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GROWTH CHARACTERISTICS OF *PSEUDOMONAS PUTIDA* STRAINS AND EFFECT OF HUMIC SUBSTANCES ON CELL DENSITY DURING BATCH CULTIVATION

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Abstract

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In this work are studied three strains of genus *Pseudomonas* which were identified as *Pseudomonas putida*. Growth characteristics of the three strains are studied using two types of broth media. The dynamics of the growth of the microorganisms were recorded by the changes in the optical density at 590 nm. There is a lot of research proving the positive influence of Humic substances on the development of plants in combination with soil isolates such as *Pseudomonas putida*. Humic fractions (humic acid, fulvic acid) at concentration of 0.1 g/l stimulated the growth of the soil bacteria strains acting as a regulator of the cell metabolism. In our experiments it is shown the influence of different concentrations of Humic fractions on the development of the test strains. It was found that there is a positive effect on the biomass density at the end of the batch cultivation at 2% as supplement of the media. For monitoring the biological effects of the studied strains is made test with Soybean and the seeds are treated with appropriate concentrations of the tested strains. This type of research is initial screening to monitor the activity of the strains, at all. It is used not only to monitor the germination of the seed but also for the entire development of the plants.

Key words: Pseudomonas putida, Humic fractions

Introduction

Pseudomonas putida are aerobic, gram-negative bacteria, general in agricultural soils, and are well adapted to growing in the rhizosphere. (Waller, 2007) They possess many traits that make them well suited as biocontrol and growth-promoting agents. These include the ability to 1) grow rapidly in vitro and to be mass produced; 2) rapidly utilize seed and root exudates; 3) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; 4) produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances); 5) compete aggressively with other microorganisms; and 6) adapt to environmental stresses (Caron and Patten, 1995). Object of the following studies will be monitoring their effect on the amount of

synthesized secondary metabolites and having appropriate concentrations for their application to the plant.

Materials and Methods

Bacterial strain

Two soil isolated bacterial strains from genus *Pseudomonas* – *Pseudomonas putida* OR2 and *Pseudomonas putida* OR5 were used for this study. The third strain *Pseudomonas putida* is deposited at NBIMCC with accession number BTCC1046.

Culture Media and Growth Characteristics

For all the experiments the microbial strains were fed batch cultivated. On Nutrient broth agar (BB-NCIPO Ltd.) the cell suspension of *Pseudomonas putida* strains were standardized with 0.9% NaCl at 10⁸ CFU/ml at optical density 590 nm

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by McFarland standard (Cat. №3414). For investigation of the growth characteristics of all strains were used two types of broth media: Nutrient Broth media (Bul-Bio ltd.) with supplementation of 1% glucose and composed media “Broth 14” with complementation of: sucrose – 1%; $(\text{NH}_4)_2\text{SO}_4$ – 3g/l; KH_2PO_4 1g/l; MgSO_4 – 0.5g/l; Yeast extract – 0.1%; bacto-peptone – 0.1%. To observe the growth profiles 2 ml standardized all suspension of each strain were inoculated in 100 ml NB and “Broth 14” and cultivated at $29 \pm 1^\circ\text{C}$ and 250 rpm in an orbital shaker (B. Braun). The bacterial growth was monitored by measuring the optical density to every 2 hours for the 34th hour batch cultivation at 590 nm.

Influence of Humic Substances

In this work were used Humic Substances of potassium humate (K-humate) isolated from a high quality product of “Humate Sakhalinsky”. This product has a stable content of active ingredients – salts of humic and fulvic acids and contains useful minerals and trace elements Si, Fe, Mg, Zn, Co, Cu, Mn. The raw material for humate production is Leonardite – highly-oxidized lignite (www.humate-sakhalin.ru).

Interacting with living organisms, Humic substances even in small quantities affect their growth by inhibiting or stimulating it. Resosphere pseudomonades are in close contact with the Humic substances in soil. The aim of this work is to characterize the growth of bacterial strains *Pseudomonas putida* with supplementation of Humic substances and standard growth media.

To prepare extraction solutions of humic acid, fulvic acid and mixture of both we used a method with some modifications from CDFA – California Department of Food and Agriculture (Page, 1983; Swift, 1996).

