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# *Citrobacter farmeri* phas32, an isolate from bean (*Phaseolus vulgaris*) farm soil with high phytase production

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## Abstract

**Introduction:** Phytase hydrolyzes phytic acid and enhances bioavailability of phosphorus and other nutritive minerals for monogastric animals, so it is commonly used as an important food additive.

**Materials and method**s**:** The aim of this study was isolation of phytase producing bacteria from one of Shushtar's bean farms, Southwest of Iran by phytase screening medium (PSM) and optimization of the growth and enzyme productive conditions by the best isolate.

**Results:** The best isolate was identified as *Citrobacter farmeri* strain phas32. Optimized conditions for phytase production by this isolate were 30˚C, pH 7, 0.25% phytic acid and 48 h incubation and phytase enzyme of phas32 had the best activity at 65˚C and pH 8.5. Enzyme unit and its molecular weight were 31 U/ml and 40 KD, respectively.

**Discussion and conclusion:** Finally, based on these results it can be concluded that the *Citrobacter farmeri* strain phas32 is potent phytase producer that can be used for large scale enzyme production.

**Key words:** Citrobacter, optimization, phytase, phytic acid

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## Introduction

Phosphorus is one of the important necessary elements in cell metabolism and growth but insoluble phosphate resources can't be utilized by humans, animals and plants (1). Phytic acid (myo-inositol hexa kis phosphate) is one of these sources that act as a storage source of phosphate, myoinositol and variety of mineral cations for seedling growth and seed germination in plants. Phytate represents approximately 75-80% of total phosphorus of plant seeds (2). Through binding and form ing insoluble complexes with important metals such as iron, zinc, magnesium and calcium as well as proteins and vitamins, phytate reduces their absorption, digestion and biological activities (3, 4).

Phytase i.e. myoinositol hexa kisphosphate phosphohydrolase (EC 3.1.3.8), is an important food additive for enhancing the bioavailability of phosphorus and other nutritive minerals in monogastric animals such as pigs, poultries and fish that hydrolyzes phytic acid to phosphoric acid and myoinositol (3). Plants, animals and microorganisms are sources of phytases while microbial resources such as bacteria, fungi and yeasts are the most important resources for phytase production (5). In order to food process and enhance minerals availability, human's and animal's diets are mostly enriched with microbial phytase (6). Phytases are also interested due to their function in remedy of iron deficiency in humans caused by phytate. The efficiency of myoinositol phosphates in prevention or treatment of cardio-vesicular disorders and malignancies has been investigated, too (7).

With regard to the importance and applications of phytase for humans and animals and advantages of bacterial phytases (6), screening of different habitats in order to find new isolates with higher production yield and unique characteristics is of great importance. Some of these studies are: Selvamohan *et al*. that have isolated *Pseudomonas* sp. as phytase producing bacterium from poultry feces (8) and Roy *et al*. isolated *Shigella* sp. CD2 from wheat rhizosphere (9). Yoon *et al*. isolated *Enterobacter* sp.4 as a phytase producing bacterium from soil near the roots of leguminous plants (10). Mopera et al, have introduced *Klebsiella pneumonia*e 9-3B with the ability of phytase production (11). Esakkiraj *et al* (5), reported that *Pseudomonas* AP-MSU 2 strain isolated from *Etroplus suretensis* can produce phytase enzyme (5). In the research of Singh *et al Sporotrichum thermophile* was reported with the ability to produce phytase following growth on sesame cake (12). Phytase production using agricultural byproducts was also reported in the study of Roopesh *et al* as *Mucor racemosus* identified with high levels of phytase production during growth on wheat bran and oilseed cakes (13). Shamna et al, have reported *Bacillus subtilis* BPTK4 with highest phytase production upon wheat bran (14).

Shushtar region in Khouzestan, Southwest of Iran, has numerous agricultural farms with long history of crop production that makes it as a good candidate to find phytase producing bacteria. Hot and humid climate of this region can harbor strains which can tolerate harsh environments and also produce more stable enzymes. The aim of this study was isolation of phytase producing bacteria from Shushtar's bean farm and optimization of growth and enzyme production conditions by the best isolate.

