World Applied Sciences Journal 10(10): 1129-1135, 2010
ISSN 1818-4952
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Novel Phytase and Cellulase Activities in Endophytic Azospirilla

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Abstract: Six nitrogen-fixing bacteria, isolated from roots of cultivated rice and wheat and also one strain from commercial biofertilizer, were identified using PCR amplification of 16S rDNA gene. *Azospirillum* strains isolating from rice (Tarom cultivar) and wheat (Golestan cultivar) roots that showed more growth in cellulose agar, CMC (Carboxymethylcellulose) (CM 23) agar, salicin agar, pectin agar, NBRIP and phytin agar media, were selected for comparison of their cell wall degrading enzyme activities. The *Azospirillum* strain isolated from rice showed CMCase and phytase activities significantly more than one isolated from wheat, 31.77% and 62.85% respectively; but the FPase activity of both strains was similar. Phytase activity and its zymogram for *Azospirillum* are reported in this study for the first time, with different activity profiles exhibited by various isolates. The reason for Tarom strain exhibiting higher cellulase and phytase activities may be related to the higher content of cellulose and phytin in the cell wall of Tarom cultivar.

Key words: *Azospirillum* · CMCase · Nitrogen fixation · PCR · Pectinase · Phytase

INTRODUCTION

Unlike most studies in the 1960s, 1970s and 1980s that largely concentrated their attention on rhizosphere bacteria as likely sources of fixed N, much of the recent interest has focused upon the possibility that bacteria within the plants themselves, i.e. "endophytic diazotrophs" [1, 2], are responsible for the observed N_2 fixation. James and Olivares [3] briefly reviewed the evidence that endophytic diazotrophs may actually fix N_2 in planta and transfer the fixed N products to their hosts. Bacteria of the genus *Azospirillum* (á-subclass of proteobacteria) are known for many years as plant growth promoting rhizobacteria (PGPR) [4]. Azospirilla have the potential to stimulate the growth and development of plants by N_2 fixation, the hormone effects, improvement of root development [5] and the enhancement of proton efflux in plant roots. They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates. Azospirilla are generally regarded as rhizosphere bacteria, but display strain-specific differences in the way they colonize roots. They predominantly colonize the root surface and only a few strains are able to infect plants [1, 6]. Some *Azospirillum* strains have specific mechanisms to interact with roots and colonize even the root interior, while others colonize the mucigel layer or injured root cortical cells [7].

The application of fluorescently labeled *rRNA*-targeted oligonucleotide probes in combination with scanning confocal microscopy confirmed that *A. brasilense* Sp245 enters the interior of root hair cells, which had apparently intact cell walls, whereas the occurrence of strain Sp7 was restricted to the rhizosphere soil, mainly to the root hair zone of that same Brazilian wheat cultivar [8]. The physiological basis for the observed invasiveness of *A. brasilense* Sp245 is not known. Since pectin is a major constituent of the primary cell wall and middle lamellae and low levels of pectinolytic and cellulolytic activities have been detected in *Azospirillum* cultures, the bacteria may eventually enter the root cortex intercellular spaces via enzymatic degradation of host cell wall middle lamellae [9-11]. Except for *A. irakense*, none of the *Azospirillum* species is able to grow on pectin as sole C-source. Recently, genes encoding a pectate lyase (pelA) and two aryl L-glucosidases (salA and salB) have been isolated in *A. irakense* by heterologous expression in *E. coli* [12, 13]. Alternatively, *Azospirillum* species may enter the root through lysed root hairs and cracks, disrupted cortical

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tissues at lateral root junctions. An *Azospirillum*-plant root association can only be successful if the bacterium is able to survive in the soil and attain significant populations on the host root system.

Phytic acids (salt of myo-inositol hexaphosphate) and their derivatives may comprise 50% of the total organic P present in soils [14]. Phytic acid is also a principal storage form of phosphorus in many seeds and pollen [15]. Oxidized inositols serve as noncellulosic cell wall components [16] and methylated inositols were shown to be involved in osmoprotection in a halophytic plant [17]. The plants may utilize phytic acid after hydrolysis of C-O-P ester bonds by several types of phosphatases, including phytase. These enzymes are normally present in soils where they originate from both micro-organisms and plant roots. The phytase is mainly associated with the root cell wall and with mucilage in apical root zones [18]. Phytase is important in mobilizing these P reserves for the growing seedlings [19] and pollen germination. It seems that Azospirilla can invade root cells through cell wall degrading enzymes including cellulase complex, pectinase and phytase.

