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Evaluation of Biochemical Properties of *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* for Possible Use as Soil Bio-Fertilizers

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Abstract

Considerable portions of used chemical fertilizers have to be substituted with more ecofriendly sources of nutrients; due to their polluting impacts on the long term of application; bio-fertilizers are promising alternatives providing nutrients in available forms with almost no hazardous effects on soil. In this study, Bacillus amyloliquefaciens and Pseudomonas fluorescens were evaluated as plant growth promoting Rhizobacteria (PGPRs) to be recommended as efficient bio-fertilizers individually and/or in mixtures. Biochemical parameters including growth promoting traits and extracellular enzymes were quantitatively determined in bacterial cultures. Phosphatases, urease, Phosphate solublization and Indole-acetic acid (IAA) and gibbrillic acid (GA) production were the most important parameters measured. Results showed that B. amyloliquefaciens was extremely distinguished in producing alkaline and acidic phosphatases reached almost 21 and 16 enzymatic units, respectively. As a growth promoting bacterium, B. amyloliquefaciens produced about 53 µmole GA/ml and 640 nmole IAA/ml. On the contrary, P. fluorescens was more efficient in inorganic phosphate souliblization and urease production than B. amyloliquefaciens. Urease units produced by P. fluorescens were up to 160 (U). In view of that, B. amyloliquefaciens and Pseudomonas fluorescens could be recommended to be applied as bioertilizers.

Keywords: Growth promoters; phosphatase; urease; plant nutrition; sustainable agriculture.

1. Introduction

As long as human population continues to increase, the world will have to withstand the rising demand for food. Maintaining crops' quality and quantity is, not only, essential to fulfill the food needs of the growing populations all over the world; but also very crucial to several industrial and economic uses. Accordingly, characteristics and amounts of applied fertilizers are key factors affecting the growth, yield, sustainability of the agricultural systems [1].

Unfortunately, intensive crop cultivation typically involves a high application rate of nutrients, and the excess amount of fertilizer that leaches from the soil affects the quality of both the environment and human health [2]. Chemical fertilizers used in agriculture are contaminating both soil and ground water. The permanent consumption of chemical fertilization leads to decline in soil fertility and quality and could cause the accumulation of heavy metals in soils and consequently in plant tissues [3].

In nature, plant-microbe interactions actively occur in rhizosphere, which apparently lets the favorable species to exist and collaborate [3,4]. Generally, inoculation with beneficial microbial species is an essential factor in agricultural practices due to loss of topsoil, reduced soil fertility, decayed plant growth, low yield index and inadequate diversity of native microflora [4]. Lately, many bio-fertilizers (microbial inoculants) and organic fertilizers (compost, manures, and humic and falvic acids) are providing natural nutrient sources for plant growth and development; such substances are recently called bio-stimulants [5]. Plant bio-stimulants can be clarified as various substances and microorganisms that improve plant growth and development. When applied to plants or the

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rhizosphere, their function is to stimulate natural processes to enhance nutrients' uptake, nutrient's efficacy, tolerance to biotic and abiotic stresses, and crops' productivity and quality [6].

Phosphorous is a very vital nutrient in plant development and crop production. Even though being abundant in soils, in both organic and inorganic forms, its availability is constricted since it is largely found in insoluble forms [7]. Availability of organic phosphate compounds could be a limitation since phosphorous is highly reactive and it will interact with other metallic elements; thus phosphorus becomes restricted and unavailable to plants [7,8]. As a result, plant growth and yield are declined. Therefore, the capability of producing extracellular enzymes to facilitate available forms of phosphate is a crucial aspect for the PGPRs effectiveness in plant nutrition [8]. Therefore, using Phosphate solubilizing microorganisms (PSM) which owning a phosphate-solubilizing ability can convert the insoluble phosphate compounds into soluble forms in soil, facilitating phosphorus to be absorbed by plant roots [9].

Many bacterial genera have been utilized as PGPR, these including *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Caulobacter*, *Chroobacteriu, Micrococcus, Pseudomonas,* and *Serratia* [10]. Among those promising growth promoting bacteria, *Bacillus* and *Pseudomonas* genera are the most commonly commercialized for being PGPRs [11].

Bacilli are Gram-positive bacteria that are universally found in agricultural soils and many of them can colonize plant roots. *Bacillus* is a rod-shaped, endospore-forming bacterium, which are commercially preferred as PGPR partially for being able to produce heat and drought-tolerant endospores [12,13].