## Materials and Methods

**Screening of phytase producing bacteria:** Soil samples were collected from three parts of plowed bean farm located in Shushtar (32° 2' 37" N / 48° 51' 24" E), Khouzestan, Iran. Samples were immediately transferred to lab and refrigerated till experiments. One gram

of soil sample was transferred to 25 ml of 2% wheat bran medium and incubated (30˚C, 150 rpm) for 3 days. Then 100 µl of supernatant was cultured on phytase screening medium (PSM) containing ($^{g}/_{l}$) glucose (15), sodium phytate (1.5), $NH_4NO_3$ (2), KCl (0.5), $MgSO_4.7H_2O$ (0.5), $MnSO_4$ (0.3), $FeSO_4.7H_2O$ (0.3) and agar (2) (pH 7.5) at 30˚C for 24 h. A subculture was subsequently prepared on PSM medium from those colonies with halo zone to gain pure culture. Pure colonies were stored on nutrient agar at 4˚C (15).

**Enzyme production assay:** In order to confirm phytase production, the isolates with clear zone on PSM agar were cultivated in 25 ml of PSM broth and incubated (30˚C, 150 rpm, 72 h). Following a centrifugation (6000 ×g, 15 min), qualitative enzyme assay was performed on the supernatant and the best isolate was selected for growth and enzyme production optimization (15). In qualitative enzyme assay, the reaction mixture contained 2 ml of supernatant and 4 ml of substrate solution (0.84% solution of sodium phytate in buffer solution, pH 5.5). The buffer solution was prepared through dissolving 0.18 g acetic acid, 3 g sodium acetate.$3H_2O$ and 0.15 g $CaCl_2.2H_2O$ in 100 ml distilled water, pH = 5.5. The reaction was done at 37˚C for 65 min and then stopped by 4 ml freshly prepared color reagent. The color reagent was prepared by mixing 25 ml ammonium molybdate solution (10 g ammonium molybdate.$4H_2O$ and 1 ml $NH_3$ (25%) in 100 ml distilled water) with 25 ml ammonium vanadate solution (0.235 g ammonium vanadate, 40 ml distilled water at 60˚C, then slowly added 2 ml nitric acid before dilution to 100 ml) and stirring while slowly adding 16.5 ml nitric acid (65%). Then the mixture cooled to room temperature and adjusted to 100 ml. The color changed following phytase activity was measured at 415 nm. In blank reaction, the color reagent was added prior to the enzyme sample. One unit of enzyme was defined as the amount of enzyme that can release 1 µmol inorganic phosphate in 1 min (16). Based on the quantitative assay, the best isolate was selected for further analysis.

**Optimization of growth condition in PSM broth medium:** To select the suitable temperature and pH for growth of selected bacterium, the growth curve of bacterium was drawn at 30˚C, 37˚C and 45˚C in PSM medium and following determination of optimum temperature, the optimum pH was obtained from pH 5.5, 7 and 8.5. Growth curve was gained by inoculating 1% inoculum equal to 0.5 McFarland turbidity to PSM, incubation while agitating at 150 rpm and reading the turbidity of culture medium at 580 nm (Bio-Rad, USA) every 2 h for 60 h. This process was done in triplicates and the absorption was read twice, i.e. 6 times of reading in each absorption determination.

**Effects of different parameters on phytase production:** In order to find the effects of culture conditions on phytase production a series of experiments was designed as follow. All of them were performed as triplicates and changing one factor in each experiment was applied. The tested parameters were temperature (30, 37 and 45˚C, (21)), pH (5.5, 7 and 8.5, (8)), phytic acid concentration (0.15, 0.25 and 0.35%, (17)) and different agricultural wastes (wheat bran, rice bran and powdered rice straw at 1% ($^{w}/_{v}$) while phytic acid and $FeSO_4.7H_2O$ was deleted, (8, 18)). The enzyme activity was assessed at 12, 24, 48, 72 and 96 h of incubation.

**Determining optimum conditions for phytase activity:** The effect of temperature, pH and protease on enzyme activity was determined in triplicate experiments. To determine the optimum temperature of phytase, enzyme

assay was performed by treating the enzyme at 30˚C, 37˚C, 45˚C, 55˚C, 65˚C and 75˚C. Similarly, phytase activity was assessed in pH 5.5, 7 and 8.5. The stability of phytase against proteases including pepsin and trypsin were also investigated. The supernatant of phytase enzyme was incubated with 0.1 $^{mg}/_{ml}$ of pepsin (Merck, Germany) or trypsin (Sigma, USA) at 37˚C for 60 min. Then, enzyme assay was performed and compared with the enzyme activity obtained from untreated supernatant (9).