The objective of present study was to isolate and identify *Azospirillum* from wheat and rice cultivars roots and investigation of their cell wall degrading enzymes activities e.g. cellulase complex, pectinase and phytase.

MATERIALS AND METHODS

Isolation of Bacteria: Fresh root samples were prepared from three cultivars of wheat (Golestan, Shirazi, Sefied) and three cultivars of rice (Tarom, Khazar, Hashemi) in Guilan province. Root samples were washed in rapidly running tap H_2O for 20 min to remove the soil particles adhering to the root surface. The washed roots were surface-sterilized with a 0.5% NaClO solution for 30 min. The roots were rinsed in sterile water at least 4 times, then cut into pieces (5-8 mm), which were macerated with forceps and introduced into semisolid NFb medium (5 gr malic acid, 0.5 gr K_2HPO_4, 0.2 gr $MgSO_4.7H_2O$, 0.1 gr NaCl, 20 mg $CaCl_2$, 2 ml trace-element solution, 2 ml alcoholic solution of Bromothymol Blue (5%), 4 ml FeEDTA, 1 ml vitamin solution, 4 gr KOH, 1.75 gr agar, 1000 ml distilled H_2O, NaOH to adjust the pH to 6.8. The trace-element solution contained: 200 mg $Na_2MoO_4.2H_2O$, 235 mg $MnSO_4.7\ H_2O$, 280 mg H_3BO_3, 8 mg $CuSO_4.5H_2O$, 24 mg $ZnSO_4.7H_2O$, 200 ml distilled H_2O. The vitamin solution contained: 10 mg biotin; 20 mg pyridoxine; 100 ml distilled H_2O) [20]. As the Azospirilla multiply in semisolid NFb medium, the disk of growth migrates closer to the surface until finally it is just below the surface (formation of pellicle).

DNA Extraction and 16S rDNA Gene PCR Amplification: Genomic DNA was obtained from pure cultures by proteinase K-sodium dodecyl sulfate (SDS) treatment followed by phenol-chloroform extraction and subsequent ethanol precipitation [21]. The bacterial DNA were prepared for PCR amplification of the 16S rDNA using forward primer (5'-AGA GGG GCC CGC GTC CGA TTA GGT AGT T-3', location 37-64 in *Azospirillum*) and reverse primer (5'-CCC GAC AGT ATC AAA TGC AGT TCC CAG GTT-3', location 436-407 in *Azospirillum*). PCR product length was 400bp. Each 25 µl of PCR reaction solution contained 2 µl (10 µM) of each primers, 2 µl of template DNA, 0.5 µl of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 µl (0.25 unit) of *Taq* DNA polymerase (Gen Fanavaran, Iran), 2.5 µl of 10X PCR buffer, 0.5 µl of 50 mM $MgCl_2$ and 15 µl sterile distilled water. PCR amplification was performed in an automated thermal cycler (BIO RAD, USA). The program includes an initial denaturation at 95°C for 4 min. Thermal cycling then proceeded with 30 cycles of 94°C for 1min, 55°C for 1 min, 72°C for 1min and a final extension at 72°C for 4 min. 5 µl of each PCR reaction solution was analyzed by 1.2% agarose gel electrophoresis. DNA fragments sizes were estimated by comparison with the standard marker 100 bp DNA ladder.

Cellulase Complex Assay: The strains were transferred to a cellulose liquid medium composed of: 10 gr cellulose, 0.004 gr $FeCl_3$, 1 gr $(NH_4)_2\ SO_4$, 0.6 gr NaCl, 0.5 gr K_2HPO_4, 0.5 gr $MgSO_4\ .7H_2O$, 0.5 gr KH_2PO_4, 0.002 gr $CaCl_2.2H_2O$, 1000 ml distilled H_2O and pH = 5-7. CMC (CM 23) liquid medium is similar to cellulose liquid medium, but instead of 10 gr cellulose, 10 gr CMC was used. FPase, CMCase and cellubiase activity was studied in supernatant with different carbon sources. Cellulose medium was used as basal medium with changing carbon sources to test cellulase activity in different carbon sources. 1ml of the bacterial isolates (OD=0.5) were inoculated in 100 ml cellulose, CMC, FP and salicin media in 250 ml conical flasks and enzyme activity were analyzed for 5-6 days [22].

