Archive of SID

http://dx.doi.org/10.22037/afb.v%vi%i.23398

Research Article

Applied Food Biotechnology

APPLIED FOOD BIOTECHNOLOGY, 2019, 6 (3):139-150
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Immobilization of Phytase Producing Probiotics in Shrimp Chitosan Cross-linked by Zinc Oxide Nanoparticles and Assay its Antibacterial Activity

Nargess Chatraei[1], Giti Emtiazi[1,2*]

1- Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran.

2- Department of Biotechnology, Faculty of Biological Science and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran.

Abstract

Background and objective: Phytase is used in human and poultry additives. This enzyme is mostly produced by *Aspergillus niger* which is a plant pathogen. Phytase from probiotics is a good candidate for the food supplements.
Materials and methods: *Bacillus coagulans*, as a probiotic, was used for phytase and phosphatase activities on phytin agar and Pikovskayas Agar. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymogram analyses of the extracted phytase enzyme were carried out. Probiotic cells with phytase activity were immobilized on chitosan extracted from shrimp shells and the efficiency was investigated and analyzed using scanning electron microscopy and Fourier transform infrared.
Results and conclusion: In this study, *Bacillus coagulans* showed intracellular phytase activities from broken cells with Soluble Index of 2.5 on phytase specific media. Iron and zinc oxide nanoparticles accelerated the enzyme activity by 25%. Cells were precipitated using ZnO-chitosan nanoparticles and the enzyme activity was investigated on gels. Chelation of chitosan-metal ion increased the positive charge density of chitosan which was expected to enhance adsorption of zinc and teichoic acid on Gram-positive *Bacillus coagulans*; approved by SEM and FTIR. Cells immobilized on ZnO-chitosan promoted the enzyme activity by 28,800 U ml^{-1} gel. The entrapped cells were resistance to high temperature and pH. This complex not only included activities against *Streptococcus agalactiae*, but also dissolved insoluble phosphate and phytin, which has made this complex a good candidate for use as additive in human and animal foods.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:
Received 18 Nov 2018
Revised 20 Feb 2019
Accepted 16 March 2019

Keywords:
- Chitosan immobilization
- Phytase
- Probiotic
- Zinc oxide nanoparticles

***Corresponding author:**
Giti Emtiazi,
Department of Biotechnology, Faculty of Biological Science and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran.

Tel: +98-31-37932457
Fax: +98-31-37932456
E-mail: emtiazi@sci.ui.ac.ir

1. Introduction

Phytases are a special group of phosphates that carry phosphate-phase hydrolyses from the phytate molecule. Phytate is the major source of phosphate storage in plant seeds. These enzymes are excreted from plants, micro-organisms and animals; of which, microbial phytases are promising and worth considering. Phytases are added to the diets of single-gastric animals such as pigs, birds and fish to enhance the absorption of phosphate in the diet as well as to decrease phosphate contamination in the environment. Nearly 20 years ago, the first commercial product of phytase was introduced to the market. Typically, phytases act as a single molecular-weight enzyme of 40-100 kDa. Up to date, two major groups of phytases have been identified, including acid phytases with an optimum pH of 5 and alkaline phytases with an optimum pH of 8. Most phytases include optimum pH and temperatures of 4.5-6.5 and 45-60°C, respectively. Some phytases include effects on a wide range of substrates and hydrolyze phytate-free metals such as phytate-calcium. Phytases vary in terms of number of phosphate groups they can release. They are usually able to release 3-5 phosphate groups from the phytate molecule. Phytate produces insoluble complex with metal cations (e.g. zinc, magnesium and iron) vitamins and proteins; therefore, decreasing adsorption of these cations [1,2]. Phytases are a group of phosphatases that degrade phytate from cereals and release phosphorus from phytin. Therefore, production of inositol derivation can be used for the treatment of diseases such as Parkinson, Alzheimer and multiple sclerosis (MS) [3]. Phytases, such as other enzymes, require conditions for more sustainability and stability. Immobilization of the phytase enzyme can be a promising

www.SID.ir

Archive of SID

solution. Some important properties of a support for immobilization in food, medical and agricultural industries include non-toxicity and biocompatibility. Moreover, the support must not pose threats to human health and environment. Biodegradability and cost-effectiveness are other desirable properties of the selective materials [4]. Phytase enzyme or phytase producing cell has been immobilized on various materials such as alginate hydrogel, carrageenan and chitosan [5-7].

Of many carriers studied for the immobilization of enzymes, chitin and chitosan are cheap and natural polymers that include excellent biodegradability, non-toxicity and biocompatibility. Their hydroxyl and amino groups can react with functional groups, which is an excellent feature for the carrier of an electrochemical enzyme biosensor. Since chitosan is produced from the hard shells at low costs, it can be interested economically and global availability. Therefore, unique properties of chitin and chitosan include biocompatibility, biodegradability to non-toxic products, non-toxicity, physiological inertness, remarkable affinity to proteins and anti-fungal, anti-bacterial, anti-tumor and anti-cholesterol properties. They include a high potential for use in various industries [8-10]. In addition to direct use of phytase enzyme, phytase producing microorganisms can be added directly to diets as probiotics due to the health benefits [3]. Studies have reported several phytase producing probiotics, including Bifidobacterium, Lactobacillus, *Saccharomyces cerevisiae*, *Yarrowia lipolitica* and *Candida tropicalis* [1,2,11-14]. These studies have often been carried out on anaerobic bacteria and yeasts. However, in the current study, the phytase activity of Bacillus is reported for the first time. Use of probiotics due to their antimicrobial or antifungal activities is of interests of many researchers [15]. The aims of the current study included immobilization of phytase producing probiotics in biocompatibility matrices such as chitosan with prebiotic effects. The probiotic strain with antibacterial activity is an aeration strain that include a high phytase activity in immobilized mode. This makes the probiotic strain a good candidate for the additives of human and animal foods.

2. Materials and methods

2.1. Culture and media

Media for the growth and solubilization of phytin test included phytase specific media (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% $FeSO_4$ and 0.001% $MnSO_4$ with 0.5% sodium phytate, pH 7) (Sigma, USA) [16,17].

2.2. Chitosan extracted from shrimp shells

The *Penaeus semisulcatus* shrimp shells were purchased from local stores. The extra tissues were separated from the shells and then washed with cold water. Shells were completely dried and then cut into pieces of nearly 0.5 mm^2. Generally, the process of converting chitin to chitosan consisted of three major steps of demineralization, deproteinization and deacetylation as follows.

Demineralization. Initially, 25 g of the prepared chitin were added to 1% hydrogen chloride solution and mixed. The mixture was agitated for 24 h at room temperature to remove the minerals. This was then washed with deionized water and dried.

Deproteinization. To remove protein contents of the sample, the previous treatment was soaked in 50 ml of 3% sodium hydroxide solution for 1 h.

Deacetylation. The remaining powder of the high-lighted process was soaked in 50% sodium hydroxide solution and boiled at 100°C for 2 h. The compound was cooled down for 30 min in a fume hood and washed with deionized water. To remove residual sodium hydroxide, the compound was boiled at 100°C for 5 min in deionized water and dried at 60°C for 12 h in an oven [18].

