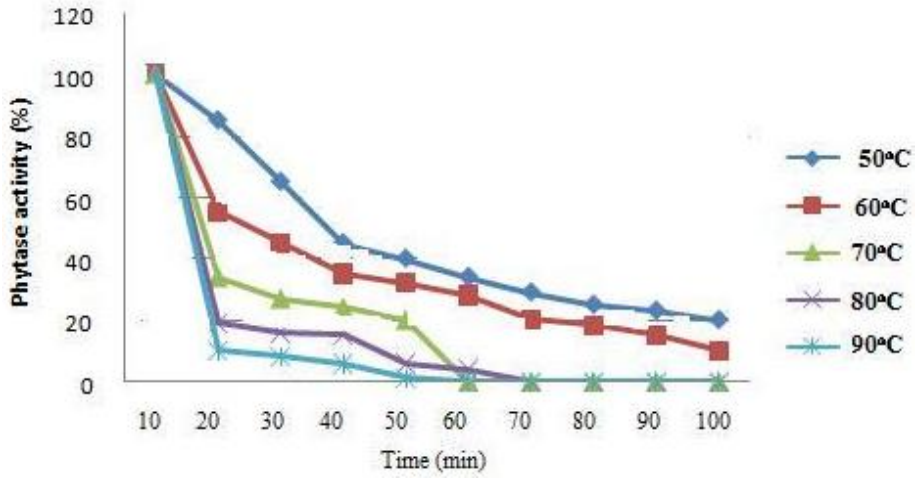

Figure 7. Study of the denaturation of phytase enzyme from *Bacillus subtilis* MJA. X-axis, Time (min); Y-axis, Phytase activity (%).

of 70°C after 10, 20, 30, 40 and 50 min resulted in an inhibition in the activity to 34, 27, 24, 20 and 2% respectively while, pre-incubation of the enzyme for more than 50 min resulted in a complete denaturation of phy-tase enzyme (Figure 7). On the other hand, it was obser-ved that the phytase enzyme activity also decreased with increasing pre-incubation temperature within the same pre-incubation time. Continuing pre-incubation of the phytase enzyme at temperature more than 70°C resulted in a complete denaturation of the phytase enzyme (Figure 7). This result is in agreement with those of previous studies (El-Toukhy, 2001; Farhat et al., 2008) which showed that the inactivation-time relationship for both extracellular and intracellular phytases obtained from *Rhizopus oligosporus* persisted after heating at 55 and 65°C.

Effect of substrate concentration on phytase enzyme activity

Phytase enzyme activity gradually was increased by increasing the substrate concentration up to 10 mM. Increasing the substrate concentration more than 10 mM resulted in an inhibition in the phytase enzyme activity. Similarly, previous studies showed that the activity of other phytases extracted and purified from different regions was inhibited by increasing substrate concentration (Roy et al., 2012).

Table 2. Effect of metal ions on enzyme activity.

Concentration (mM)	Phytase activity (U/ml x 10^3)			
	Mg^{+2}	Ca^{+2}	Cu^{+2}	Fe^{+2}
0.5	0.7	0.85	0.29	0.34
1	0.9	1.2	0.24	0.28
1.5	0.5	0.7	0.19	0.24
2	0.42	0.64	0.18	0.14
5	0.23	0.3	0.03	0.05
10	0.02	0.05	0.001	0.004

According to Lineweaver-Burk, the maximum velocity rate (V_{max}) was found to be 510 U/mg, and this result was similar to the results obtained by Sasirekha et al. (2012). The Michaelis-Menten constant (K_m) in our investigation was found to be 0.485 mM. This result was close to that obtained in other investigations (Shamna et al., 2012).

Effect of enzyme concentration on phytase enzyme activity

The maximum activity of phytase (420 U/ml) was obtained by using phytase concentration of 0.1 gm/ml. These results are similar to the results in other studies (Escobin-Mopera et al., 2012).

Effect of different divalent metal ions on phytase enzyme activity

Table 2 shows that Cu^{2+} or Fe^{2+} showed an inhibition effect on the phytase activity and the effect was dose dependent. On the other hand, cations such as Mg^{2+} or Ca^{2+} showed an increase in the phytase activity till addition of 1.5 mM where the phytase activity began to decrease. Our results are in strong agreement with the results of previous studies (El-Toukhy, 2001; Park et al., 2012).

Immobilization of Bacillus cells for phytase production

Figure 8 shows that agar-agar immobilized matrix provided the highest enzyme activity of about 800 U/ml and relatively higher than the activity of free cells which was found to be 320 U/ml during production time period of 24 h. The polyacrylamide matrix showed a less activity compared to agar-agar matrix which was found to be 517 U/ml. Finally, calcium alginate matrix showed the lowest activity as compared to the other two matrixes, which was found to be about 480 U/ml (Figure 8). Adinarayana et al. (2005) studied the effect of calcium alginate, agar-agar, and polyacrylamide matrices and found that calcium alginate was an effective and suitable matrix for higher alkaline protease productivity compared to the other matrices studied. Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not the least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (Abd-El-Haleem et al., 2003; Singh and Satyanarayana, 2011).

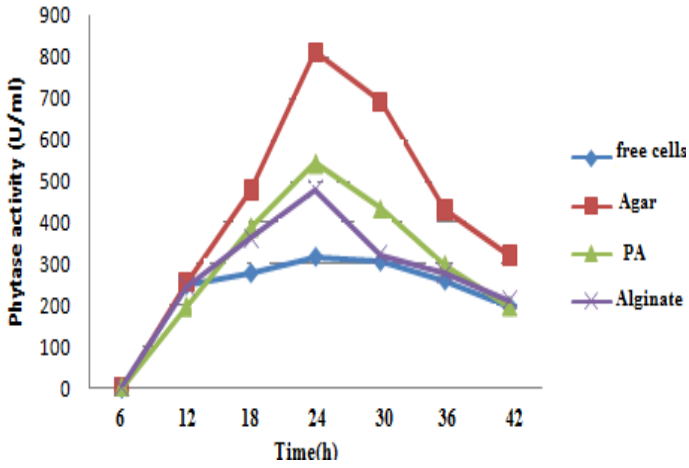

Figure 8. Time course of phytase production by immobilized cells of *Bacillus subtilis* MJA in different matrices. PA, Polyacrylamide matrix; agar, Sodium alginate. The best matrix for high phytase production is agar, polyacrylamide and then sodium alginate respectively. Immobilization increased the enzyme activity compared to free phytase.

Conclusion

It can be concluded from the results obtained from our investigation on the phytase obtained from *B. subtilis* MJA, that the enzyme is suitable for biotechnical purpose. Also, as a phytate-degrading enzyme, it can be considered a promising candidate to be used in many applications especially in food industries. In addition, phytase production by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of cultivation, in addition to some specific advantages such as long life-term stability, and reusability.

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