Pectinase (Polygalacturonase) Assay: Pectinase activity was determined by measuring the release of reducing groups using the DNS assay. The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2 M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture supernatant, was incubated at 40ºC for 10 min [23].

Phytase Assay and Zymogram: One ml of the bacterial isolates (OD=0.5) were inoculated in 100 ml NBRIP and phytin broth in 250 ml conical flasks and enzyme activity were analyzed for 6-7 days. Phytase assay was determined by measuring the amount of librated inorganic phosphate. The reaction mixture consisted of 0.8 ml of acetate buffer (0.2 M, pH 5.5) containing 1 mM Na-phytate and 0.2 ml of both media supernatant. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. 1 ml aliquot was analyzed for inorganic phosphate liberated by the method of Harland and Harland. One enzyme unit (U) was defined as the amount of enzyme liberating 1 nmol of inorganic phosphate in 1 min [24]. For zymogram, samples were mixed with sample loading buffer (2.5 mM Tris buffer (pH 6.8) containing 0.05% bromphenol blue, 10% glycerol) in a microtube. The samples were resolved by SDS-PAGE on a 10% separating gel topped with a 4% stacking gel [25]. Phytase activity was detected by incubating the gels for 16 h in a 0.1 M sodium acetate buffer (pH 5.0) containing 0.4% (w/v) sodium phytate. Activity bands were visualized by immersing the gel in a 2% (w/v) aqueous cobalt chloride solution. After a 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Phytase activity was evident as zones of clearing in an opaque background [26].

RESULTS AND DISCUSSION

Isolation and Identification of Endophytic Azospirilla: Six different *Azospirillum* strains were isolated from wheat and rice roots and another starin was isolated from commercial. Strains were identified through biochemical tests and amplification of 16S rDNA gene by PCR (Fig. 1). All eight isolates were able to reduce NO_3^- and had catalase. All isolates fixed nitrogen under microaerophilic condition, when malate, citrate and L-rhamnnose were used as the only sources of carbon. All the isolates formed pellicle near the semisolid NFb medium surface too. In the case of molecular identification, similar 400 bp band was observed for all isolates. The result verifies that all bacteria were used in this experiment are *Azospirillum* according to 16S rDNA patterns.

Enzyme Activities: The enzymes which were investigated in present study consisted of CMCase, filterpaper hydrolase (Fpaes), cellubiase, pectinase and phytase.

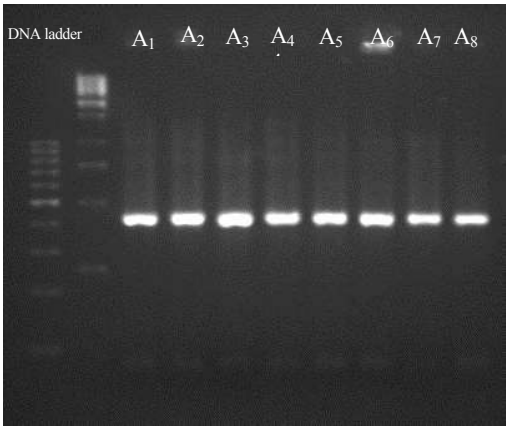

Fig. 1: Typical agarose gel of 16s rDNAs prepared from *Azospirillum brasilense* (ATCC=A1) and *Azospirillum* strains isolated from Tarom (A_5) and Golestan (A_6). The amplified DNA shows a band of 400bp.

At first, all strains tested for cellulase, pectinase and phytase activities on cellulose agar, CMC agar, salicin agar, pectin agar, NBRIP and phytin agar respectively. *Azospirillum* strains isolating from rice (Tarom cultivar) and wheat (Golestan cultivar) roots that showed more growth in cellulose agar, CMC (Carboxymethylcellulose) (CM 23) agar, salicin agar, pectin agar, NBRIP and phytin agar media, were selected for comparison of their cell wall degrading enzyme activities. Interestingly no strain had pectinase activity, but that isolated from rice showed more CMC ase and phytase activities on CMC and phytin broth.