2.3. Qualitative and quantitative assays of phytate degrading ability and estimation of bacterial growth

Diameter of the halo zone around the colony in Phytas specific agar medium (PSM) was measured. The strain was placed on the medium using sterile toothpicks and incubated at 28°C for 14 days. The halo zones and sizes of the colonies were recorded daily [19]. The following formula was used to calculate the Solubility Index (SI):

$$\mathbf{SI} = \frac{\textbf{colony diameter} + \textbf{halo zone diameter}}{\textbf{colony diameter}}$$

The higher solubilization index means the higher ability of dissolving phytin [16]. Phytate degrading ability of the strain was assessed by measuring the quantity of released inorganic phosphate in the phytase specific media. The bacteria was inoculated in 50 ml of phytase specific media and incubated at 37°C for three days at 200 rpm using rotary shaker. To measure the quantity of released inorganic phosphate 0.5 ml of the culture supernatant, 0.5 ml of molybdite-vanadate reagent and 4 ml of distilled water were used. After 10 min, the absorbance was measured at 450 nm and the concentration of released inorganic phosphate was calculated based on the standard curve. To cultivate *Bacillus* (*B.) coagulans* strain and estimate the bacterial growth, the bacterial strain was inoculated into 50 ml of phytase specific media and incubated at 37°C for one week at 200 rpm using rotary shaker. The optical density (OD) was measured at 600 nm during the week. To dissolve the residual insoluble phosphate, 1 N HCl solutions were added to each sample (1:1 v:v). Furthermore, pH values were measured during the growth of the bacterial strain [16].

www.SID.ir

Archive of SID

2.4. Phytase production

Since the maximum growth of this strain was seen after 30 h; therefore, phytase production was assessed at the maximum bacterial growth of 30 h. The isolated strain was inoculated in 50 ml of phytase specific media in a flask and incubated at 37°C for 30 h at 200 rpm using rotary shaker. Broken cell suspensions were used for phytase activity.

2.5. Enzyme assay

Phytase activity was assessed by measuring the quantity of released inorganic phosphate based on a method by Harland and Harland [20]. To assess the phytase activity, 0.2 ml of the broken cell suspension (cells were broken using ultrasound by 4 times destruction in 30 s) was separately added to the tube and then mixed with 0.8 ml of acetate buffer solution (0.2 M, pH 5.2, containing 1 mM of phytin). This was incubated at 37°C for 30 min. Then, quantities of the released phosphorus were calculated using vanadate molybdate reagent after 30 min. One enzyme unit (IU) was suggested as the quantity of the enzyme releasing 1 μmol of inorganic phosphate in 1 min under the assay conditions.

2.6. The SDS-PAGE and phytase zymogram analyses

Briefly, loading buffer was added to the sample in a microtube. The loading buffer contained 62.5 mM of tris buffer (pH 6.8), 0.05% of bromophenol blue, 0.72 M of 2-b-mercaptoethanol, 10% of glycerol and 2% of sodium dodecyl sulfate. This microtube was heated using boiling water bath for 5 min to denature samples. This was followed by electrophoresis on 4% stacking and 10% separating gels. Gels were stained by submerging in 0.1% (w v^{-1}) of Coomassie brilliant blue to detect protein bands and distained in an aqueous methanol (40%) and acetic acid (10%) solution [21]. The modified zymogram method was based on a method by Bae et al. [21]. To achieve zymograms, gels were soaked in 1% of Triton X-100 at room temperature for 1 h and then in 0.1 M of sodium acetate buffer (pH 5.0) at 4°C for 1 h. To assess the phytase activity, gels were incubated in a 0.1 M sodium acetate buffer solution (pH 5.0) containing 0.4% (w v^{-1}) of sodium phytate for 16 h. Then, gels were immersed in a 2% (w v^{-1}) aqueous cobalt chloride solution at room temperature for 5 min. The cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w v^{-1}) aqueous ammonium molybdate and 0.42% (w v^{-1}) ammonium vanadate solutions. Protein bands with phytase activity were visualized by submerging the gels in a Coomassie brilliant blue solution and distained as described previously.

2.7. Cell entrapment with chitosan

To entrap cells with phytase activity, 1 ml of chitosan (0.5%) and 1 ml of Tripolyphosphate (TPP) solutions (0.5%) were respectively added to the tube per ml of the cell suspension. This resulted in gel formation and cell entrapment within 30 min [19]. To entrap cells in chitosan cross-linked with zinc oxide nanoparticles (CEC-Z), 1 mg l^{-1} of the zinc oxide nanoparticles was added to the cell suspension and mixed well. Then, chitosan solution was added to the mixture and mixed well. In this step, tripolyphosphate was added to the mixture and mixed well and set for 30 min to form the gel.

2.8. The FTIR analyses of cross-linked ZnO

Fourier transform infrared spectroscopy (ATR-FTIR) (JASCO, FT/IR-6300, Japan) was carryout to characterize the chemical structure of chitosan cross-linked with zinc oxide nanoparticles, compared with control sample (chitosan gels without nanoparticles).

2.9. The SEM images of the cell entrapment

Morphology of the cells entrapped in chitosan gels and chitosan cross-linked with zinc oxide nanoparticles was studied using scanning electron microscopy at 12.0 kV (SEM) (Philips, XL30, the Netherlands) [22].

2.10. Phytase activity analyses of the entrapped cells

First, 0.2 ml of chitosan and 0.2 ml of tripolyphosphate solution were added to 0.2 ml of the cell suspension and set for 30 min. Supernatant was removed and 0.8 ml of acetate buffer (0.2 M, pH 5.2, containing 1 mM of phytin) was added to the suspension and incubated at 37°C for 30 min. Quantity of the released phosphorus was calculated using vanadate molybdate reagent [19].

2.11. The antibacterial activity

To assess the antibacterial activity of CEC-Z, microbial suspensions of four pathogens were prepared. These pathogens included *Staphylococcus aureus*, *Streptococcus (S.) agalactiae*, *S. mutants* and *S. pyogenes*. Several colonies of fresh and 24-h cultures of the bacteria were transferred to Mueller-Hinton broth (MHB) culture media to achieve 0.5 McFarland standard (opacity equivalent to 1.5×10^8 bacteria ml^{-1}). Microbial suspensions were prepared on MHA culture media. A well was created in each of the four culture media to assess the inhibitory zone of the CEC-Z. Then, 50 μl of CEC-Z were added to the wells per plate. All plates were incubated at 37°C for 24 h. After this time, formation of inhibitory zones was assessed and the zone diameters were recorded [23].

