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Extraction and Molecular Evaluation of Phytase-producing Bacteria from Soil of Alfalfa and Clover Fields of Isfahan

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ABSTRACT

The main storage form of phosphorus is phytate (Myo-inositol Hexakisphosphate) in legume crops, such as clover and alfalfa, which are of high importance in terms of nutrition for humans and animals. In animals, due to a lack of phytase enzymes in their intestines, it is not possible to break the phytic acid (a nutritional constituent). Hence, phytic acid acts as an anti-nutritional chelating agent for various metal ions like Ca, Mg, Fe, Zn, *etc*., so it reduces the nutritive quality of food. Since phytase is an important enzyme in the food/feed industry, the objective of this study is to isolate phytase-producing bacteria cells to analyze phytate molecules. The present study was conducted in the laboratory of the Sana Institute of Higher Education. In this study, 8 soil samples of alfalfa and clover fields located in Isfahan (Khomeini Shahr and Morche Khort Regions) were collected and several bacteria isolates were separated using differential media. To examine the phytase activity, the isolated bacteria on the specific media fortified with phytate were cultivated and positive phytate bacteria were identified using morphological traits, biochemical tests, and 16srRNA sequencing determination. The data obtained from quantitative properties showed that two isolates of B1 and D1 have 17- and 20-mm size of zone diameters, respectively. Based on morphological properties, the B1 bacteria showed a big size of the colony, with a bump hanging in the margin surrounding the colony and white pigment, which was gram-positive. However, the D1 sample indicated a small colony size, with a wavy margin, smooth bump, and creamy pigment which was gram-positive. By biochemical recognition test, among all bacteria cells, two bacteria colonies were distinguished concerning the phytase activity and were recognized as *Bacillus* sp. In addition, the 16srRNA sequencing analysis showed that one strain belongs to *Bacillus paralicheniformis* (95%) and the other one is related to *Bacillus endophyticus* (95%), both of which are found in soil usually.

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Introduction

Phosphor (P) is the necessary component of the biological membrane and nucleic acid metabolism in plants, making up for about 0.2% of a plant's dry weight. Phosphor is a key macronutrient of nucleic acids, phospholipids, and ATP, which after Nitrogen (N) is the second most frequently limiting molecule for plant growth and it is not possible for plants to grow without this reliable supply of this nutrient. Moreover, main biological processes like photosynthesis and respiration activate using mineral phosphate or organic derivatives (Raghothama and Karthikeyan, 2005).

Phytic acid (as phytate) is the main storage form which constitutes 1-5 weight percent in cereal, legumes, oil seeds, and nuts (Gupta *et al*., 2015). Phytic acid ($C_6H_{12}SO_{24}P_6$) includes 12 protons with a replacement which can be seen in different forms. Phytase expression explains the specific group of phosphatase enzymes which can phytate hydrolysis and release the minimum of one phosphate group from this material (Haefner *et al*., 2005).

To access P in plants, the two steps of conversion of insoluble forms of inorganic P to soluble forms are used since, first, the application of Phosphate Solubilizing Bacteria (PSB) is of great importance to release phosphates meanly by the release of organic acids (Haefner *et al*., 2005). Then, the obtained organic P forms (as phytases) which are predominant in most soil (10-50% of total P) are converted to phytases (Myo-inositol hexakisphosphate phosphohydrolases) through the mineralization process to produce available P for plants (Sarikhani *et al*., 2016).

Numerous studies have shown that phytases exist among microorganisms, plants, and animals, and can be abundantly extracted from fungi, yeasts, and bacteria (Shieh and Ware, 1968). There are significant populations of phosphate-solubilizing bacteria in soil and in plant rhizosphere, which include aerobic and anaerobic species. In addition, the population of phosphate-solubilizing bacteria in the rhizosphere is much higher compared to the non-rhizosphere soil (Rodriguez and Fraga, 1999; Whitelaw, 2000).

Considering the importance of the phytase enzyme in organic phosphate dissolution and the need of monogastric animals to use these food resources, phytase production and its addition as a food additive increase the absorption of phosphate in the diet and also reduce phosphate pollution in the environment is of high interest (Koinetzny and Greiner, 2004). In addition, phytase can be used in the pharmaceutical industry to treat Alzheimer's disease, reduce cholesterol, prevent the formation of kidney stones, help bread production, papermaking, and soil amendment, and increase plant growth as well (Mittal *et al*., 2011; Parhamfar *et al*., 2015).