Azospirillum isolated from Golestan cultivar (wheat) was grown on CMC, cellulose and salicin agar for studies of cell wall degrading enzymes activities. As it is shown in Fig. 2, maximum CMCase activity was 1.32 u/ml during 5 days growing on CMC broth, but it was 1.88 u/ml when it was grown on cellulose broth. The activity of cellubiase was low in all media for both strains isolated from Golestan and Tarom cultivars. The activity of FPase was low in CMC broth. However almost 0.8 u/ml FPase was shown on cellulose and FP broth. The cellulase activity of *Azospirillum* isolated from Tarom was shown in Fig. 3. As comparison with *Azospirillum* isolated from Golestan cultivar, the one isolated from Tarom had 31.77% high CMCase activity in FP, CMC and cellulose broth, but it did not have high cellubiase or FPase activities in any tested media. The cell wall of plant cells are mainly composed of cellulose, which is embedded in an amorphous polysaccharide matrix of hemicelluloses, pectin and some glycols and proteins [27]. Also oxidized inositols serve as noncellulosic cell wall components [16].

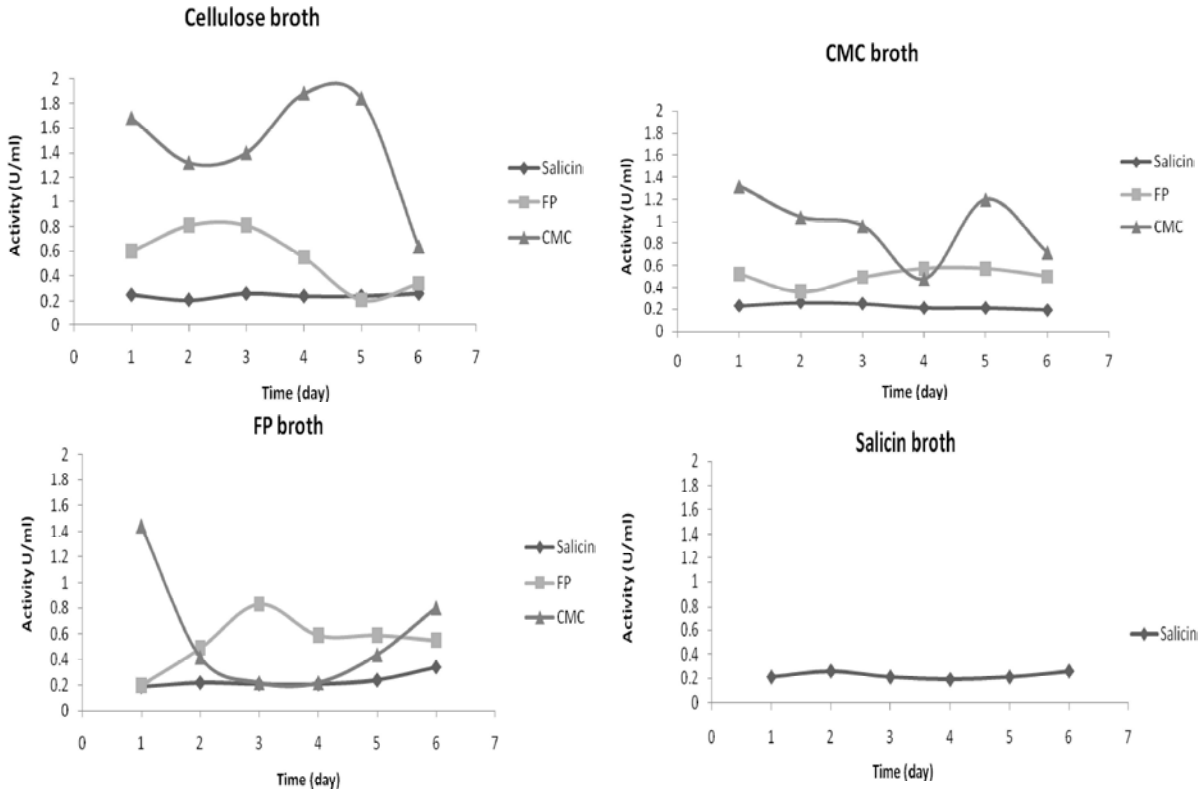

Fig. 2: The cellulase enzyme complex activity of *Azospirillum* strain isolated from Golestan. Three substrates including CMC, FP and salicin added to the filtrate of different media (A,B,C,D) which incubated at 30 °C for 6 days.