Bacillus amyloliquefaciens strains are often known to serve as plant growth promoting bacteria (PGPB). Due to their biofertilizer and biocontrol potentiality, they are becoming increasingly important as a natural alternative to various agrochemicals [14]. In opposition to expensive polluting chemical fertilizers, *B. amyloliquefaciens* can play a part in sufficient plant nutrition and reduced environmental impacts on soil fertility [13].

Pseudomonas fluorescens comprises a group of common, nonpathogenic bacteria which inhabit soil and plant surface environments. It is a familiar Gram-negative, rod-shaped bacterium [15]. Because of being well adapted in soils, *P. fluorescens* have been investigated extensively to be utilized in agricultural practices that require survival of bacteria in the soil. *P. fluorescens* is one of the PGPRs that promote plant growth, ease nutrient uptake and enhance yield of many crops [16].

Considering the good impact of PGPRs in terms of biofertilization, biocontrol, and bioremediation, all of which exert a positive influence on crop productivity and ecosystem functioning, **the aim of the present study** was to investigate the two bacterial strains: *B. amyloliquefaciens* and *P. fluorescens* for their potential ability to be used as biofertilizers. Accordingly, they were evaluated for their biochemical activities such as: phosphate solublization, growth promoters' production and providing extracellular enzymes; in order to understand their role in transforming the unavailable forms of vital nutrients like phosphate into their available forms.

2. Materials and Methods

The two Bacterial strains (*B. amyloliquefaciens* and *P. fluorescens*) were provided from Soil, Water and Environment Research Institute (SWERI), Agricultural Research Center as isolated and identified by Kasim *et al.* [17] and Saleh *et al.* [18].

The two strains were inoculated individually on nutrient broth medium, and were grown for 48h on a shaker incubator at 28-30 ºC [17]. Then, the enriched strains were inoculated at a concentration of 1% v/v, cultivated into 50 ml of specific media designated to enhance extracellular enzymatic production. The cultures were incubated on a shaker incubator at 30ºC for 48h [19]. After incubation, the cell-free supernatants were subjected to the quantitative determination of desired enzymatic activities including; amylase, cellulase, phosphatases, protease, and urease [20].

The enriched cultures were inoculated, at a concentration of 1% v/v, into 50ml of yeast extract peptone agar medium supplemented with 0.5 % Na-carboxymethyl cellulose and incubated on a shaker incubator at 30 ºC for 48h. In order to determine cellulolase (E.C 3.2.1.4) activity, the method of Deng and Tabatabai [19] was applied. Then 5ml of the bacterial culture was incubated with 20 ml of 2% carboxymethyl cellulose (CMC) in 50 mM sodium acetate buffer (pH 5.5) at 30 °C for 24h. The supernatant was targeted to reducing sugar analysis using dinitrosalicyclic acid (DNS) method.

Amylase (E.C 3.2.1.1) activity was measured according to Ross [20]. 10ml of 50 mM phosphate buffer (pH 7) and 10ml of 2 % soluble starch solution were added to 5ml of bacterial culture and incubated at 30 °C for 24h; and then the reducing sugars were measured by DNS method as described before [19].

Urease (E.C 3.5.1.5) activity in soil was established according to Tabatabai and Bremner [21] as followed: 5ml of bacterial culture was mixed with

10 ml of 10 % urea solution and 20 ml of 0.1 M citrate buffer (pH 6.7) and incubated at 30 °C for 24h. Urease activity was expressed as nmole of NH_4^+/g soil/h.

Alkaline phosphatase (E.C 3.1.3.1) and acidic phosphatase (E.C 3.1.3.2) activities were measured according to the technique originated by Tabatabai and Bremner [22]. 1ml of the supernatant was mixed with 4ml modified universal buffer (MUB) solution (pH 6.5 for acidic phosphatase or pH 11 for alkaline phosphatase). 1ml of 5mM p-nitrophenylphosphate solution was added, mixed well and incubated at 37°C for 1h. After incubation, 1ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added and mixed thoroughly. This mixture was filtered and the absorbance was measured at 420 nm. One phosphatase unit was defined as the amount of enzyme that released 1 nmole of p-nitrophenol /ml/ h.

Phosphate solubilization ability of bacteria was determined by growing bacterial strains on the NBRIP broth medium as described by Nautiyal [23] and after incubation inorganic phosphate was determined using ascorbate method as described by Murphy and Riley [24] and Watanabe and Olse [25]; 1ml of supernatant was added, then 2.5ml of the freshly prepared color reagent were added and the final volume was completed to 100ml with distilled water. The blue color developed was measured spectophotometrically at 880 nm.