**Investigation of nitrogen fixation:** Nitrogen fixation test was done according to Dobereiner *et al*. (19).

**Partial purification of phytase:** The supernatant obtained from cultivation of the isolate in PSM broth at optimum condition was centrifuged at 14000 ×g for 30 min. The proteins were precipitated through adding ammonium sulfate up to 85% saturation and incubation at 4˚C for 4 h by agitation. The pellet was then harvested by centrifugation at 14000 ×g for 30 min. The obtained pellet was resuspended in 20 mM Tris-HCl buffer (pH 8) and was dialyzed for one day in same buffer (20).

**Protein concentration and molecular weight of enzyme:** To determine the protein concentration in the supernatant obtained from optimum enzyme production conditions, the Bradford method was used. In this method, standard protein solution (Bovine Serum Albumin) 1 $^{mg}/_{ml}$ was used (21). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Sigma- Aldrich, SDS (L3771), acrylamide/bisacrylamide (a2917), temed (t9281), APS (A3678)) was performed in order to determine the molecular weight of produced phytase, as described by Laemmli using 10% acrylamide gel. Proteins were stained with Coomassie brilliant blue R-250 (22).

**Identification of phytase producing isolate:** The selected isolate was identified based on molecular and phenotypic methods. In molecular identification, DNA was extracted by DNA extraction kit (Cinnagen, Iran) and the 16S rRNA gene was amplified by FD1 (5-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3) and RP1 (5-CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT-3) primers (23). PCR reaction mixture was composed of 1X buffer concentration, 0.7 $^{\rho mol}/_{\mu l}$ primers, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1.5 u Taq DNA polymerase and 2 µl of template DNA in 25µl final volume. PCR reaction was performed in thermal cycler (Bio-Rad, USA), based on the following thermal cycling program: denaturation (94˚C, 5 min), 30 cycles composed of denaturation (94˚C, 60s), annealing (58.1˚C, 40s) and extension (72˚C, 150s) and a final extension at 72˚C for 20 min. The PCR product was electrophoresed in 1% agarose containing DNA safe stain and 1kb DNA ladder (Sinaclon (Cat. No**. PR901645)** and then sequenced (Macrogen, Korea) and edited by Bioedit software version 7.2.2. Then, its similarity with existing data in gene bank was searched by BLAST algorithm of NCBI. Phenotypical identification was done based on Bergey´s manual of systematic bacteriology (24).

## Results

As a result of primary screening, 6 phytase producing bacteria were isolated from plowed bean farm in Shushtar, but following their cultivation in PSM broth and phosphate content measurement, only four isolates had the ability of releasing phosphate. In enzyme assay analysis on 3 days culture of bacteria (Table 1), it was determined that phas32 isolate has the best potential of enzyme production, so it was selected for further analysis.

Table 1- Enzyme assay of 4 phytase producing isolates.

| Isolate name | Phas11 | Phas32 | Phas12 | Phas31 |
|---|---|---|---|---|
| Enzyme activity ($^{U}/_{ml}$) | 22.15 | 39.38 | 13.92 | 20 |

Figure 1 represents the growth curve of phas32 strain at 30˚C, 37˚C and 45˚C. This isolate could not grow at 45˚C and its optimum temperature was 37˚C. In such a manner that maximum growth was achieved after 22 h of incubation.

In the experiment of culturing at different pH (Figure 2), it was concluded that phas32 could grow in acidic, alkaline and neutral pH, but alkaline pH was more favorable for bacterial growth than others.

Enzyme production by phas32 isolate was investigated at 30˚C, 37˚C and 45˚C during 96 h. As represented in Figure 3, this isolate has the most enzyme production at 30˚C after 24 h and 72 h with 31 $^{U}/_{ml}$ yield of enzyme at these conditions. Furthermore, phas32 had the most enzyme production at this temperature and pH 7 after 24 h (Figure 4).