3. Results and discussion

3.1. Microorganism

B. coagulans was provided by Persian Type Culture Collection (PTCC). This bacteria is a Gram-positive, catalase positive, spore-forming, motile, and a facultative anaerobe with optimum temperature of 50°C for growth. In other studies, the *B. coagulans* has been introduced as probiotics [24]. This strain grows on PVK media and phytin agar.

www.SID.ir

Archive of SID

3.2. Qualitative and quantitative assay of phytate degrading ability and estimation of bacterial growth

The solubilization index (SI) was calculated as 2.5 improved by 2 weeks of incubation (Table 1). As shown in Fig. 1a less phosphorus was released at 30°C with shaking at 125 rpm. While the best activity of phytin hydrolyzing enzyme was seen at 37°C within 30 h (Fig. 1b). Increased release of phosphorus was shown during the growth. By increasing the incubation temperature, the bacterial strain produced further soluble phosphate as shown in Fig. 1. Since the pH of media was not changed on the media, the soluble phosphate was resulted from the phytase activity.

3.3. Phytase zymogram from cell extracts

Since the cell-free extracts did not include phytase activities, results indicated that this enzyme was intercellular. Furthermore, cell membrane extractions did not show any activities which approved the presence of enzyme inside the cells. Results from the zymogram of the intracellular extracts containing *B. coagulans* phytase showed that the highlighted bands with phytase activity included an 86-kDa molecular weight (Fig. 2).

Table 1. Solubilization index of *Bacillus coagulans* on phytin agar. Halo zones were measured for 14 days. The SI increased after four days.

Day	Colony measurement (mm)	Halo zone measurement (mm)	Solubilization Index
1	0	0	0
2	4	6	2.5
3	6	9	2.5
4	7	11	2.5714
9	12	20	2.6667
14	13	25	2.9231

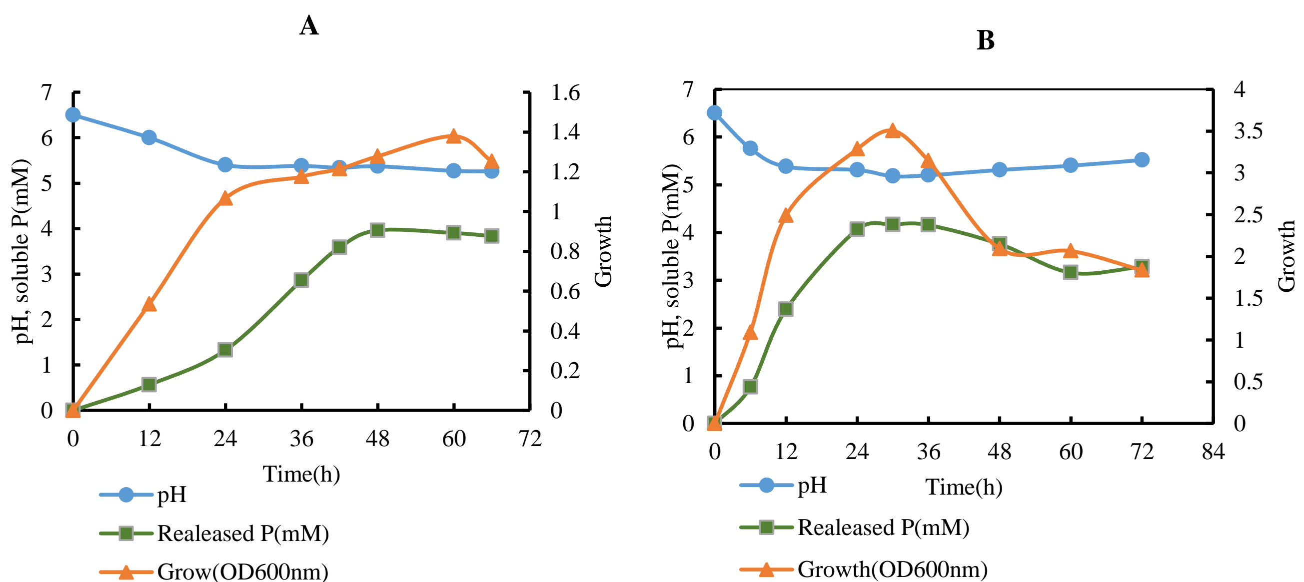


Figure 1. Incubation of *Bacillus coagulans* on phytase specific medium under various conditions. a) Incubation at 30°C with shaking at 125 rpm; b) Incubation at 37°C with shaking at 200 rpm. As figures show, best activities of the phytin hydrolyzing enzyme were seen at 37°C within 30 h

www.SID.ir

Archive of SID

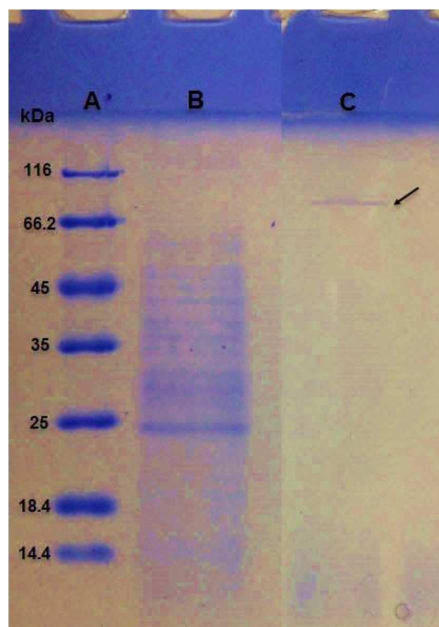


Figure 2. SDS-PAGE gel and zymogram of the intracellular extracts containing phytase enzyme of BH4. A) Protein marker; B) SDS-PAGE bands; proteins in cell extracts of BH4; C) Zymogram gel

3.4. Cell entrapment with chitosan cross-linked to ZnO nanoparticles (CEC-Z) for phytase activity

The enzyme can be immobilized by physically entrapping into a polymer or gel matrix. The pore size is calculated as the enzyme is preserved while the substrate and product are able to pass through the matrix. However, inaccuracies may occur during the immobilization process as results of changes in pH and temperature or addition of solvents. Matrices used to entrap enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicon and resin [25]. In the present study, chitosan was used to form the gel. Shrimp chitosan was solved using acetic acid 1% and the soluble chitosan was gelated using 0.5% TPP. The probiotics were harvested from the supernatants using zinc cross-linked chitosan from shrimp wastes. Nearly 10^8 bacterial cells per ml were entrapped using shrimp chitosan by addition of zinc oxide nanoparticles (1 mg l^{-1}). The possible schematic formation of gel and bacterial entrapment are shown in Fig. 3. The FTIR data showed a shift in wavenumbers 3267 to 3392 cm^{-1} and the wavenumber 524 cm^{-1} showed the amide interaction to ZnO (Fig. 4). Almost all bacterial cells (10^8 ml^{-1}) were entrapped using chitosan-ZnO polymer. The SEM images (Fig. 5a) showed that the nanoparticles of chitosan gel (Chitosan gel without nanoparticles) are not sufficiently coherent. While the nanoparticles of chitosan gel were further compact and better immobilized when ZnO cross-links were used in chitosan to form chitosan-cell polymer (Fig. 5b). The probiotics were entrapped on extracted chitosan using cross-links of ZnO nanoparticles. The antibacterial activities of this polymer have been studied (Table 2). The most effects of this polymer were seen on *S. agalactiae* (Table 2). Two of the most important characteristics of matrices for immobilization for food, medical and agricultural uses include non-toxicity and biocompatibility. Furthermore, these matrices must not include threats to human health and environment. Biodegradability and economically are other desirable properties of the selected matrices.