In previous studies, the potential of phosphate-solubilizing bacteria extracted from forest soil was investigated, and the results showed that the ability of *Pseudomonas* species to dissolve different sources of phosphate was more than other isolated bacteria (Teimouri *et al*., 2020). In another study, the phytase enzyme was produced and optimized by *Pseudomonas* bacteria from bird droppings. The results showed that the isolated bacterium had a high phytase activity, creating a halo of 23 mm in the specific phytase culture medium (Hosseinkhani and Hosseinkhani, 2009). The biochemical and molecular traits of phytase-producing bacteria extracted from soil samples of India's fields were investigated showing that from 42 isolated bacteria, three samples had remarkable phytase activity in an agar medium named *Lysinibacillus*, known as PSM (Dev *et al*., 2016).

Considering the significance of phytase-producing bacteria, and the lack of data on the presence of phytase-producing bacteria and their characterization from the soil of alfalfa and clover fields of Khomeini Shahr and Morche Khort of Isfahan Province, the objective of this research is to isolate and identify the native phytase-producing bacteria from the aforementioned fields to use them for further physical conditions and nutrient sources.

Materials and Methods

Sampling

Eight soil samples were collected from a depth of 10-30 cm in the plant rhizosphere. Soil sampling was performed from clover and forage fields from Khomeini Shahr and Margh Baharesh Morche Khort of Isfahan Province. Each sample was poured into sterile plastic bags and transferred to the laboratory quickly conserving in the refrigerator at 4°C. Samples from forage and clover fields were selected as indicators after the mixture. After the transformation of samples to the laboratory, one gr of soil sample was added to sterile distilled water. For dilution, the 3-10 and 4-10 dilution series were used and from each sample, 100 µl was cultivated on Piko Vs Kaya (PVK) medium, consisting of 1% glucose, 0.5% ammonium sulfate, 0.02% potassium chloride, 0.01 % magnesium sulfate, 0.02% sodium chloride, 0.1% yeast extract, 1.5% agar, 0.002% iron sulfate, 0.002% manganese sulfate, and 0.5%

sodium phytate. The diameter of the light zone around bacterial colonies was measured on a solid medium of PVK with the phosphorus of sodium phytate after 7-14 days at 30°C. Two samples with more diameters were selected for the next stages.

Biochemical Tests

Isolated bacteria were recognized by using gram staining, citrate tests, methyl red, nitrate reduction test, H2S indole, urease, Voges-Proskauer, and glucose methods based on a paper guide (Borgi *et al*., 2014).

Molecular Recognition

To purify the bacterial DNA, the method of boiling was used according to the protocol of McCT Company. In this method, 100 µl of samples of the bacterial colony was boiled at 100°C for 15 min under complete sterilized conditions since one colony was enough for each 4-5 cc of liquid medium. To confirm the existence of DNA, two or three colonies from each sample were added to 30 µl of distilled water. To enhance the concentration of the sample, they were boiled at 100°C for 15 min. Then, the mixture was centrifuged at 10000 rpm for 2 min. The primers of 27-F:5'-AGTTTGATCMTGGCTCAG-3' and 1525-R:5'-AAGGAGGTGWTCCARCC-3' were utilized for the amplification of the 16srRNA gene. The PCR reaction in 25 µl volume consisted of 7 µl of sterilized distilled water, one µl of each primer, 6 µl of DNA, and 10 µl of master mix (Amplicon Company). The PCR reaction was performed on the thermocycler (Eppendorf, Germany) with primary denaturation at 95 °C for 5 min and 35 cycles, main denaturation at 94 °C for 30 min, annealing at 58 °C for 1 min, extension at 72°C for 1 min, and final extension at 72 °C for 10 min. Then, the PCR products were electrophoresed on 1.5% agarose gel followed by staining with a representative of Redsafe. To determine the band size, the gel documentation was used after electrophoresis representing that the presence of a single band indicates that the PCR is being successful. Then, for DNA sequencing, the 75 µl of PCR product with forward and reverse primers were sent to Kosar Kohan Gene Company.

Data Analysis

The obtained sequences were aligned in the NCBI site and their similarity level was evaluated with other species that exist in the studied databases. The Genomic Workbench CLC 6.8.1 version was used to draw a phylogenetic tree and survey their relationships.

Results and Discussion

In the present study, the qualitative screening of insoluble organic phosphate solubilizing bacteria was performed based on the size of the zone diameter of samples in a PVK medium. Among 12 samples, two isolates were selected which had a bigger zone diameter (Fig. 1).

