Vol. 2, No. 2: 36-40.
(2014).
ISSN: 2313-3937

WORLD JOURNAL OF EXPERIMENTAL BIOSCIENCES

Research article

Detection of hydrolytic enzymes produced by *Azospirillum brasiliense* isolated from root soil

Hala M. Radif [1], Shatha S. Hassan[1]*

ABSTRACT

Many samples of plant roots soil were collected from Iraq soils, fifty two isolates were obtained from these samples. The ability of *Azospirillum brasiliense* to produce hydrolytic enzymes such as protease, chitinase, lipase, pectinase and phosphatase was screened. Most isolates produced these hysrolytic enzymes. *A. brasiliense* (Aw-1) showed the maximum ability to produce these enzymes hydrolyses ratio (5.2 mm) for chitinase, (4.2 mm) for pectinase and complete hydrolyses of protease, pectinase and phosphatase. This isolate was isolated from soil collected from wheat roots.

Keywords: *Azospirillum brasiliense,* **chitinase, protease, pectinase, phosphatase.**

Citation: Radif HM, Hassan SS. (2014) Detection of hydrolytic enzymes produced by *Azospirillum brasiliense* isolated from root soil. *World J Exp Biosci* **2**: 36-40.

Received October 23, 2014; Accepted November 4, 2014; Published November 5, 2014.

INTRODUCTION

Soil microorganisms, *Azospirillum*، *Azotobacter* and Enterobacter are defined as a plant growth promoting bacteria (PGPB) [1]. These bacteria encourage plant growth by promoting the outbreak of secondary roots. PGPB also produce many secondary metabolites and hydrolytic enzyme, which act as anti-fungal cell wall and can degrade the structural matrix of fungal cell walls [2]. Hydrolytic enzymes break down protein, carbohydrate and fat molecules into their simplest units [3].

Lytic enzymes are one of the important mechanisms that are involved in the antagonistic activity of bio-control agent [4]. Enzymes are major components of biological soil processes such as the degradation of organic compounds, their mineralization and the liberation or recycling of nutrients including nitrogen, phosphorus, sulphur and other essential metals [5]. Among these, chitinase plays a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal pathogens

*Correspondence: mohannadnasif@yahoo.com
Department of Biology, College of Science, University of baghdad, Baghdad, Iraq
Full list of author information is available at the end of the article

[6]. *Azospirillum* lysates hydrolyze various chitin substrates, which result in release of free sugars. The expressed protein also had anti-fungal activity as demonstrated by inhibition of growth of the plant pathogenic fungus, Rhizoctonia solani [7].

Large number of soil microorganisms produces lytic enzymes such as *Azospirillum, Azotobacter, Bacillus, Pseudomonas, Acetobacter* and fungi [8]. Pectinase are a group of enzymes that hydrolyze the pectic substances, present mostly in plants, microorganisms and animals. Microorganisms are producing pectinase such as *B. subtilis* [9], *Streptomyces* sp. and *Azospirillum* sp. [10].

Several study reported the ability of several microorganisms such as *Achromobacter* sp., *Alkaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., Actinomycetes, Aspergillusniger, *Candida cylindracea*, *Mucur miehi* and *Rhizopusoryzae* to produce lipase [10].

Enzyme activity can be indirect assessment by the activity of a specific group of microorganisms in the soil [11]. Enzymes can serve as sensitive indicators of soil quality [12]. The aim of this study is to determine the ability of *A. brasiliense* isolated from soils of plant roots to produce some hydrolytic enzymes, which are important for improvement of soil and plant growth.

MATERIALS AND METHODS

Sample collection

Sixty-eight samples of soil surround plant roots (wheat, maize, barley and rice) were collected from Baghdad, Mousl and Babylon in sterile containers and transported to the laboratory and preserved at 4oC until using.