Nutrient broth supplemented with 0.1 % tryptophan was used for production of indole-3-acetic acid (IAA) and gibberellic acid (GA) by bacterial strains. Indole-3-acetic acid (IAA) was determined with the method described by Mahadevan and Chandramohan [26]. Culture supernatant was acidified to pH 3.0 with 1N HCl, then 1ml was added to 2ml of Salkowski reagent freshly prepared as follow: 2 ml of 0.5 M $FeCl_3$ were mixed with 98 ml of 35 % perchloric acid in a dark glass bottle. The intensity of the color developed was measured at 530 nm. Gibberellic acid (GA) was determined according to Bruckner and Blechschmidt [27], Culture supernatant was acidified to pH 2.5 with 1N HCl. Gibberellic acid was measured at 254 nm. A standard curve was estimated using different concentrations of GA3 and expressed as mmole GA3/ml.

All experiments and analytical determinations were replicated at least three times and the presented data are the mean values. The obtained results were subjected to one way (ANOVA) analysis of variance analysis to determine the significance between treatments using CoStat software (CoHort software, California, USA) [28].

3. Results and Discussion

In the present work, bacterial extracellular enzymes activities of the considered two bacterial strains *B. amyloliquefaciens* and *P. fluorescens* used in this study are presented in Table (1). Bacterial cellulase activity was examined to confirm that the bacterial strains have the least possible cellulolytic effect. In order to employ them as inoculants in fertilization, they have to be safe for planted seeds and have no lytic effects on the components of seeds or young seedlings. Results show that *B. amyloliquefaciens* and *P. fluorescens* didn't have any cellulolytic activity. Amylase activity was measured for the same previous reason; however amylolytic activities of bacteria could be utilized in industrial applications including polysaccharides hydrolysis in fermentation processes.

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Table (1): Enzymatic activity of the bacterial strains: *P. fluorescens* and *B. amyloliquefaciens*

Bacterial extracellular enzymatic activity (nmole/ml/h)					
Treatment	**Cellulase**	**Amylase (nmole Glu /ml/h)**	**Urease (nmole NH_3 /ml/h)**	**Alkaline Phosphatese (nmole PNP/ml/h)**	**Acidic Phosphatase (nmolePNP/ml/h)**
P. fluorescens	---	1.438 [a]	159.007 [a]	5.861 [b]	3.406 [b]
B. amyloliquefaciens	---	1.280 [a]	90.681 [b]	20.863 [a]	16.090 [a]
LSD	**---**	**0.457**	**14.561**	**4.56**	**4.42**

The mean values with different small letters indicate significant differences ($p \leq 0.05$).

B. amyloliquefaciens productivity of alkaline and acidic phosphatases was very distinguished. It reached almost three to five folds of the enzymatic units produced by *P. fluorescens* as illustrated in Figure (1). Depending on the ability to produce phosphatases and consequently provide phosphate in available forms for plants, *B. amyloliquefaciens* can be recommended as an effective PGPR that could be used in fertilization.

According to [29], the application of *Bacillus*-based fertilizers to soil can enhance the plant-available forms of nutrients in rhizospheres, control disease-causing pathogenic microbial growth and induce pest defense systems. Moreover, the secretion of phosphatases and organic acids from *Bacillus* spp. acidifies the surrounding environment to facilitate the conversion of insoluble phosphate into free phosphate that could be absorbed by plants [30].

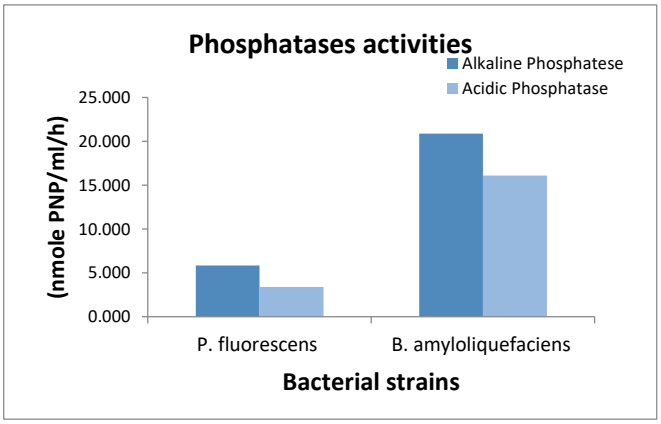

Figure (1): Alkaline and acidic phosphatases activity (nmoles PNP/ml/h) of bacterial strains: *P. fluorescens* and *B. amyloliquefaciens.*