Gene expression

Chromosomal DNA from strain *Pseudomonas putida* OR2 and OR5 was isolated with NucleoSpin[®] tissue isolation kit (Macherey-Nagel) according to the description of manufacturer. 16S rDNA from the strains was amplified with universal (27F or 1492R) primers by using Ready-To-Go PCR Beads (GE Healthcare Live Sciences). PCR products were visualized in a 1% agarose gel with 4 μl SYBR[®] Safe DNA Gel Stain by UVP trans illuminator system. Obtained PCR product was used as a template DNA for two-time repeated standard sequencing procedure. The sequence of amplified product was analyzed by a commercial provider (Macrogen Inc., Korea). The resulting sequence was further processed with the program Chromas 2 • 3 (Chromas version 2 • 3, <http://www.technelysium.com.au/chromas.html>) and compared with the available nucleotide database from GenBank using the BLAST program (www.ncbi.nlm.nih.gov/blast).

Detection of enzyme activity

The enzyme production of the isolates was observed via API ZYM system (Biomérieux, France) according to the manufacturer’s instruction.

Testing of studied strains on Soybean *in vivo*

For the experiments were used 20th and 72th hour cultures of the three strains *Pseudomonas putida*. The bacterial suspension was introduced as 2% working solution. The samples were centrifuged at 4500 rpm for 20 minutes. The obtained free cell supernatant was applied of three concentrations – native, 10x and 20x. All tests were prepared in triple. The soybean seeds (“Tu chan” Ltd., L20120830, China) were sodden into solution for 24 hours. After that they were placed on a petri dishes with filter paper soaked with 5 ml of each solution. The seeds were allowed to sprout for 3 days. Water was used as a control.

Results and Discussion

Gene analysis

In the blast analysis at GenBank DNA database of strain OR2 were observed 96% similarity with *Pseudomonas putida* (27F) and 99% (1492R) similarity with strain *Pseudomonas plecoglossicida*. In the blast analysis of strain OR5 were observed 83% similarity with strain *Pseudomonas putida* (27F) and 99% (1492R) similarity again with strain *Pseudomonas putida*.

Enzyme activity

The biochemical characteristics and the effect at presence of Humic substances in cultural media on the enzyme activity of studied strain were examined with the API ZYM system (Biomérieux, France). Some strong alkaline phosphatase, leucine aminopeptidase, acid phosphatase, and esterase (C4) activities were detected. Weak positive reactions were observed for valine aminopeptidase, phosphohydrolase, and esterase lipase (C-8) in the majority of strains tested. In some cases Humic substances enhance the effects of the enzyme, while in others it is suppressed. Most strongly defined enzyme activity of all strains has *Pseudomonas putida* BTCC1046. Almost equal activities with a few exceptions have been observed in *Pseudomonas putida* OR5 and OR2. The results are presented in Figure 1.

Dynamics of accumulation of biomass

The results for the growth of the strains in the different variants of the broth media as relative optical units corresponding to the accumulation of biomass are presented in Figure 2 (A, B, C). A typical curve of bacterial growth with