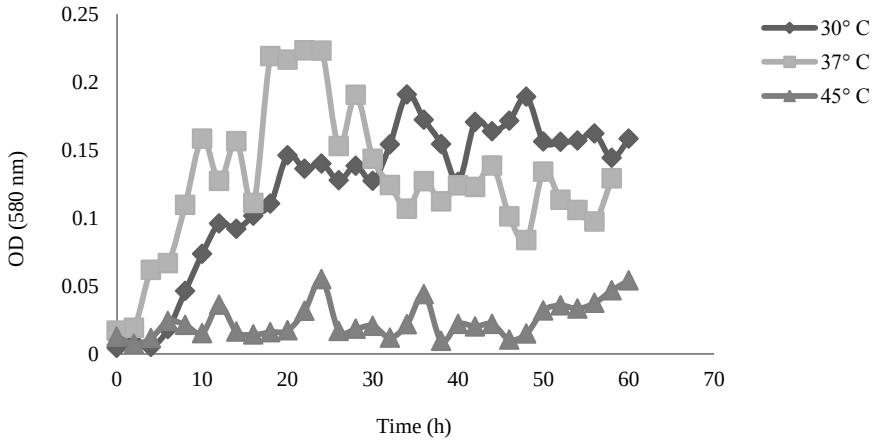


Fig. 1-Growth curve of phas32 isolate at different temperatures

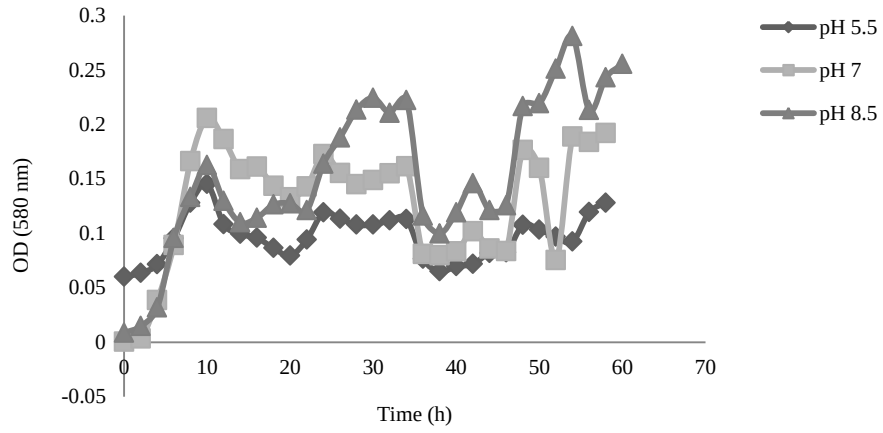


Fig. 2-Growth curve of phas32 isolate at different pH

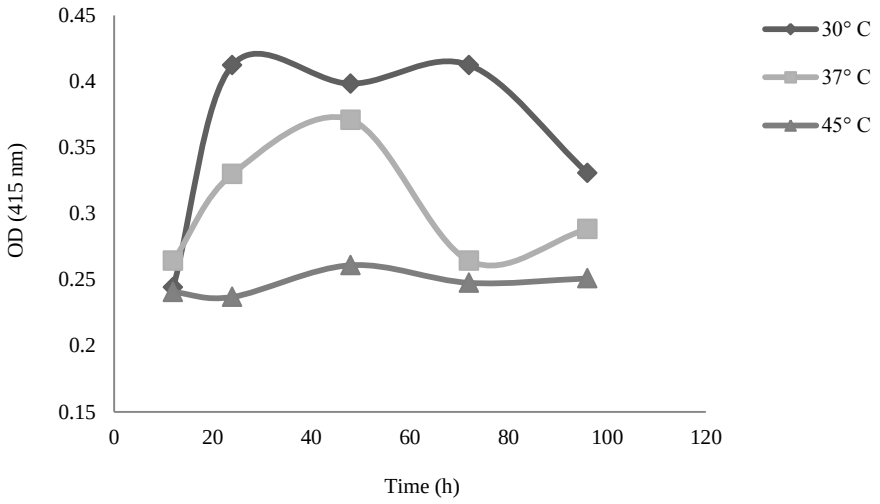


Fig. 3- Enzyme production by phas32 isolate at different temperatures

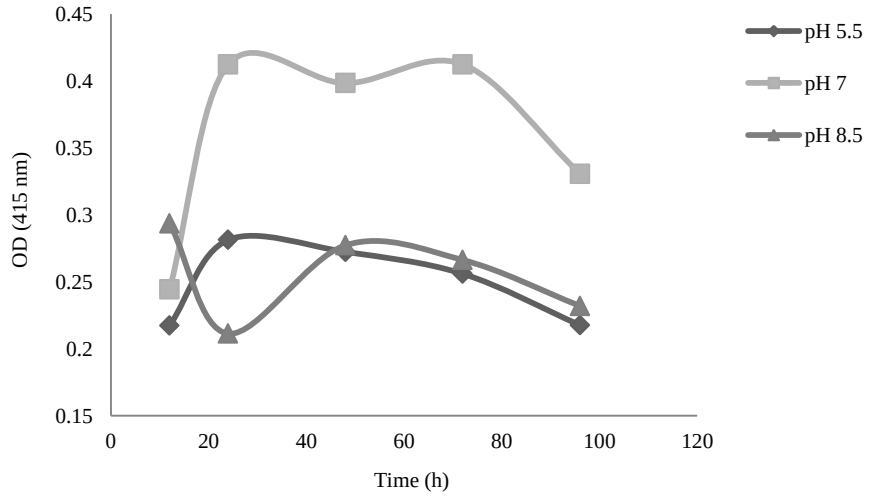


Fig. 4- Phytase production by phas32 isolate at different pH

In response to different concentrations of phytic acid, it was found that phas32 can produce highest amount of phytase at 0.25% of phytic acid (Figure 5). In addition, phytase production in PSM prepared with different agricultural wastes was also evaluated. As a result, wheat bran induced the most enzyme production after 24 h of incubation (Figure 6).

In the experiment for thermal stability of produced phytase, the supernatant was treated under 30-75˚C. As it obviously evidenced in Figure 7, the enzyme was active and stable till 65˚C but its activity was reduced at 75˚C.