Isolation of *Azospirillum*

One gram of each sample was added to 9 ml of distilled water. Serial dilution with sterile distilled water up to 10-8dilutions was done. One ml of the diluted sample (10^{-6} to 10^{-8}) was inoculated in test tube contain 9 ml of selective nitrogen free bromothymol blue semisolid media, consisted of 5.0 gm malic acid, 0.5 gm K_2HPO_4, 0.2 gm $MgSO_4$.7 H2O, 0.1 gm NaCl, 0.002 gm $CaCl_2$, 0.002 $Na_2MoO_4.2H_2O$, 0.01 Na_2MoO_4.2H2O, 0.015 $FeCl_3.6H_2O$, 0.0001 Biotin, 4.5 KOH, 1.75 agar and 2 ml bromothymol blue all these components was completed to 1 liter with sterile distilled water [13]. All tubes were incubated at 32 ^oC for 48 h. The change of color of nitrogen free bromothymol blue semisolid medium from green to blue with formation of white pellet after 24 h and raise to the top after 48 h confirmed to grow of *Azospirillum*. The bacteria was purified on Rogo Congo medium (5.0 gm malic acid, 0.5 gm K2HPO4, 0.2 gm MgSO4.7 H_2O, 0.5 gm NaCl, 0.5 gm yeast extract, 0.015 gm $FeCl_3.6H_2O$, 4.8 gm KOH, 20.0 gm agar dissolved in 1 litter distilled water) and incubated at 37 ^oC for 72 h [14] and preserved on Nitrogen free bromothymol blue semisolid or agar medium

Identificationof *Azospirillum*

Azospirillum Isolates were identified by morphological features, microscopic examination and biochemical tests. The last included growth in 3% NaCl and at pH 6 and 7.5, oxidase and catalase test, requirement of biotin for bacterial growth and consuming of different carbon sources in bacterial growth.

Determination the production of hydrolytic enzymes (hydrolases)

The following enzymes was detected on special agar media, the diameter of hydrolysis zones and bacterial colony were measured and the ratio of hydrolysis zone/colony diameter was obtained and considered as a semi-quantitative measurement for enzyme production.

Production of protease

Milk agar medium consisted of 10 % Skim milk and 2 % agar [15], the medium was inoculated with 24 h age of bacterial culture and incubated at 30 ^oC for 24h.

Production of pectinase

Pectine agar medium consisted of 0.1 gm pectin, 0.5 gm yeast extract, 0.01 gm $MgSO_4.7H_2O$, 0.6 gm KH_2PO_4, 0.5 gm NaCl ,0.1 gm K_2HPO_4 and 2 gm agar all these materials were dissolved in 100 ml distilled water [16]. The medium was inoculated with 24 h of bacterial culture and incubated at 30 ^oC for 3-7 days.

Production of lipase

Two methods were applied for detection lipase. Detection on mineral salt agar medium consisted of 0.5 gm NH_4Cl, 4 gm NaCl, 0.5 gm KH_2PO_4, 1 gm Na_2HPO4, 0.5 gm $MgSO_4$ and 1 ml olive oil, the mixture was completed to 1 litter with distilled water [8]. This medium was inoculated with 24 h of bacterial growth and incubated at 30 ^oC for 3-7 days. Appearance of bacterial culture and reduction of oil drops of medium indicates a positive result.

Detection of enzyme activity can be applied on brain heart infusion agar medium. Brain heart infusion agar prepared according to the manufacturer constructions with addition of % 2 agar and %1 of Tween 80. The medium was inoculated with 24 h of bacteria and incubated at 30 ^oC for 3-7 days, a positive result was observed as turbidity around colonies.

Production of chitinase

Chitin agar medium consisted of 0.1 gm colloidal chitin, 0.5 gm yeast extract, 0.01 gm MgSO4.7 H2O, 0.6 gm KH_2PO4, 0.5 gm NaCl, 0.1 gm K_2HPO_4 and 2

gm agar the materials were dissolved in 100 ml distilled water [16] was inoculated with 24 h of bacterial culture and incubated at 30 ^{o}C for 3-7 days. This experiment was done under laboratory conditions.