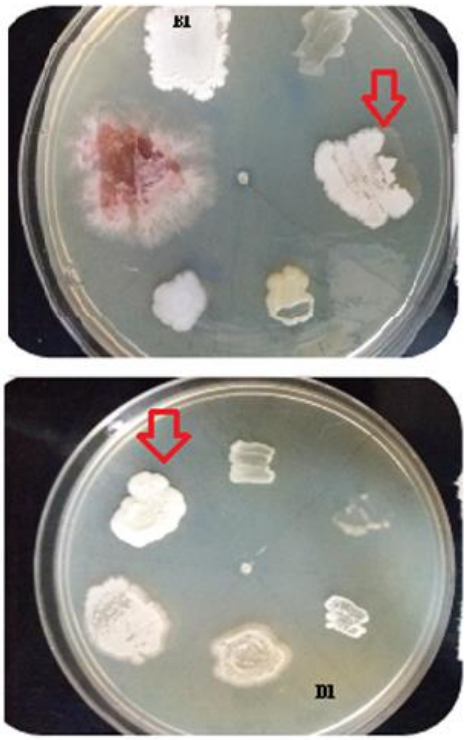

Fig. 1. Isolated bacterial colonies from soil samples on PVK medium.

The size of the zone diameter of two isolates B1 and D1 were 17 and 20 mm, respectively. After the assessment of quantitative traits, the bacterial samples of B1 and D1 were recognized according to macroscopic and morphological properties of samples such as colony size, pigment production, colony shape, the shape of the margin surrounding the colony, colony bump, and gram staining (Table 1).

The determining characteristics of the B1 bacterial sample were the big size of the colony

hanging in the bump margin surrounding it, its white pigment, and being gram-positive.

Table 1. The morphological traits of B1 and D1 bacterial samples.

BN	CS	MSC	Bum	Pig	GS
B1	Multi-piece	Big	Positive	White	Smooth
D1	Bump	Small	Positive	White	Smooth

BN= Bacteria Name, CS= Colony Size, MSC= Margin Surrounding the cClony, Bum= Bump, Pig= Pigment, GS= Gram Staining

However, the D1 sample showed a small colony size with a wavy margin, smooth bump, and creamy pigment, which was gram-positive. In a previous study, the identification and characterization of the phosphate-solubilizing bacterium from an alfalfa rhizosphere in a heavy metal-contaminated reclamation area in Shanxi, China, was carried out based on morphological observations, 16S rRNA sequencing, cellular fatty acid composition analysis, and biological test. The results showed that the H22, Y11, and Y34 were identified as *Pseudomonas sp*., while Y14 and S32 were identified as *Pantoea* sp. (Chen *et al*., 2019). In another study by Teimouri *et al*. (2004), nine phosphate-solubilizing bacteria were isolated on a specific medium using consecutive dilutions and were recognized by staining methods and biochemical assays.
In the present study, the results obtained from different parameters of biochemical tests in two samples of B1 and D1 were shown in Table 2.

Table 2. Biochemical properties in B1 and D1 samples.

Assays	Bacteria Name	
	D1	B1
Mobility	+	+
Aerobic growth	-	-
Non-aerobic growth	+	+
Urea test	+	-
Nitrate reduction	+	+
H2S	+	-
Indole	-	-
Citrate consumption	-	+
Red methyl	-	+
Voges-Proskauer	+	-
Glucose	+	+

After DNA extraction and colony PCR at 58°C, the band with 1500 bp (base pair) length on agarose gel 1.5% appeared which confirmed the weight of the 16srRNA gene (Fig. 2). To evaluate the phylogenetic relationship between the samples, the four clades were obtained using CLC Software, version 6.8.1 (Fig. 3). In the first group, two strains were located as *Bacillus endophyticus* and B1 sample, since the B1 sample showed 95% bootstrap with *Bacillus endophyticus*. In the second group, the D1 sample was located with *Bacillus paralicheniformis* with 95% bootstrap. The bacteria of *Bacillus licheniformis* and *Bacillus sonorensis* were considered as the out-groups (Fig. 3).

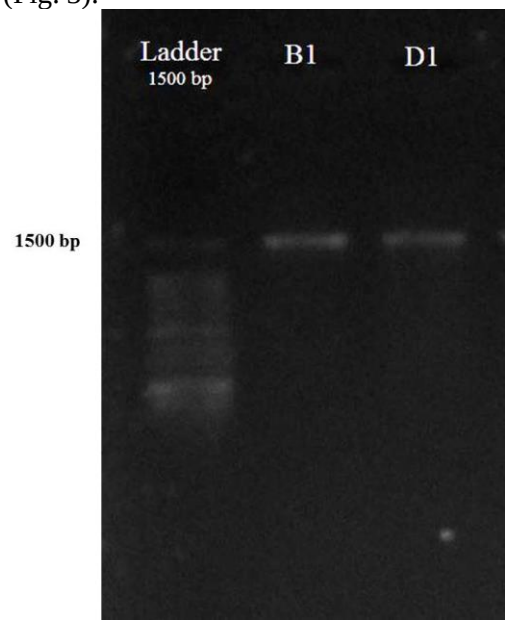

Fig. 2. Results obtained from the colony PCR of B1 and D1 samples at an annealing temperature of 58 centigrade degrees on 1.5% agarose gel.