The enzyme activity at different pH including 5.5, 7 and 8.5 was also investigated. However, this enzyme was active in acidic, alkaline and neutral pH, but had the best activity in alkaline pH (Figure 8).

Protease treatment of enzyme revealed that it is sensitive to pepsin and trypsin (Figure 9) and its activity was greatly reduced following these treatments.

The results of phenotypic identification revealed phas32 as *Citrobacter* sp. The 16S rRNA sequence of this isolate had 98% similarity with *Citrobacter farmeri*. The sequence of this isolate was registered in gene bank with accession number KF796627 (Figure 10).

The result of SDS-PAGE (Figure 11) showed that the obtained phytase had 40 KD weight and protein concentration of the supernatant was 0.103 $^{mg}/_{ml}$.

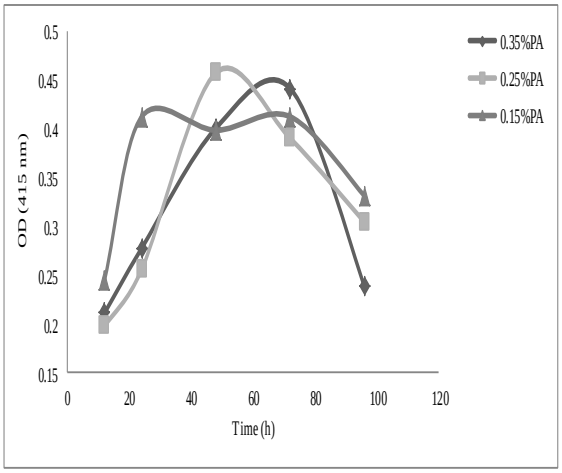


Fig. 5- Enzyme production by phas32 at different phytic acid concentrations.

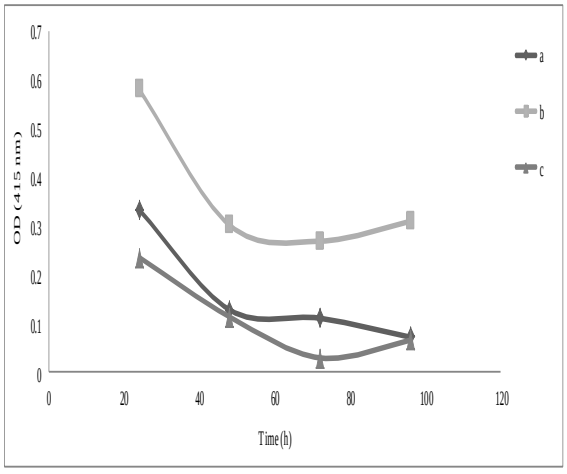


Fig. 6- Enzyme production by phas32 isolate in medium containing different agricultural substrates, a) Rice bran; b) Wheat bran and c) Powdered rice straw.