Table 2. Inhibitory effects of *Bacillus coagulanc* entrapped on chitosan cross-linked to ZnO on Gram-positive pathogens

Pathogen strain	Inhibition zone (mm)
Staphylococcus aureus	3.2
Streptococcus agalactiae	5
Streptococcus mutants	2.5
Streptococcus pyogenes	3.7

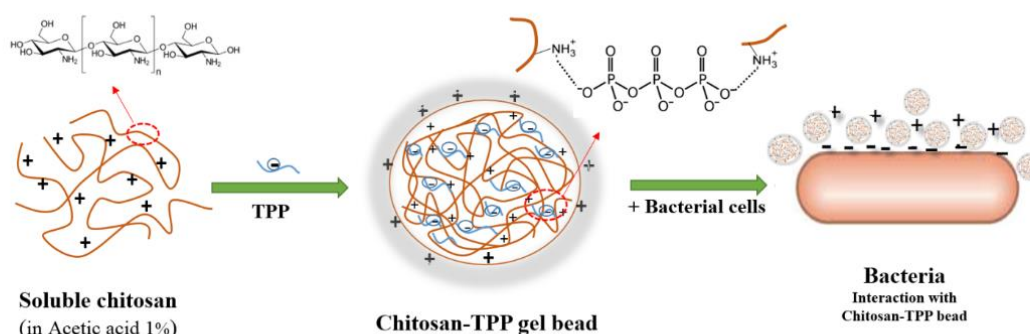


Figure 3. Interaction between chitosan and TPP, formation of chitosan-TPP gelation and their association with bacterial surfaces

www.SID.ir

Archive of SID

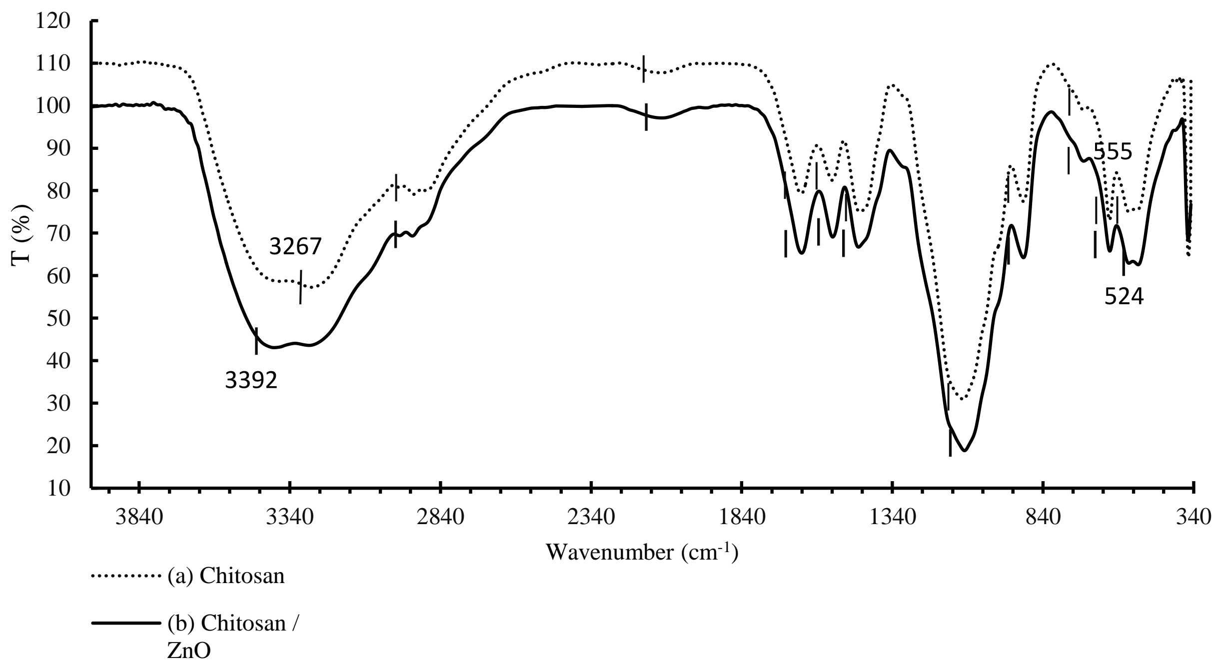
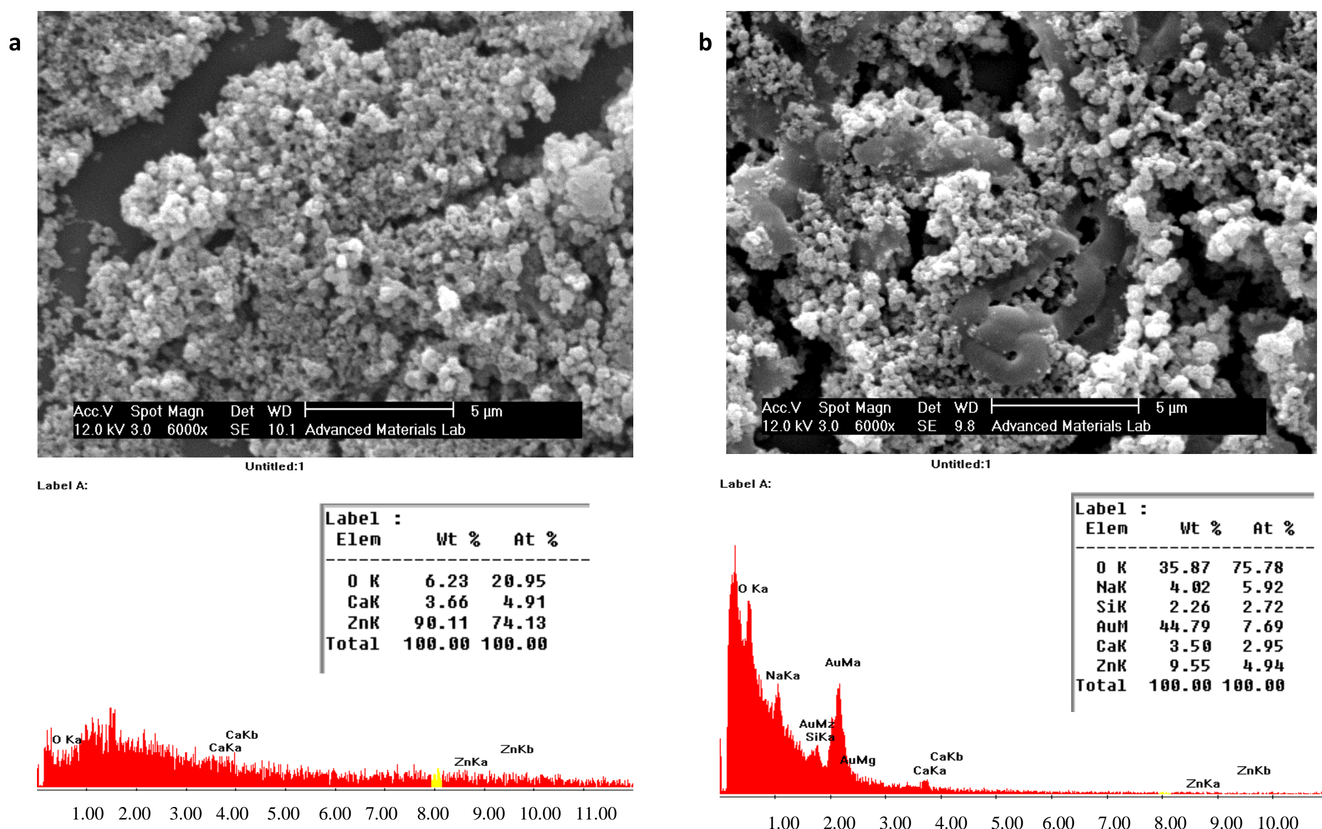