Production of phosphatase

Phosphate agar medium consisted of 10 gm glucose, 5 gm Tri-basic phosphate, 0.5 gm $(NH4)_2$ SO4, 0.2 gm KCl, 0.1 gm $MgSO_4.7H_2O$, trace $MnSO_4$, trace $FeSO_4$, 0.5 gm yeast extract and 15 gm agar, these materials were completed to one litter of distilled water. The medium was inoculated with 24 h of bacteria growth and incubated at 30 ^{o}C for 3 – 5 days. Appearance of bacterial growth and light clear zone around the growth indicates a positive result.

RESULTS AND DISCUSSIONS

Isolates of *Azospirillum*

Fifty two isolates of bacteria were obtained from sixty eight samples of soil after culturing on *Azospirillum* selective medium (nitrogen free bromothymol blue). Seventeen isolates obtained from soil of wheat, fourteen isolates obtained from soil of barley, twelve and nine isolates were obtained from soil of rice and maize, respectively.

While, no isolate was obtained from soil of vegetables (Spinach, onions and Radish). The conversion of nitrogen free bromothymol blue semi solid medium color from green to blue with formation of white color pellicles down the surface after 24 h and raised to the top after 48 h was indicating the positive result and confirming the growth of *Azospirillum*. Bacteria were purified by culturing on RC medium. The result appeared scarlet, red and pink color which small colonies on the medium **Fig. 1**, which indicated growth of *Azospirillum*.

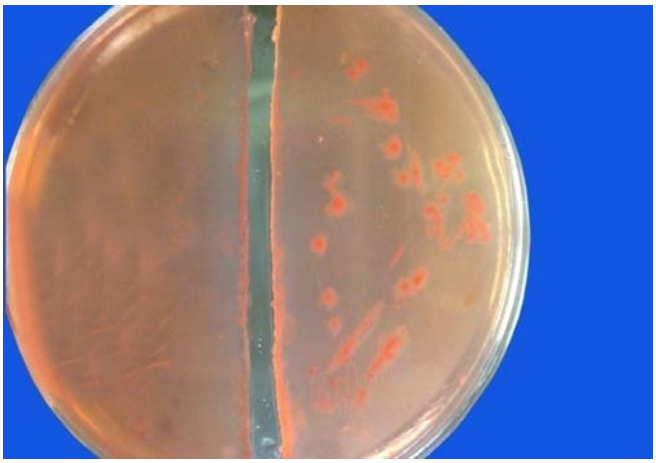

Fig. 1 Colonies of *Azospirillum* on RC medium.

In present study, *A. brasiliense* isolates were identified according to their morphological features and the results of biochemical tests (**table 1** and **2**). General microscopic examination of these isolates showed Gram negative bacteria, slightly – curved rods with pointed ends, vibriod, ovoid and pleomorphic shape. *Azospirillum* isolates were able to grow on 3 % NaCl, but unable to grow at higher concentration, and able to grow at pH 6 and 7.5. *Azospirillum* isolates were unable to utilize glucose, sucrose, maltose, lactose, trehalose and mannitol. While, utilize Pectine was variable (**table. 2**). *A. brasiliense* isolates done not required to biotin for its growth.

The ability of isolates to produce hydrolytic enzymes

Azospirillum is known as plant growth promoting bacteria enhance plant growth through various forms, such as synthesis of pathogen depressing substances and fungal cell wall hydrolyzing enzymes such as cellulase, chitinase, pectinase, protease and lipase to protect plant from disease [17].

In this study, most *Azospirillum* isolates were able to produce hydrolytic enzymes. **Table. 3** showed the isolates have high ability to produce hydrolytic enzymes. The hydrolysis zones of chitin ranged between 1.7- 5.2 mm. *A. brasiliense* isolates (Aw-1, Aw-5, Aw-6, Aw-13, Ar-3 and Am-5) that isolated from wheat, rice and maize gave superior hydrolysis zones compared with others, this may be attributed to the adaptation of bacteria in the soil to chitin which is generally found in soil as waste of insects and other organisms, these wastes induce the microorganisms to produce chitinase.