On the other hand, *P. fluorescens* was superior in hydrolyzing urea via producing urease enzyme which is one of the most important hydrolytic enzymes and is involved in the nitrogen cycle in soil. *P. fluorescens* produced around 160 enzymatic units (**nmole NH_3 /ml/h**); which were about twice the amount produced by *B. amyloliquefaciens*. In this regard, it was reported that *P. fluorescens*, as one of the promising PGPRs, is a vital component of soil fertility and plant growth promotion due to its enzymatic activity specially phosphatases and urease [31]. In addition, *P. fluorescens* is well-known for its ability to promote plant development and reduce a range of plant diseases because of its biochemical activities [16].

Furthermore, *P. fluorescens* was a bit better in phosphate solubilization than *B. amyloliquefaciens* as presented in Table (2) and demonstrated in Figure (2); which is a very important characteristic in bacteria used as biofertilizers. *P. fluorescens* is well-known for its ability to transfer phosphorus from an insoluble to a soluble state. Acidification, chelation, and exchange reactions are all familiar mechanisms for the conversion of unavailable forms of phosphate to available forms. P solubilizing characteristics were found in *Pseudomonas* species obtained from soil and rhizospheres of several crops [16,32,33].

Table (2): Growth promoters production of the bacterial strains: *P. fluorescens* and *B. amyloliquefaciens*

	Bacterial Growth promoters				
Treatment	**IAA production without Tryptophan precursor (µmole IAA/ml)**	**IAA production with Tryptophan precursor (µmole IAA/ml)**	**GA production without Tryptophan precursor (µmole GA/ml)**	**GA production with Tryptophan precursor (µmole GA/ml)**	**Phoshate Solublization (µmole PO_4 /ml)**
P. fluorescens	0.763 [b]	0.435 [b]	31.791 [b]	28.120 [b]	1.339 [a]
B. amyloliquefaciens	0.986 [a]	0.643 [a]	52.669 [a]	46.605 [a]	0.948 [b]
LSD	**0.0877**	**0.0975**	**7.306**	**3.753**	**0.0663**

The mean values with different small letters indicate significant differences ($p \leq 0.05$)

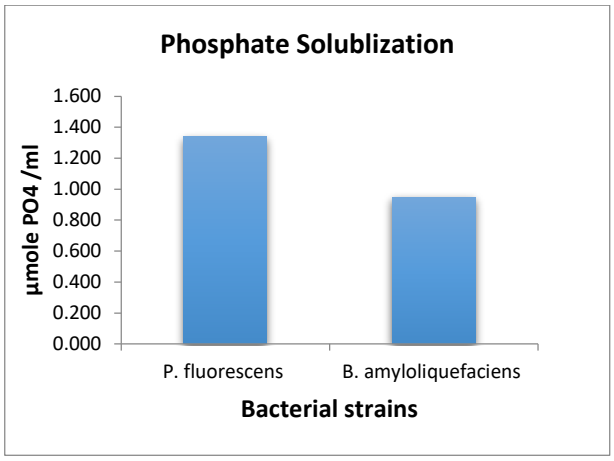

Figure (2): Phosphate solublization ability of bacterial strains: *P. fluorescens* and *B. amyloliquefaciens.*

Plant-growth-promoting substances, such as IAA, gibberellins and cytokinins, are synthesized by PGPRs like *Bacillus* and *Pseudomonas* spp. and increase root and shoot cell division and elongation [10,34]. In this concern, bacterial production of two phytohormones: indole acetic acid (IAA) and gibberellic acid (GA) were measured for the tested two bacterial strains (*B. amyloliquefaciens* and *P. fluorescens*), representing plant growth promoting traits production, as shown in Table (2) and illustrated in Figure (3).