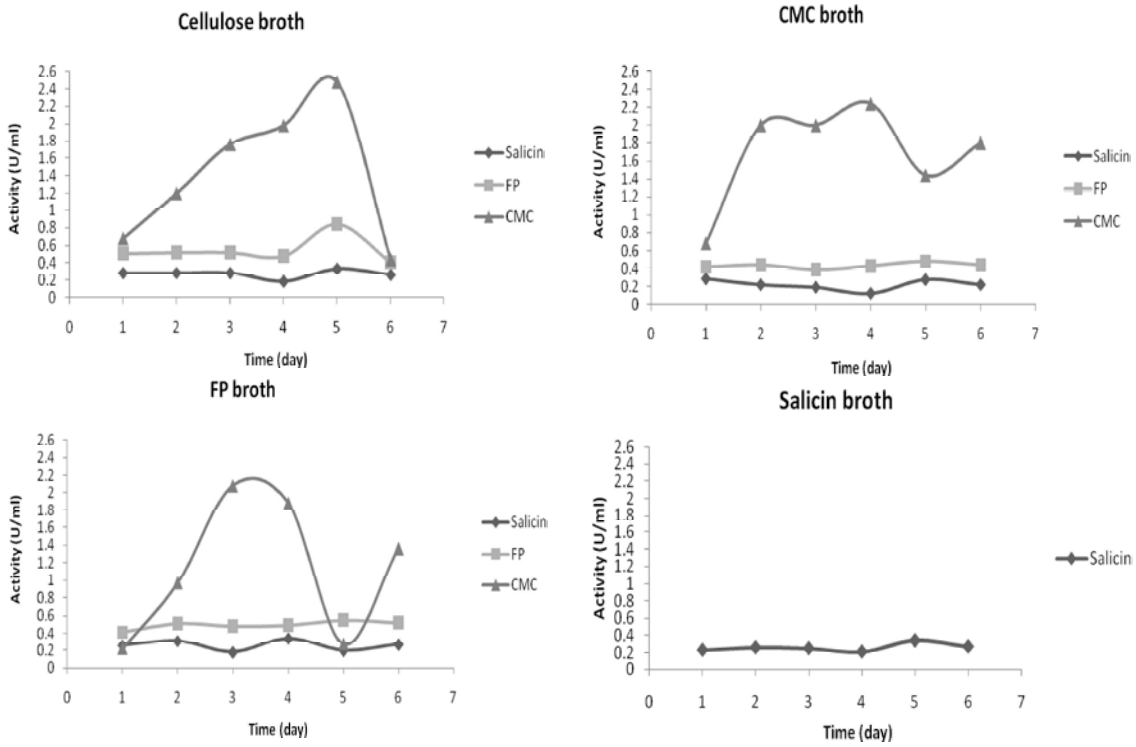

Fig. 3: The cellulase enzyme complex activity of *Azospirillum* strain isolated from Tarom. Three substrates including CMC, FP and salicin added to the filtrate of different media which incubated at 30 °C for 6 days