Figure 4. a) FTIR of chitosan; b) Chitosan cross-links with ZnO nanoparticles



Label :

Elem	Wt %	At %
O K	6.23	20.95
CaK	3.66	4.91
ZnK	90.11	74.13
Total	100.00	100.00

Label :

Elem	Wt %	At %
O K	35.87	75.78
NaK	4.02	5.92
SiK	2.26	2.72
AuM	44.79	7.69
CaK	3.50	2.95
ZnK	9.55	4.94
Total	100.00	100.00

Figure 5. The SEM image and EDXS of BH4 strain immobilized on chitosan-TPP gel beads. a) Chitosan gel treated with zinc oxide (1 mg l^{-1}); b) Chitosan gel without nanoparticles. Based on the elemental dispersion analysis (using EDXS device attached to SEM), where zinc oxide nanoparticles are further cross-linked, the particles are further coherent and better immobilized.

www.SID.ir

Archive of SID

Of numerous carriers for the enzymes fixation, chitin and chitosan have been interested by many researchers [4,26]. Chitin and chitosan are natural poly amino saccharides. Chitin is one of the most abundant renewable sources of organic compounds worldwide. Since chitosan is produced from hard shells (mainly crabs and shrimps) as wastes in seafood industries at low costs, it can be used globally [9,27,28]. Chitosan is susceptible to chemical changes due to hydroxyl and reactive amino groups. The solubility in acidic solutions, on one hand, and the accumulation with poly ions, on the other hand, have resulted chitosan to include a certain degree in gel formation. Enzymes have been stabilized on chitosan based substrates in many studies [8]. In the recent study, the chitosan-TPP gel was successfully used to fix and entrap phytase producing bacteria and the enzyme activity was set to approximately 90%. In other studies, this matrix has been used as a stabilization bed for enzymes such as catalase, pectinase, trypsin and proteinase as well as stabilization of bacteria as coating for a better bacterial encapsulation in alginate calcium particles [8,29]. Lata et al. showed that the phytase activity yield of *Aspergillus heteromorphus* included 82% and 87% respectively when entrapped in calcium alginate and carrageenan [6]. Zhang et al. entrapped phytase on altered alginate hydrogels using a transesterifica-tion/silicification method. The immobilized phytase enzyme activity was preserved up to 94% [5]. However, this is a difficult, long and expensive method. Moreover, several chemical reactions are used. Therefore, the method used in the present study was relatively cheap, easy to use, biocompatible and highly active (approximately 90%), compared to other methods. In a study, chitosan with glutaraldehyde as a cross-linking agent was used to immobilize the Geobacillus phytase enzyme. Enzyme was bound to chitosan particles using glutaraldehyde with covalent bonds. In this method, the phytase activity was preserved to eight cycles and resistant to high proteolytic activities, temperatures and pH [7]. Quan et al. immobilized phytase producing *Candida krusei* yeast cells on alginate beads and showed that the immobilization efficiency after an acidification treatment included 77%; preserved for one month. The phytase immobilized cells were further stable at various pH and temperatures [30]. In general, use of chitosan, especially with cross-linking agents, can be an appropriate and effective method for the immobilization of enzymes, including phytase.

3.5. Phytase activity of CEC-Z

Phytase activity of cells entrapped on 0.5% polymers from shrimp shells are shown in Fig. 6. Enzyme activity of the non-immobilized bacteria with 10^8 cells ml^{-1} included 277 U ml^{-1}. This activity was 100 times greater in immobilized cells (CEC) because of the concentration and accumulation of the bacteria. In the case of immobilization with chitosan extracted from shrimp shells, the enzyme activity was highly preserved and calculated as nearly 25,000 U ml^{-1} of CEC. Therefore, the efficiency was estimated at approximately 90%. Commercial chitosan includes a low efficiency for the entrapment, which is possibly due to the high purity of commercial chitosan (Fig. 6). Zinc oxide nanoparticles cross-linked with bacterial cells and chitosan promote the phytase activity, compared to other ferric and copper oxide nanoparticles (Fig. 7). The phytase activity was improved to 28,800 U ml^{-1} using cells immobilized on ZnO-chitosan with 10^8 cells ml^{-1} of gel and the phytase activity of CEC-Z were stable against dryness (Fig. 7). The use of zinc oxide nanoparticles during immobilization can compensate for reduced efficiency caused by immobilization. Furthermore, these nanoparticles include catalytic activities for this strain. If treatment is carried out with these nanoparticles during immobilization, it creates further consistent immobilized supports due to the presence of negative bonds of ZnO with chitosan cross-links; as shown in SEM images (Fig. 5). This can increase phytase activity. The phytase activity of CEC was stable at high temperatures (45°C) (Fig. 8). Results showed that use of zinc oxide nanoparticles (1 mg l^{-1}) could increase the activity of phytase to 100% and preserve high levels of enzymatic activities under dry conditions. The summarized uses of this complex for human and animal feeds are shown in Fig. 9. The SEM images showed that the best immobilization occurred when nano-ZnO was used. Stability of the complex was constant at high temperatures. Onem et al. showed that chitosan treatment with nanomagnets could greatly enhance the enzyme stability and that the enzyme stabilized in chitosan particles included high resistances to high temperatures [22]; similar to that shown in the current study. Vasile et al. demonstrated that cross-linked ZnO nanoparticle with chitosan created an appropriate matrix for immobilization because the entire solution was entrapped in ZnO-chitosan gels. They also showed that ZnO-chitosan gels included great antimicrobial properties, inhibiting growth of *S. aureus* and *Pseudomonas aeruginosa* [31].