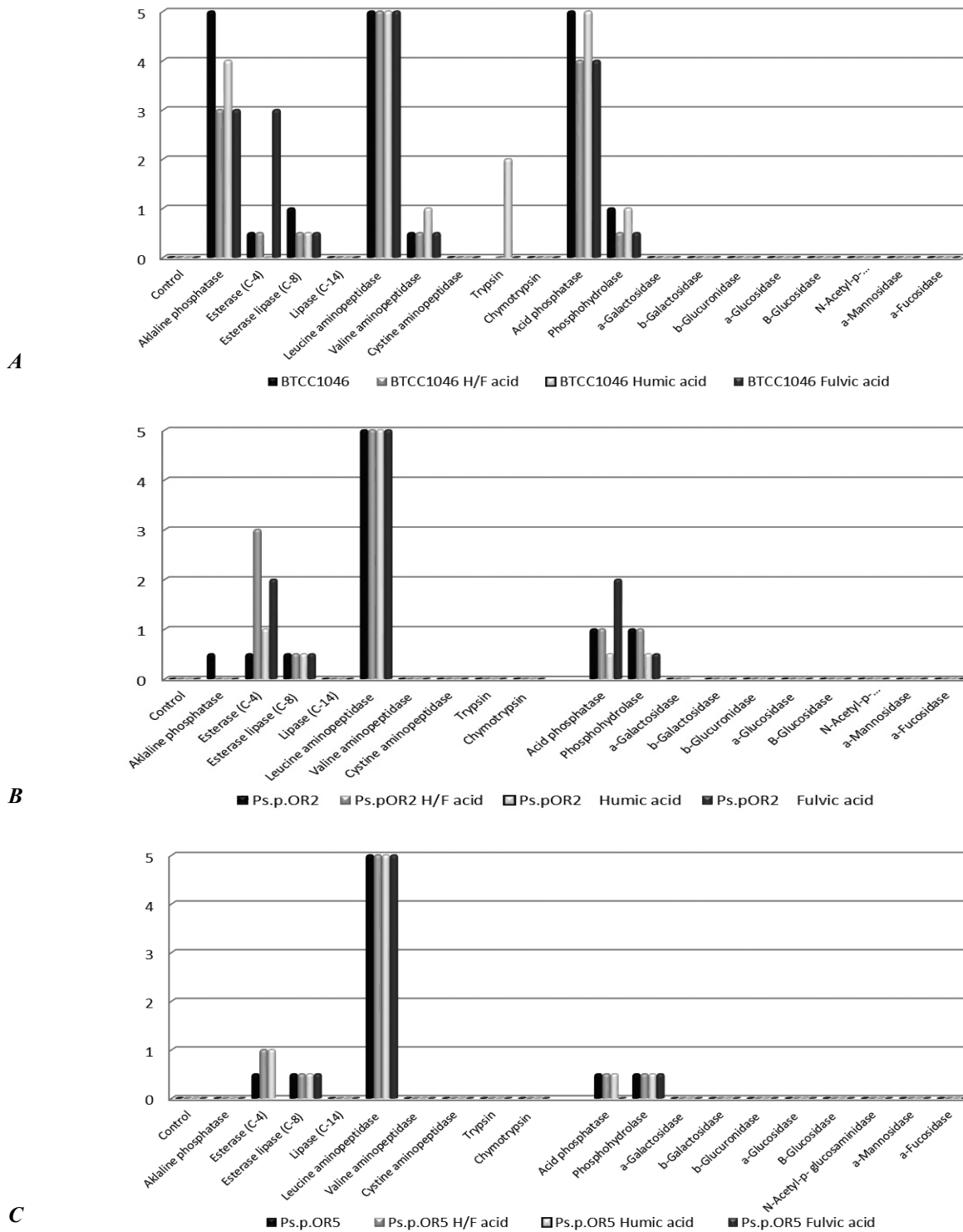


Fig. 1. Biochemical characteristics of the tested strains by API ZYM system.
A – *Ps. putida* BTCC1046, B – *Ps. putida* OR2, C – *Ps. putida* OR5

pronounced exponential phase can be observed. The lag phase for the three tested strains last 4 to 6 hours. The duration of exponential phase is between the 8th and 20th hour for all strain tested, and passes into a stationary phase at 20th to 24th hour. Most high optical density is reached at strain *Ps. putida* OR2 in the presence of “Broth 14’”. *Pseudomonas putida* OR5 and *Ps. putida* BTCC1046 marked better growth