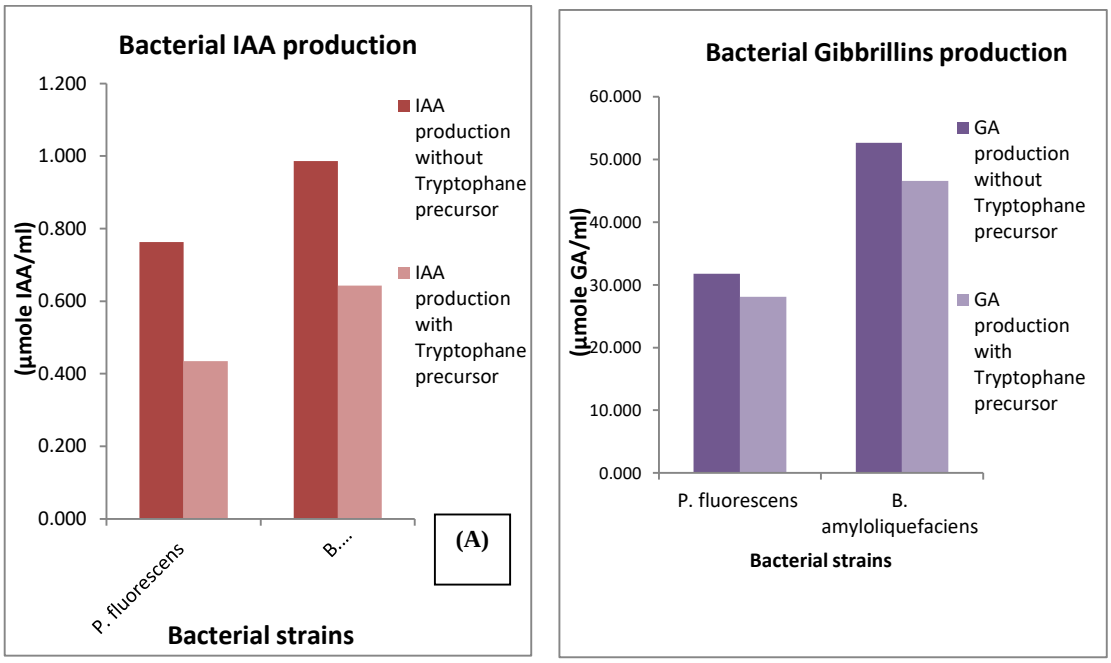

Figure (3): (A): Indole acetic acid (IAA) productivity (µmoles IAA/ml) and (B): Gibbrillic acid(GA) productivity (µmoles GA/ml) of bacterial strains: *P. fluorescens* and *B. amyloliquefaciens*

In agreement with Shao et al. who mentioned that *B. amyloliquefaciens* can produce phytohormones and antibiotics that promote plant growth directly or indirectly [35]; in the current research, it was found that *B. amyloliquefaciens* was outstanding in IAA and GA production compared with *P. fluorescens*. Kumar et al. stated that, *B. amyloliquefaciens* strains can produce substances with auxin (IAA)-like bioactivity [11]; in addition, it was observed that IAA-like compounds were present in the culture filtrates of *B. amyloliquefaciens* as detected by enzyme-linked immunosorbent assay tests with IAA-specific antibodies [11,36]. Besides, the presence of IAA was demonstrated using analyses via GC-MS performed with culture filtrates of *B. amyloliquefaciens* [37].

Because of the existence of tryptophan in the bacterial environment like growth medium or soil, encourages the synthesis of indole-3-acetic acid (IAA) and gibberellic acid (GA) as repeatedly mentioned in numerous researches [37,38], two sets of growth media for measuring IAA and GA production were prepared, one with Tryptophan as a precursor and the other one with no tryptophan. Results showed that contrary for what was expected, tryptophan didn't enhance the productivity of tested phytohormones for the two strains *B. amyloliquefaciens* and *P. fluorescens.* As noticed in the obtained results, *B. amyloliquefaciens* was better in producing both IAA and GA.

Among several species of PGPBs, *Pseudomonas* and *Bacillus* spp. have been identified as the predominant communities, and afew of PGPBs have been commercialized due to their survival within a diverse range of biotic and abiotic environments [30,31]. Indeed, *Bacillus*-based bio-fertilizers are more active compared to *Pseudomonas*-based bio-fertilizers due to the more effective metabolite production and spore-forming character of *Bacillus* spp., which enhances the viability of cells in commercially formulated products [39]. The N fixation, P solubilization, plant growth promoting hormones, and enzymes section of *Bacillus* spp. confirm their fertilizing effects on plants to improve the growth and yield of crops [30].

4. Conclusion

As demonstrated in this study, the selected strains ***P. fluorescens* and *B. amyloliquefaciens*** can provide plant growth promoters which are known by enhancing plant growth and development. Due to their beneficial aspects, those bacteria are recommended as potential bio-fertilizers and they may be used individually and/or in mixtures. Further investigations on applying those strains with plants are recommended.

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