www.SID.ir

Archive of SID

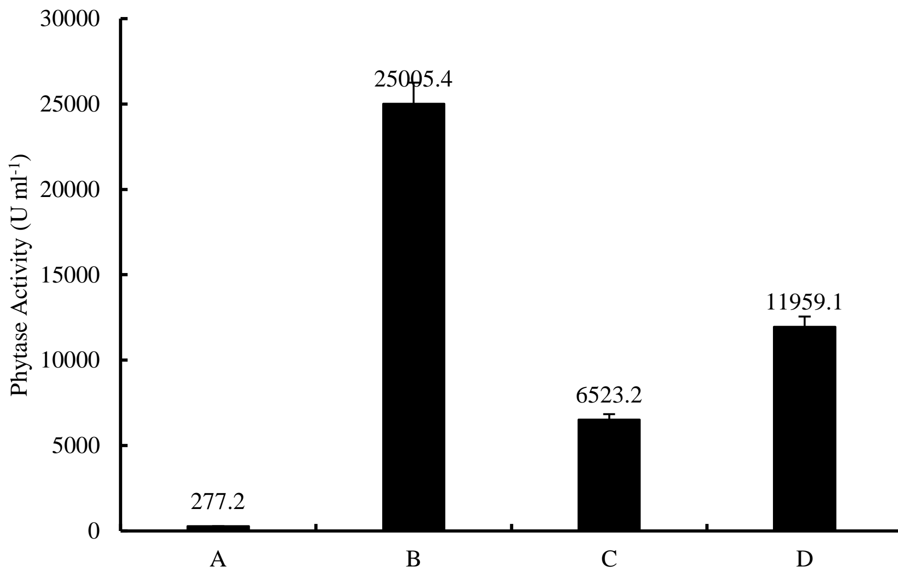


Figure 6. Phytase activity immobilized on chitosan. a) Non immobilized enzyme; b) Immobilized enzyme on shrimp chitosan (0.5%); c) Immobilized enzyme on commercial chitosan (0.3%); d) Immobilized enzyme on commercial chitosan (0.5%)

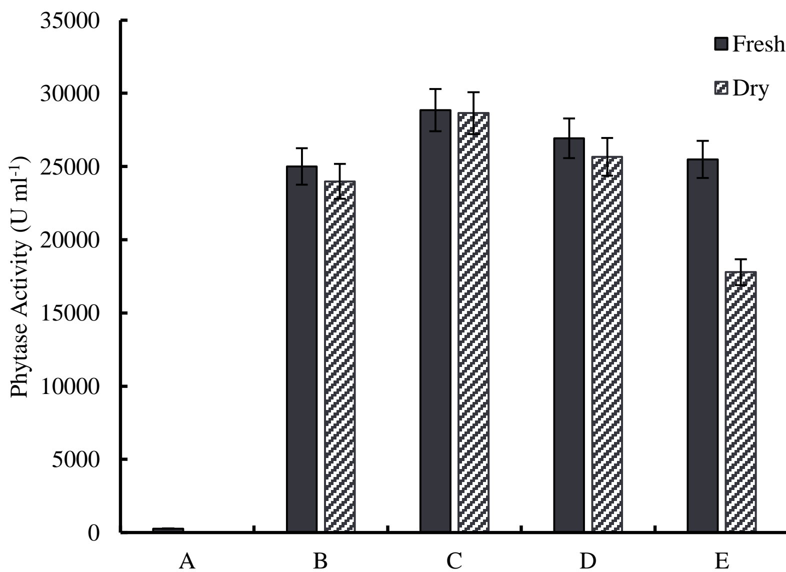


Figure 7. Effects of nanoparticles on phytase activity. a) Fresh non-immobilized enzyme with cells, b) Immobilized enzyme or cells on chitosan, c) Chitosan cross-linked with ZnO nanoparticles, d) Chitosan cross-linked with Cu nanoparticles, e) Chitosan cross-linked with Fe nanoparticles. The CEC-Z included higher phytase activities and were resistance to dryness, compared to other ferric and copper oxide nanoparticles

www.SID.ir

Archive of SID

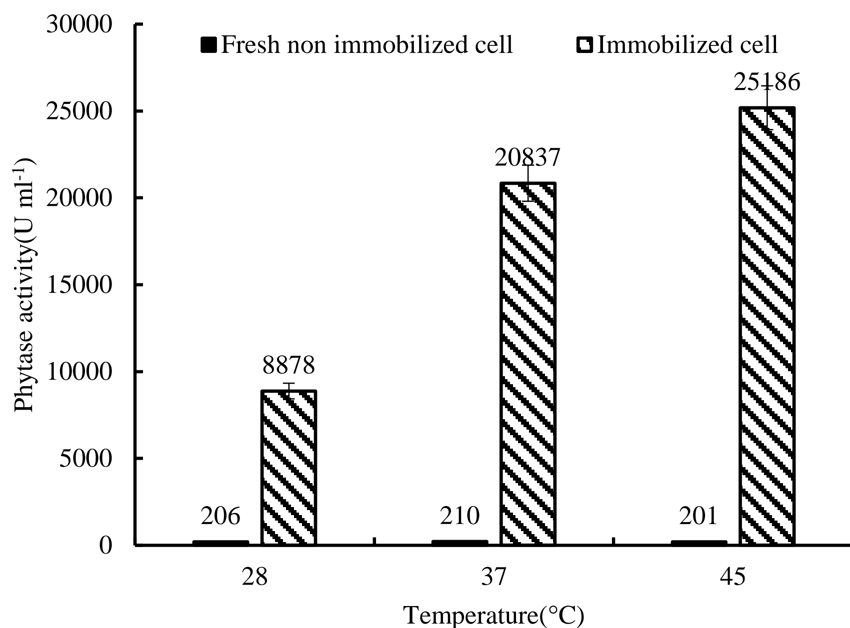


Figure 8. Effects of temperature on phytase activity. Cell entrapment with chitosan included higher phytase activities at higher temperatures

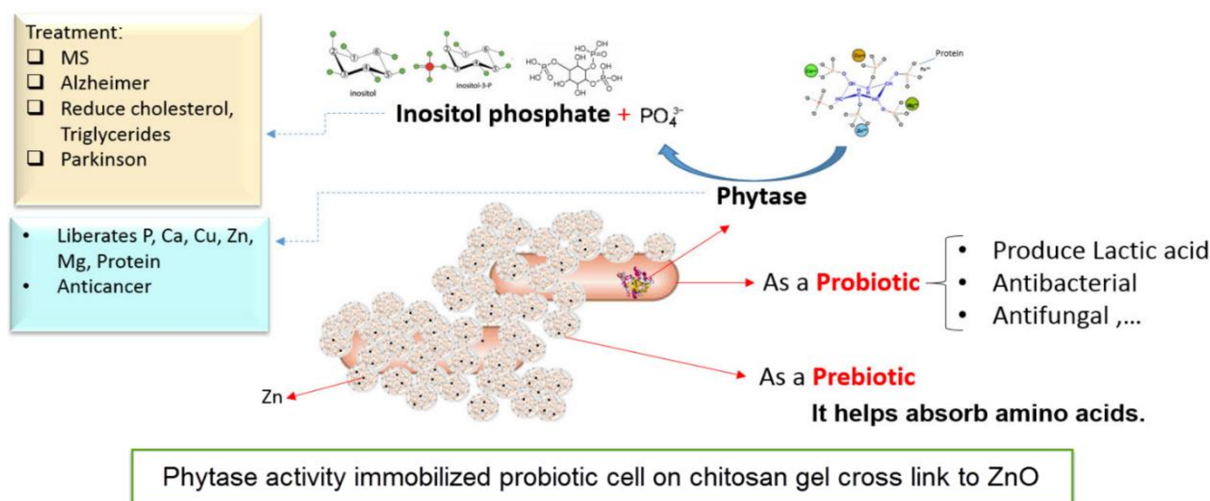


Figure 9. Summary of the polymer complex**.** Chelation of chitosan-ZnO increased the positive charge density of chitosan which was expected to enhance adsorption of zinc and this complex adsorbed the teichoic acid on Gram-positive *Bacillus coagulans*