The hydrolysis zones of pectin ranged between (1.1-4.2) mm. *A. brasiliense* isolates (Aw-1, Aw-10, Aw-13, Aw-16, Ab-7, Ab-12 and Ar-3) that isolated from wheat, barley and maize gave superior hydrolysis ratio compared with others. This may be attributed to the adaptation of bacteria in the soil to pectin and use it as a sole carbon source to obtain the energy for bacterial growth. All bacterial isolates were able to produce protease with complete hydrolysis and able to produce lipase enzyme with the appearance of bacterial growth and the presence of turbidity around colonies.

Forty fife isolates were able to produce phosphatase; this is indicating that such isolates have the ability to dissolve phosphate by this enzyme. The presence of such bacteria in the soil has a positive effect to plant growth.

Phosphorus is a component of the complex nucleic acid structure of plants, which regulates protein synthesis, important in cell division and development of new tissues. It also associates with complex energy transformation in the plant. That promotes root growth in winter, stimulates tiller and

Table 1 Cultural and morphological characteristics of *Azospirillum* isolates

Bacteria	Morphological Characteristics on (RC) medium		Cells properties		Biochemical tests					
	Density of growth	color	Shape	Motility	Gram stain	Oxidase	Catalase	Growth in %3 Nacl	Growth in pH(6)	Growth in pH(7.5)
Azospirillum isolates	Light or moderate	Red pink,deep read and scarlet	Curved rod,ovoid. ,vibriod and pleomorphic	Vibrating	Gv¯e	+	+	+	+	+

Table 2 Biochemical tests distinguishing *A. brasiliense* isolates.

Characteristic	Result
Glucose	-
Maltose	-
Lactose	-
Sucrose	-
Trehalos	-
Mannitol	-
Pectine	v
Biotin requirement	-

-, Negative; v, variable

often hastens maturity [18]. Phosphatase enzyme helps plants to obtain the inorganic phosphate they need, soil phosphatase plays a major role in the mineralization process of organic phosphorus substrates. The activity of soil phosphatase can be influenced by numerous factors and soil properties and forming systems play a key role among them [19]. Soil acid phosphatase plays a vital role in controlling phosphorus mineralization and its activity reflects in soils [15].

Table 3 Hydrolytic enzymes produce by *A. brasiliens* isolates

Bacterial isolates	Hydrolysis ratio(diameter of clearzone /colonydiameter) mm		Protease*	Lipase	Phosphatase
	Chitinase	Pectinase			
Aw-1	5.2	4.2	+	+	+
Aw-13	5	4.1	+	+	+
Ar-3	5	4	+	+	+
Aw-5	5	3.9	+	+	+
Am-5	5	3.3	+	+	+
Aw-6	5	2.5	+	+	+
Aw-3	4.8	3.8	+	+	+
Ab-2	4.8	3.7	+	+	+
Ab-11	4.2	3.9	+	+	+
Ab-3	4.1	4	+	+	+
Ab-7	4.1	4.1	+	+	+
Ab-12	4	3.9	+	+	+
Aw-10	4.3	3.2	+	+	+
Aw-8	4.5	3.3	+	+	+
Ar-2	3.8	3.3	+	+	+

Enzyme production and nitrogen fixation by free immobilized and co-immobilized inoculants of *Trichoderma harizanum* and *A. brasiliense* enhanced tomato seeding growth due to the synergistic effect of both *Azospirillum* and Trichoderma [20]. Rhizospheric bacterial community belonged to genus *Proteobacteria*, *Bacillus* and Pseudomonas promoting plant growth and play an ecological important activities like production of hydrolytic enzymes including cellulose, pectinase, protease, chitinase and lipase, indicated beneficial relationship between rhizobacteria and Zea mays [21].

Conflict of interest:

The authors declare that they have no conflict of interests.