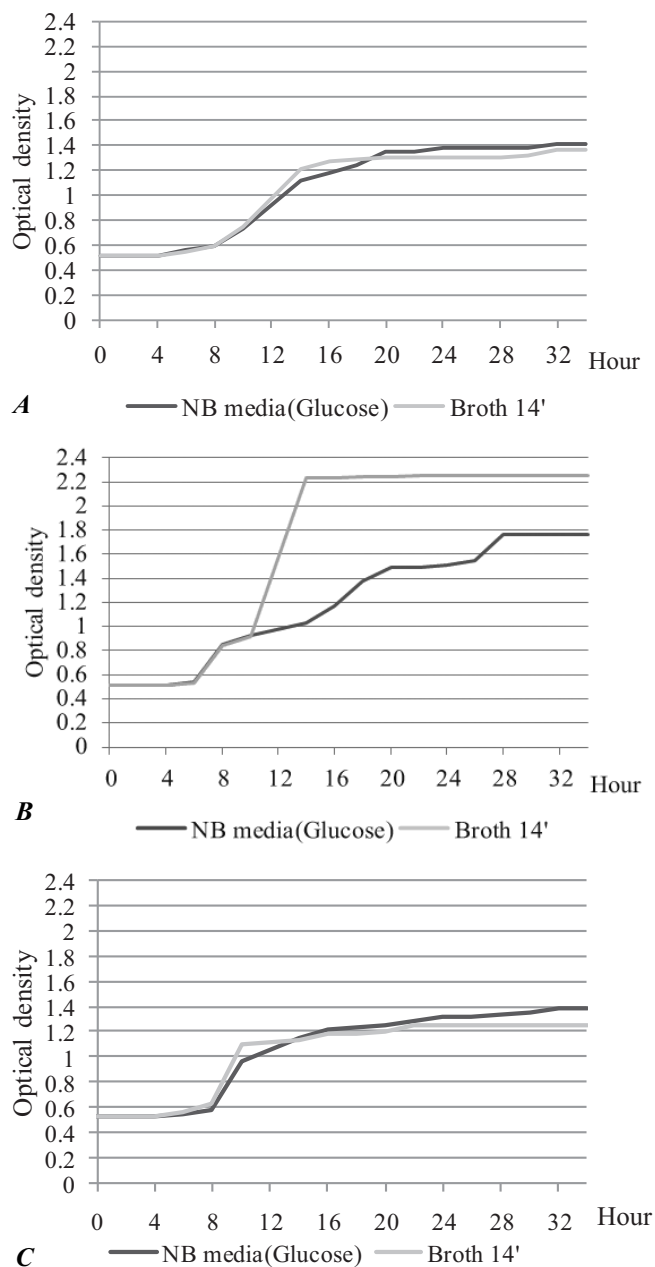


Fig. 2. Biomass accumulation (OD) – A) *Ps. putida* BTCC1046, B) *Ps. putida* OR2, C) *Ps. putida* OR5

characteristics at NB media. In conclusion from an economic perspective NB media is more suitable for biomass accumulation of strains.

At Figure 3 are presented results of 24th hour batch cultivation of *Pseudomonas putida* strains in a NB media in presence of Humic substances. The extraction solutions of humic acid, fulvic acid and mixture of humic/fulvic acids were diluted of 100 x than added to the media. The amount of added Humic substances to the NB media is 1:4. In all three strains the presence of Humic substances has no negative effect on their development. The extraction solutions of fulvic acid and mixture of humic/fulvic acid gave increase of the optical density up to 10% for *Ps. putida* OR5 strain.

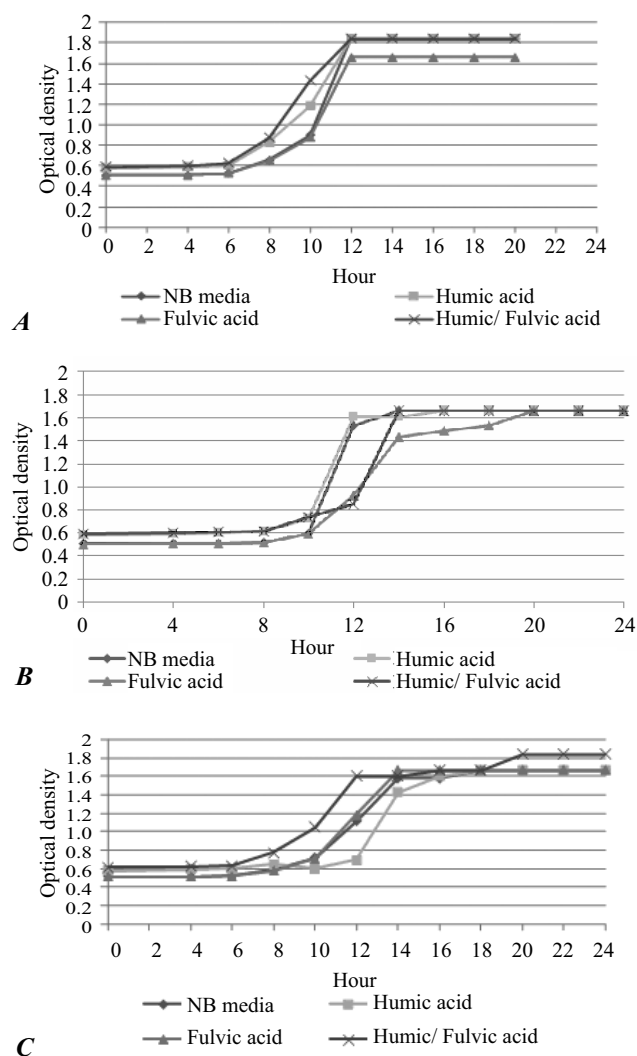


Fig. 3. Biomass accumulation (OD) in presence of Humic substances: A) *Ps. putida* BTCC1046 B) *Ps. putida* OR2, C) *Ps. putida* OR5