4. Conclusion

During the past decade, use of microbial phytase in poultry diets has increased. However, concerns over phosphorus pollution by releasing phytate-bound P and reduce P excretion in environment has increased as well. It can be concluded from the current study that supplementation of foods by phytases derived from probiotics and immobilized on prebiotic chitosan could benefit health as an alternative additive to antibiotics. Furthermore, chitosan diets include positive effects on growth in broiler chickens. These effects are characterized by hypo-cholesterolemic, anti-inflammatory, antimicrobial, antioxidant and antitumor effects when fed as a dietary additive in farm animals. Since bacterial strains used in this study are recognized as safe, their phytase activity higher the values of these strains. In summary, addition of probiotic bacteria with phytase activity (immobilized in

www.SID.ir

Archive of SID

prebiotics matrix with high efficiency) to foods can improve human and animal health.

5. Acknowledgements

The authors acknowledge supports from Scientific Research Foundation of the University of Isfahan.

6. Conflict of interest

The authors declare no conflict of interest.

References

1. Ries EF, Alves Macedo G. Improvement of phytase activity by a new *Saccharomyces cerevisiae* strain using statistical optimization. Enzyme Res. 2011; 2011: 1-6. doi: 10.4061/2011/796394.

2. Haros M, Carlsson NG, Almgren A, Larsson Alminger M, Sandberg AS, Andlid T. Phytate degradation by human gut isolated *Bifidobacterium pseudocatenulatum* ATCC27919 and its probiotic potential. Int J Food Microbiol. 2009; 135(1): 7-14. doi: 10.1016/j.ijfoodmicro.2009.07.015.

3. Afinah S, Yazid A, Anis Shobirin M, Shuhaimi M. Phytase: Application in food industry. Int Food Res J. 2010; 17(1): 13-21.

4. Tischer W, Wedekind F. Immobilized Enzymes: Methods and Applications. Biocatalysis-from Discovery to Application. 200: Springer, Berline, Heidelberg, 1999: 95-126. doi: 10.1007/3-540-68116-7_4.

5. Zhang W, Xu F. Hierarchical composites promoting immobilization and stabilization of phytase via trans esterification/silification of modulated alginate hydrogels. ACS Sustain Chem Eng. 2015; 3(11): 2694-2703. doi: 10.1021/acssuschemeng.5b00536.

6. Lata S, Rastogi S, Kapoor A, Imran M. Immobilization of phytase produced by fungal strain *Aspergillus heteromorphus* MTCC 10685. Res J Pharm Biol Chem Sci. 2014; 5(3): 1615-1631.

7. Sirin Y, Akatin MY, Colak A, Saglam Ertunga N. Dephytinization of food stuffs by phytase of *Geobacillus* sp. TF16 immobilized in chitosan and calcium-alginate. Int J Food Prop. 2017; 20(12): 2911-2922. doi: 10.1080/10942912.2016.1261151.

8. Krajewska B. Application of chitin-and chitosan-based materials for enzyme immobilizations: A review. Enzyme Microb Technol. 2004; 35(2): 126-139. doi: 10.1016/j.enzmictec.2003.12.013.

9. Tharanathan RN, Kittur FS. Chitin the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr. 2003; 43(1): 61-87. doi: 10.1080/10408690390826455.

10. Badawy ME, Rabea EI, Marei GI. Preparation and characterizations of chitosan/citral nanoemulsions and their antimicrobial activity. Appl Food Biotechnol. 2018; 5(2): 69-78. doi: 10.22037/afb.v5i2.19005.

11. Haros M, Bielecka M, Sanz Y. Phytase activity as a novel metabolic feature in Bifidobacterium. FEMS Microbiol Lett. 2005; 247(2): 231-239. doi: 10.1016/j.femsle.2005.05.008.

12. Khodaii Z, Natanzi MM, Naseri MH, Goudarzvand M, Dodsons H, Snelling AM. Phytase activity of lactic acid bacteria isolated from dairy and pharmaceutical probiotic products. Int J Ent Pathog. 2013; 1: 12-16. doi: 10.17795/ijep9359.

13. Nuobariene L, Arneborg N, Hansen ÅS, editors. Phytase active yeasts isolated from bakery sourdoughs. 9th Baltic conference on food science and technology “Food for consumer well-being”; 2014. 223-227

14. Hirimuthugoda NY, Chi Z, Wu L. Probiotic yeasts with phytase activity identified from the gastrointestinal tract of sea cucumbers. SPC Beche de Mer Inf Bull. 2007; 26: 31-33.

15. Moroni AV, Dal Bello F, Arendt EK. Sourdough in gluten-free bread-making: An ancient technology to solve a novel issue?. Food Microbiol. 2009; 26(7): 676-684. doi: 10.1016/j.fm.2009.07.001.

16. Hosseinkhani B, Emtiazi G, Nahvi I. Analysis of phytase producing bacteria (*Pseudomonas* sp.) from poultry faeces and optimization of this enzyme production. Afr J Biotechnol. 2009; 8(17): 4229-4232.

17. Sasirekha B, Bedashree T, Champa K. Statistical optimization of medium components for improved phytase production by *Pseudomonas aeruginosa*. Int J Chem Tech Res. 2012; 4: 891-895.

18. Majidi NS, Emtiazi G, Esfahani SS. Flow cytometry detection of bacterial cell entrapment within the chitosan hydrogel and antibacterial property of extracted chitosan. J Med Bacteriol. 2016; 5(3-4): 9-14.

19. Rodrıguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv.1999; 17(4): 319-339. doi: 10.1016/S0734-9750(99)00014-2.

20. Quan C, Zhang L, Wang Y, Ohta Y. Production of phytase in a low phosphate medium by a novel yeast *Candida krusei*. J Biosci Bioeng. 2001; 92(2): 154-160. doi: 10.1016/S1389-1723(01)80217-6.

21. Bae H, Yanke L, Cheng K-J, Selinger L. A novel staining method for detecting phytase activity. J Microbiol Methods. 1999; 39(1):17-22. doi: 10.1016/S0167-7012(99)00096-2.