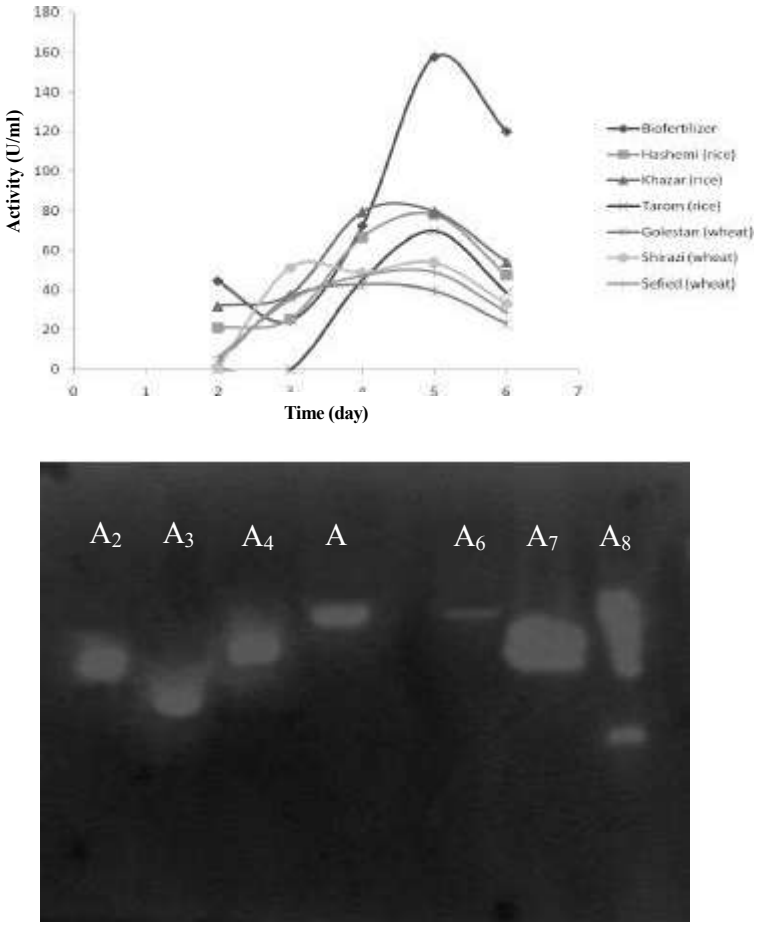

Fig. 4: The phytase zymogram of phytase (B) and zymogram of phytase (B) in *Azospirillum* isolates

The physiological basis for the observed invasiveness of *A. brasilense* Sp245 is not known. Since pectin is a major constituent of the primary cell wall and middle lamellae and low levels of pectinolytic and cellulolytic activities have been detected in *Azospirillum* cultures, the bacteria may eventually enter the root cortex intercellular spaces via enzymatic degradation of host cell wall middle lamellae [9-11]. Khammas *et al*. [28] proposed that, except for *A. irakense*, none of the *Azospirillum* species is able to grow on pectin as sole C-source, but Elbeltagy *et al.* [29] showed *Azospirillum lipoferum* isolated of rice had pectinase. There are two general types of wall based on the relative amounts of pectic polysaccharides and the structure and amounts of hemicellulosic polysaccharides. Type I walls [30], which typically contain xyloglucan and/or glucomannan and 20-35% pectin, are found in all dicotyledons, the non-graminaceous monocotyledons (e.g. Liliidae) and gymnosperms (e.g. Douglas fir). Type II walls are present in the Poaceae (e.g. rice and barley) and are rich in arabinoxylan, but contain <10% pectin [30].

There is no obvious evidence for producing cellulase by *Azospirillum* and the reports of pectinolytic activity of *Azospirillum brasilense* are contradictory. However, it is also possible that *Azospirillum* does not produce the complete enzyme set required for the infection, but induces plant to produce these enzymes by e.g. phytohormonal effects. Gafny *et al*. [31] reported that attachment of *A. brasilense* to pear millet and maize roots, respectively, was stimulated by lectin-like substances present in the root exudates. Involvement of a root lectin was also suggested in binding of wheat germ agglutinin by *A. brasilense* and *A. lipoferum* cells [1].

Mostajeral *et al.* [32] demonstrated that plant cell wall is important in the plant-*Azospirillum* interaction and the involvement of polysaccharide degrading enzymes in the root infection process by *Azospirillum* is possible. Also, their findings indicated that probably the rate of enzyme activities and its magnitude of plant growth related to inoculated seedlings with homologus and endophytic strains.

The most amount of phytase production was for biofertilizer strain and after five days. The activity of phytase in *Azospirillum* isolated from Golestan was shown in Fig. 4A. The phytase activity in Golestan strain was 42.8 u/ml during 4 days and after it dropped. The strain isolated from Tarom had 62.85% more phytase activity, 69.7 u/ml.

Phytase activity and its zymogram for *Azospirillum* are reported in this study for the first time, with different activity profiles exhibited by various isolates (Fig. 4B).

Phytate is an abundant plant constituent comprising 1 to 5% (w/w) of edible legumes, cereals, oil seeds, pollens and nuts. Like filamentous fungi, phytate-degrading enzymes of yeasts, gram-negative and gram-positive bacteria were also identified and characterized. Rice bran is rather low in inorganic phosphate and has relatively high phytate content. Therefore, the high phytate content in the medium might trigger the biosynthesis of phytate-degrading enzyme in these soil bacteria [33]. It was recognized that phytin-inosit was absorbed by barley or rice plant in the same magnitude as that of phytin-P. This fact seemed to suggest that phytin was absorbed by root without any dephosphorylation [34].

It is included that Tarom strain exhibiting higher cellulase and phytase activities may be related to the higher content of cellulose and phytin in the cell wall of Tarom cultivar in comparison with Golestan. Therefore this strain which have some of cell wall degrading enzymes and phytase can facilitate your colonization on host cell wall and cause fixing nitrogen efficiently. Also produced phytase by both of strains can raise the rate of soluble phosphorous.

ACKNOWLEDGMENT

This study was supported by the University of Isfahan. The authors are grateful for their support.

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