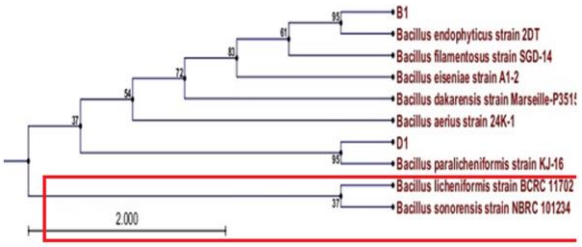

Fig. 3. The phylogenetic tree shows the relationships between 16srRNA sequences of the studied isolated B1 and D1. The numbers on each node of the clade indicate the bootstrap value (%). The red frame showed the strains as the out-groups.

Since plants produce less phytase enzyme, the presence of phytase-producing bacteria causes the increase of available phosphorus and the growth of plants (Ranjan *et al*., 2013). Recently,

researchers have introduced different habitats for phytase-producing bacteria, including the remains of cattle and birds, cereal and legume fields, corrupt fruits and vegetables, and rhizospheric soil (Mittal *et al*., 2011). In a study, the tri-calcium phosphate-solubilizing bacteria from wheat and barley fields in Marvdasht City were isolated and their results showed that the most amount and activity of bacteria were isolated from the bare field (Naseri *et al*., 2015). In addition, based on the obtained result, the isolated bacteria could be used as biological fertilizer for better plant growth (Naseri *et al*., 2015).

According to another study, the isolation and recognition of phytase enzyme-producing bacteria were performed based on the existence of a clear zone using the biochemical and morphological traits, the production rate of bacteria, and the rate of phosphate solubilizing on four media, such as PSM, PVK, NBRIP (National Botanical Research Institute's phosphate growth medium), and NBRIY. The results showed the role of *Bacillus subtilis* as phytase-producing bacteria followed by PVK medium as the most appropriate medium for the isolation of phytase-producing bacteria (Parhamfar *et al*., 2017). In another study by Nazemi (2013), the gene expression of isolated bacillus species from the soil of the mountainous region of Tonekabon City was evaluated using molecular methods resulting in the isolation of the *Bacillus subtilis* strain of STR with an emphasis on the importance of the identification and isolation of phytase producing bacillus and purifying the protein.

In a similar study, 65 phytase-producing bacteria from Himalayan soil were extracted based on phytate analysis. Among them, three efficient bacteria, i.e., *Acromobacter sp., Tetrathiobacter sp.,* and *Bacillus sp.* exhibited the ability to grow at an extended pH (Kumar Singh *et al*., 2013). In addition, the bacterial strains of phytase-producing bacteria from different soils were screened, and among the obtained 162 colonies, isolate C43 indicated the highest phytase activity compared to the other samples.

The purification of *Bacilli* resistant to heat was carried out aiming at the assessment of cellular phytase in *Bacillus* sp. by Choi *et al*., (2001). The data indicated that the optimum phytase enzyme activity was obtained at pH= 6.5-8.5 at 40°C, and if 10 mM $CaCl_2$ was added, the optimum phytase enzyme activity was higher at pH= 6-9.5 at 60°C. Also, the enzyme activity was prevented by using EDTA and metal ions, such as barium, cadmium, cobalt, copper, mercury, and chromium (Choi *et al*., 2001).

According to the results of various research studies, it is suggested to increase these bacteria's activity and make use of them to create biological fertilizer aimed at industrial usage through isolation of phytase-producing bacteria from different fields, determination of optimum medium to produce phytase enzymes, isolation of the most activated bacteria with genetic modification, and gene cloning of phytase-producing enzyme.

In conclusion, recognition and application of the existence of the microorganism in the soil are of great importance for commercialization in different industries. Furthermore, the obtained result of this study resulted in the isolation of *Bacillus endophyticus* and *Bacillus paralienshiformis* as gram-positive bacteria and optimum sources for commercial level in the future.

Conflicts of interest

The authors have declared no conflicts of interest.

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