22. Onem H, Cicek S, Nadaroglu H. Immobilization of a thermostable phytase from Pinar melkior (*Lactarius piperatus*) onto magnetite chitosan nanoparticles. CyTA J Food. 2016; 14(1): 74-83. doi: 10.1080/19476337.2015.1045942

23. Qamar MU, Saleem S, Arshad U, Rasheed MF, Ejaz H, Shahzad N, Jahan S. Antibacterial efficacy of manuka honey against new Delhi metallo-β-lactamase producing gram negative bacteria isolated from blood cultures. Pak J Zool. 2017; 49(6): 1997-2003. doi: 10.17582/journal.pjz/2017.49.6.1997.2003.

24. Khorasani AC, Shojaosadati SA. Improvement of probiotic survival in fruit juice and under gastrointestinal conditions using pectin-nanochitin-nanolignocellulose as a novel prebiotic gastrointestinal-resistant matrix. Appl Food Biotechnol. 2017; 4(3): 179-191. doi: 10.22037/afb.v4i3.17337.

www.SID.ir

Archive of SID

25. Brena B, Gonzalez Pombo P, Batista Viera F. Immobilization of Enzymes: A Literature Survey. Immobilization of Enzymes and Cells. 1051: Springer, New York, 2013: pp.15-31.
doi: 10.1007/978-1-62703-550-7_2.

26. Peter MG. Applications and environmental aspects of chitin and chitosan. J Macromol Sci A. 1995; 32(4): 629-640.
doi: 10.1080/10601329508010276.

27. Singla A, Chawla M. Chitosan: Some pharmaceutical and biological aspects-an update. J Pharm Pharmacol. 2001; 53(8): 1047-1067.
doi: 10.1211/0022357011776441.

28. Dutta PK, Ravikumar M, Dutta J. Chitin and chitosan for versatile applications. J Macromol Sci C. 2002; 42(3): 307-354.
doi: 10.1081/MC-120006451.

29. Chavarri M, Maranon I, Ares R, Ibanez FC, Marzo F, del Carmen Villaran M. Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int J Food Microbiol. 2010; 142(1): 185-189.
doi: 10.1016/j.ijfoodmicro.2010.06.022.

30. Quan C, Fan S, Ohta Y. Immobilization of *Candida krusei* cells producing phytase in alginate gel beads: An application of the preparation of myo-inositol phosphates. Appl Microbiol Biotechnol. 2003; 62(1): 41-47.
doi: 10.1007/s00253-003-1247-1.

31. Vasile BS, Oprea O, Voicu G, Ficai A, Andronescu E, Teodorescu A, Holban A. Synthesis and characterization of a novel controlled release zinc oxide/gentamicin-chitosan composite with potential applications in wounds care. Int J Pharm. 2014; 463(2): 161-169.
doi: 10.1016/j.ijpharm.2013.11.035.

www.SID.ir

Archive of SID

http://dx.doi.org/10.22037/afb.v%vi%i.23398

Research Article

Applied Food Biotechnology

APPLIED FOOD BIOTECHNOLOGY, 2019, 6 (3): 150-139
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

تثبیت پروبیوتیک تولیدکننده فیتاز در کیتوزان میگو دارای اتصال عرضی با نانوذرات اکسید روی و بررسی فعالیت ضد باکتریایی آن

نرگس چترائی[۱]، گیتی امتیازی[*۱و۲]

۱- گروه زیست شناسی، دانشکده علوم، دانشگاه اصفهان، اصفهان، ایران.
۲- گروه زیست فناوری، دانشکده علوم و فناوری زیستی، دانشگاه شهید اشرفی اصفهانی، اصفهان، ایران.

چکیده

سابقه و هدف: فیتاز در افزودنی‌های انسانی و طیور مورد استفاده قرار می‌گیرد. این آنزیم اغلب توسط *آسپرژیلوس نایجر* که یک عامل بیماریزای گیاهی[۱] است تولید می‌شود. فیتاز تولید شده توسط زیست‌یار[۲] انتخاب (کاندید) مناسبی برای مکمل‌های غذایی است.

مواد و روش ها: *باسیلوس کواگولانس* به عنوان یک زیست‌یار، برای فعالیت فیتاز و فسفاتازها در فیتین آگار و پیکووسکایاس آگار مورد استفاده قرار گرفت. ژل الکتروفورز سدیم دودسیل سولفات پلی آکریلامید و آنالیز زیموگرام آنزیم فیتاز استخراج شده انجام شد. سلول‌های زیست‌یار دارای فعالیت فیتاز بر کیتوزان استخراج شده از پوسته میگو تثبیت شدند و کارایی آن با میکروسکوپ الکترونی روبشی[۳] و طیف بینی مادون قرمز تبدیل فوریه[۴] مورد مطالعه قرارگرفت.

یافته‌ها و نتیجه‌گیری: در این تحقیق، باکتری *باسیلوس کواگولانس* فعالیت فیتازی داخل سلولی به دست آمده از سلول‌های شکسته، با شاخص حلالیت ۲/۵ بر روی محیط کشت اختصاصی فیتاز را نشان داد. نانو ذرات آهن و اکسید روی، فعالیت آنزیم را ۲۵٪ افزایش دادند. این سلول‌ها با ذرات کیتوزان-ZnO رسوب داده شدند و فعالیت آنزیمی آن بر روی ژل بررسی شد. چنگالش یون فلز-کیتوزان[۵] چگالی بار مثبت کیتوزان را افزایش داد که انتظار می‌رفت جذب کیتوزان به تیکوئیک اسید *باسیلوس کواگولانس* را افزایش دهد؛ با اطلاعات توسط میکروسکوپ الکترونی روبشی و طیف بینی مادون قرمز تبدیل فوریه تایید شد. سلول‌های تثبیت شده روی کیتوزان-اکسید روی، فعالیت آنزیمی را تا ۲۸۸۰۰ U ml^{-1} gel ارتقا داد. سلول‌های به دام افتاده، به درجه حرارت بالا و pH مقاوم بودند. این کمپلکس نه فقط ضد *استرپتوکوکوس آگالاکتیه* است، بلکه فیتین و فسفات نامحلول را نیز حل می کند که این کمپلکس را کاندید مناسبی به عنوان افزودنی برای خوراک انسان و حیوان می‌کند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۱۸ نوامبر ۲۰۱۸
داوری ۲۰ فوریه ۲۰۱۹
پذیرش ۱۶ مارچ ۲۰۱۹

واژگان کلیدی

- تثبیت کیتوزان
- فیتاز
- زیست‌یار
- نانوذرات اکسید روی

***نویسنده مسئول**

گیتی امتیازی،
گروه زیست فناوری، دانشکده علوم و فناوری زیستی ، دانشگاه شهید اشرفی اصفهانی، اصفهان، ایران.

تلفن: ۰۹۸-۳۱-۳۷۹۳۲۴۵۷+
نمابر: ۰۹۸-۳۱-۳۷۹۳۲۴۵۶+
پست الکترونیک:
emtiazi@sci.ui.ac.ir

[1] Plant pathogen
[2] Probiotic
3. Scanning Electron Microscopy (SEM)
4. Fourier Transform Infrared (FT IR)
[5] Chelation of chitosan-metal ion

www.SID.ir