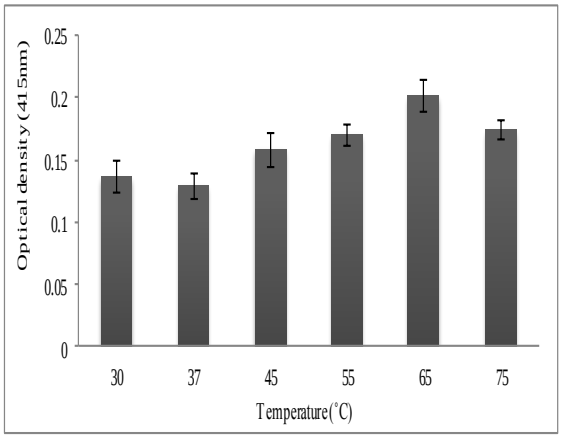


Fig. 7- Effect of temperature on phytase activity.

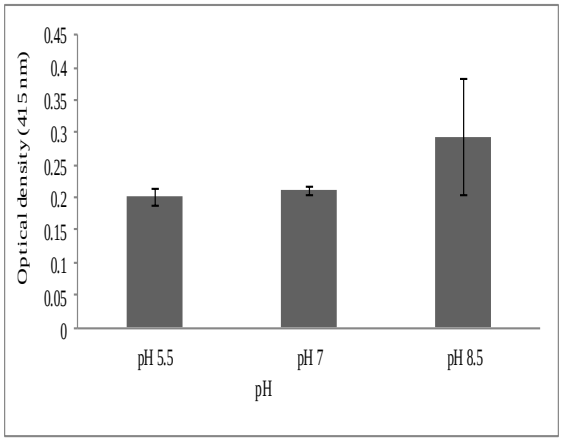


Fig. 8- Stability and activity of phytase at different pH.

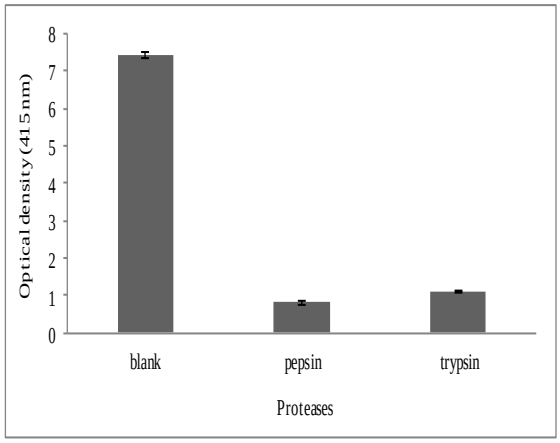


Fig. 9- Effect of protease treatment on phytase activity

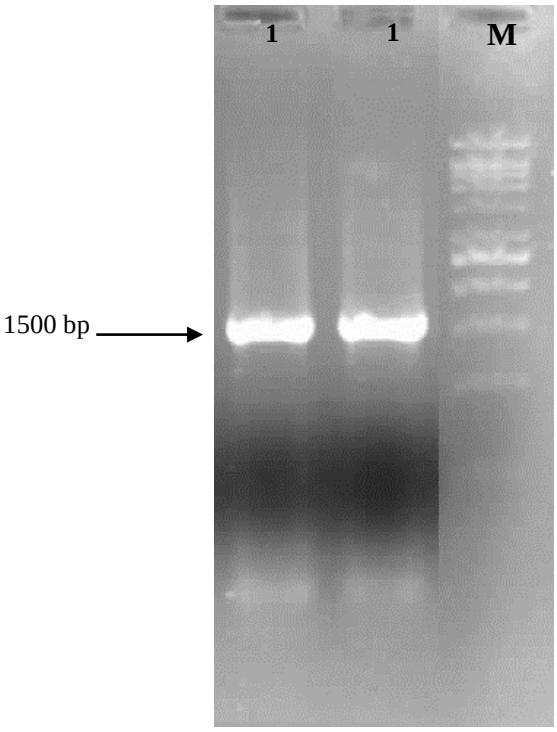


Fig. 10- Electrophoresis of PCR product. M: 1Kb DNA Marker, 1: PCR product of 16S rRNA sequence of phas32 isolate.