Study of the effect of the strains on the growth of soybean seeds

The data for the biometric measurements from the experiment with test seeds soybean are presented in Figure 4 (A, B, C). The results demonstrate that *Ps. putida* OR5 and *Ps. putida* BTCC 1046 strains cultivated for 72th hour, at 20 x concentration increase the length of the root in comparison with the control. *Pseudomonas putida* OR2

shows values closed to those of the control and in most variants observed inhibition of the seed development. All of the native filtrates have negative effect on seed germination and root development. The high concentration of dilutions conducted (10 x), also provide lower indications of the root growth. Similar experiments with soybeans in the literature are not found and therefore are not described.

Pseudomonas putida are good producers of secondary metabolites as IAA, Zeatin, Gibberellins etc (Umang Bharucha, 2011).

Conclusion

All three studied strains of species *Pseudomonas putida* are producers of enzyme activities which can improve the growth characteristics of tested plant Soybean. No inhibition of the growth of the bacteria in the range of the Humic substances concentrations tested was found. The results obtained in the experiments showed that Humic substances, being biologically active, are capable of regulating the growth of microorganisms. A combination of bacterial and humic compositions applied to plants would give a positive effect on their development.

The enzyme studies in this article show *Pseudomonas putida* strains ability to display phosphatase, aminopeptidase, phosphohydrolase enzyme activities which makes them suitable for development of enzyme products. These are important to improve the seed treatment in the early stages of germination and root formation.

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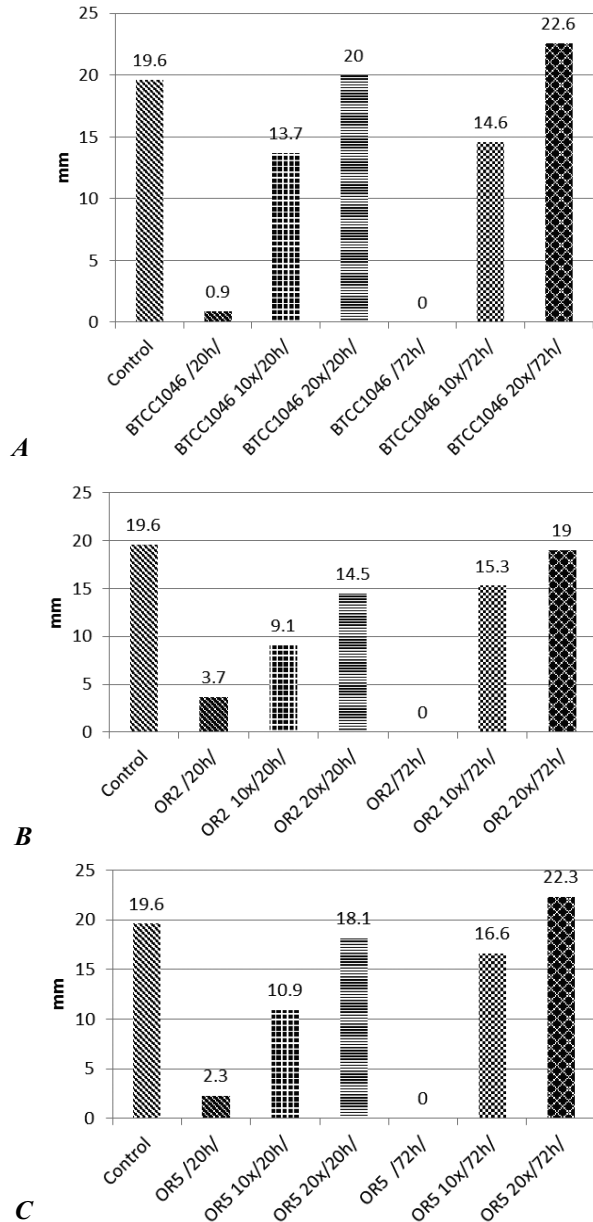


Fig. 4. Effect of the tested bacterial strains A – *Ps. putida* BTCC1046, B – *Ps. putida* OR2, C – *Ps. putida* OR5 on the seed germination and root growth of a Soybean