REFERENCES

1. **Dobereiner J, Day L. (1976)** Associative symbiosis in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. In *proceeding First International Symposium on Nitrogen Fixation*, WE Newton, C.J Nyman, eds, Washington state University press, Pullman: 518-538.
2. **Zarrin F, Saleemi M, Sultan T, Aslam M, Riaz-Ur-Rehman. (2009)** Antifungal activity of plant growth-promoting rhizobacteria isolated *Rhizoctonia solani* in wheat . *Afr J Biotechnol* **8:**219-225.
3. **Campbell NA, Reece JB. (2005).***Biology* .7thed . San Francisco ipearson Education.
4. Kim SK, Kim YT, Byn HG, park PJ, Ito H**. (2001)** purification and characterization of antioxidative peptide from B ovine skin. *J BiochemMol Biol* **34**:219-224.
5. **Baldrian P. (2008)** Enzymes of saprotrophic Basidiomycetes. In: Boddy L, Frankland J, van West P. eds. *Ecology of Saprotrophic Basidiomycetes.* Academic press, New York:19-41.
6. **Someya N, Tsuchiya K, Yoslida T, Noguchi MT, Akutsu K, Sawada H. (2007)** Co-inoculation of an antibiotic-producing bacterium and altic enzyme-caused by Fusarium oxysporum f.sp.lycopersici. *Biocontrol Sci* **12**:1-6.
7. **Jayaraj J. (2004)**Transfer of a plant chitinase gene into nitrogen-fixing *Azospirillum* and study of its expression. *Can J Microbiol* **50**:13-50.
8. **Ghafil JA, Hassan SS.** (2014) Effect of cultural conditions on lipase production from Pseudomonas aeruginosa isolated from Iraqi soil. *World J Exp Biosci* **2**: 13-18.
9. **QiangW, Xue-Rong F, Zhao-zhe H, Jian C. (2007)** Optimization bio-scouring condition of cotton Knitted fabrics with an alkaline pectinase from *Bacillus subtilis* WSHB04-02 by using Response surface methodology. *Biochem Eng J* **34**:107-113.
10. **Suneetha V. (2011)** Screening, characterization and optimization of microbial pectinase. *soil Enzymology, Soil Biology*-22, Shukla G, varma A. eds. *Springer* verlag Berlin Heidelberg, **1**:329-337.
11. **Ghafil JA. (2013)** Extraction and purification of chitinase from Bacillus subtilis. *World J Exp Biosci* **1**:5-9.
12. **Dick RP, Break well DP, Turco R.F. (1996)** Soil enzyme activities and biodiversity measurements as integrative microbiological indicators . In: Doran JW, Jones A.J. eds. *Methods for Assessing Soil Quality*. Soil Science Society of America, Madiso: 247-271.
13. **Krieg NR, Dobereiner J. (1984)** Genus *Azospirollum*. Inikrieg NR, Holt JG. eds. *Bergey's Manual of systematic Bacteriology*, Williams and wilkins, Baltimore- London, **1**:94-104.
14. **Rodriguez-caceres EA. (1982)** Improved. Medium for isolation of *Azospirillum* spp. *Appl Environ Microbiol* **44**:990-991.
15. **Huang W, Liu J, Zhou G, Zhang D, Deng Q. (2011)** Effect of precipitation on soil acid phosphatase activity in three successional forests in southern China. *Biogeociences* **8**:1901-1910.
16. **Sampson MN, Gooday GW. (1998)**.Involvement of chitinase of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiol* **144**:2189-2194.
17. **Kamney AA, Lelie D. (2000)** Chemical and biological parameters as tools to evaluate and improve heavy metal phytomediation. *J Trace elements med biology* **18**:355-364.
18. **Muchhal US, Pardo JM, Raghothama KG. (1996)** Phosphate transporters from the higher plant *Arabidopsis thaliana*. *Proc Natl Acad Sci* USA **93**:10519-10523.
19. **Sarapatka B. (2003)** Phosphate activities (ACP, ALP) in agroecosystem soil. Thesis submitted to *Swedish University of Agricultural Sciences.*
20. **Al-Katany MH. (2010)** Enzyme production and nitrogen fixation by free immobilized and coimmobilized inoculants of *Trichoderma harzianum* and *Azospirillum brasiliense* and their possible role in growth promotion of tomato. *Food technol Biotechnol* **48(2):**161-174.

Author affiliation:

1. Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.