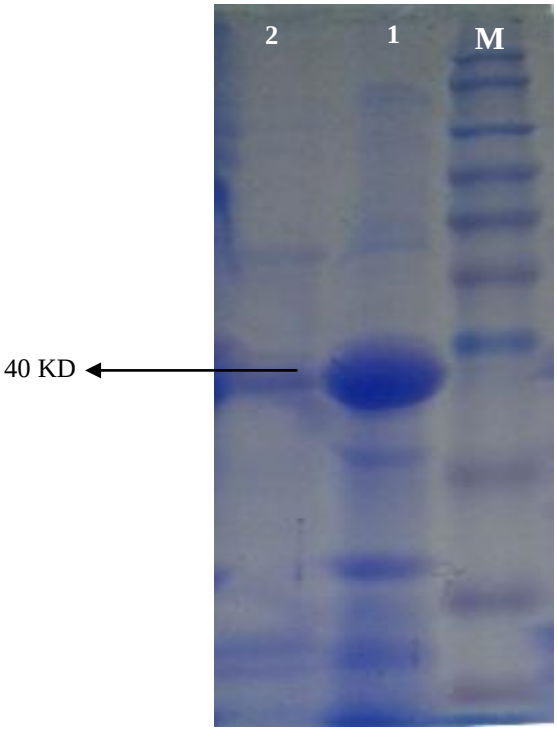


Fig. 11. SDS-PAGE result. M: ladder (10.5-175 KD), 1: phytase production in PSM broth, 2: low phytase production in PSM medium that had $KH_2PO_4$ instead of phytic acid.

## Discussion and Conclusion

In recent years, there is an increasing interest to phytases for utilization in humans' food processing, especially due to the reduction of phytate content of food and increasing of mineral absorption (25). As the aim of this study, the bean farm rhizosphere was screened for phytase producing bacteria and finally 4 phytase producing isolates were confirmed through screening in PSM agar and then PSM broth. The difference between the results of screening on PSM agar and PSM broth is related to this fact that acid production by bacteria can also produce halo zone and the potential of phytase production must be confirmed by enzyme assay. Also Mittal *et al*. (15) found that some bacterial species that had been produced halo zone in PSM medium due to acid production, didn't show any enzyme activity in PSM broth in second screening. The most potent isolate that was selected for further study was identified as *Citrobacter farmeri* phas32, a member of Enterobacteriaceae family. Similarly, Yoon *et al*. (10) isolated a phytase producing bacterium from soil near the roots of leguminous plants and identified it as *Enterobacter* sp.4. In the research of Mopera et al (2012) *Klebsiella pneumonia*e 9-3B had the ability to produce phytase in medium with phytic acid as carbon and phosphorus sources (11).

More favorable growth of the selected bacterium at alkaline pH can be explained as this fact that during growth of these species, acid production occurs that leads to pH change. This change of pH was evidenced for us during bacterial culture, so, if the initial pH was adjusted to basic pH, this acid production has less effect on bacterial growth. Esakkiraj *et al* (5), reported *Pseudomonas* AP-MSU 2 strain isolated from *Etroplus suretensis* fish with 30˚C and pH 7 as optimum conditions for phytase production. In the report of Hosseinkahni et

al (2009) *Pseudomonas* sp. showed maximum phytase production at 28˚C and 180 rpm in 72 h (26). In large scale production of bacterial enzymes, selection of substrate that be accessible and cost effective is of great importance. So, the effect of different agricultural wastes on phytase production potential of *Citrobacter farmeri* phas32 was investigated. As a result, wheat bran has yielded most enzyme production by this isolate after 24h. This is because wheat bran had a low content of inorganic phosphate and relatively had high phytate content. Therefore, the high amount of phytate in environment may stimulate biosynthesis of phytase in this isolate. Wheat bran due to its unique nature was more effective than sodium phytate in phytase production. Low degree hydrolysis of wheat bran ensures that phytase production is induced continuously during fermentation, thus leading to increased production of phytase (27).

Phytase production using agricultural waste has advantages, particularly in reducing the production cost. Many researchers have reported appropriate use of agricultural waste for phytase production for different bacterial strains. Phytase production by *Sporotrichum thermophile* using sesame cake has been reported (12). Similarly, high levels of phytase production with wheat bran and oilseed cakes have been observed in *Mucor racemosus* (13). Also, *Bacillus subtilis* BPTK4 showed the highest phytase production upon wheat bran substrate among three substrates including wheat bran, rice bran and sugar cane bagasse (14).

Thermal stability of enzymes is an important subject in enzyme application in industry. Temperature can affect variety of metabolic processes such as protein denaturation, enzyme inhibition, induction or inhibition of particular metabolite production, cell death, etc. With regard to this fact that phytases are used for processing animal meal, tolerance to higher temperature is an advantage for this enzyme. So, this enzyme had potentiality for application in pellet production for poultry and fish meals. Optimum temperature of Phytase obtained from *Mitsuokella jalaludinii* was 55˚C till 60˚C (28). *Shigella* sp. CD2 that was isolated from wheat rhizospheric zone had pH 5.5 and 60˚C as optimum pH and temperature, respectively (9).

Alike, pH of manufacturing environment also plays a significant role in metabolite production (15). The phytase of *Citrobacter farmeri* phas32 was sensitive to acidic pH and also protease that is a weakness of this enzyme. So it can't be used as a food additive while only can be used for pretreatment of high phytic acid food. Unlike the protease sensitivity of phytase of this isolate, Kim *et al* (29) isolated a phytase enzyme from *Citrobacter braakii* YH-15 that was resistant to proteases such as trypsin, pepsin, papain, pancreatin and elastase. Also, *Shigella* sp. CD2 phytase was tolerant to pepsin and trypsin (9).

Similar to the molecular weight that was reported in the present study, in the study of Shamna et al (2012), electrophoresis analysis of *Bacillus subtilis* BPTK4 phytase showed that the phytase enzyme of this isolate was 40 KD (14). Also, Greiner *et al*. (30) isolated phytase enzyme by 40 KD molecular weight.

Based on the obtained results in this study it can be concluded that bean farm soil is a habitat for phytase producing bacteria that can be used in screening studies to find new strains. The *Citrobacter farmeri* strain phas32 that has been introduced in this study in comparison with other studies has some advantages that make it suitable for industrial production of phytase and also

pretreatment of animal's food. It is suggested that more studies for large scale production of this enzyme be regarded.

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# *سیتروباکتر فارمری* phas 32، جدایه‌ای از خاک مزرعه کشت لوبیا با توانایی بسیار در تولید آنزیم فیتاز

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## چکیده

**مقدمه:** فیتاز، فیتیک‌اسید را هیدرولیز می‌کند و دسترسی زیستی فسفر و دیگر مواد معدنی مغذی را برای حیوانات تک‌معده‌ای افزایش می‌دهد.بنابراین، به‌شکل افزودنی غذایی مهم استفاده می‌شود.

**مواد و روش‌ها:** هدف مطالعه حاضر، جداسازی باکتری‌های تولیدکننده فیتاز از مزرعه لوبیای شوشتر، جنوب‌غرب ایران، با استفاده از محیط غربال‌گری فیتاز (PSM) و بهینه‌سازی رشد و شرایط تولید آنزیم با بهترین جدایه است.

**نتایج:** بهترین جدایه، *سیتروباکتر فارمری* سویه phas32 شناسایی شد. دمای ۳۰ درجه سانتی‌گراد، اسیدیته ۷، ۰/۲۵ درصد فیتیک‌اسید و ۴۸ ساعت انکوباسیون، شرایط بهینه‌سازی‌شده برای تولید فیتاز توسط این جدایه است و آنزیم فیتاز سویه phas32، بهترین فعالیت را در دمای ۶۵ درجه سانتی‌گراد و اسیدیته ۸/۵ دارد. فعالیت و وزن مولکولی آنزیم این باکتری به‌ترتیب ۳۱ U/ml و ۴۰ کیلودالتون است.

**بحث و نتیجه‌گیری:** بر اساس نتایج پژوهش حاضر، نتیجه‌گیری می‌شود که *سیتروباکتر فارمری* سویه phas32 تولیدکننده فیتاز است که امکان استفاده از آن برای تولید آنزیم در مقیاس زیاد وجود دارد.

**واژه‌های کلیدی:** *سیتروباکتر*، بهینه‌سازی، فیتاز، فیتیک‌اسید

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