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ANALYSIS OF BIOFILM PRODUCTION, SWARMING AND SWIMMING MOTILITY OF *PSEUDOMONAS* STRAINS

ÉVA TAMÁS[a], GYÖNGYVÉR MARA[b], ÉVA LASLO[a], ÉVA GYÖRGY[b], BEÁTA ÁBRAHÁM[b] and SZABOLCS LÁNYI[b]

ABSTRACT. The main aim of our study is the development of bacterial biopreparates based on organic nitrogen and phosphorus mobilizing microorganisms, in order to replace the mineral fertilizers. The *Pseudomonas* strains isolated from Borsáros Raised Bog natural reserve were analyzed for the biofilm production ability through crystal violet staining, and for bacterial motility on low agar plates. The studied characteristics are beneficial in the rhizosphere colonization and give a higher survival rate of the selected bacteria in a harsh environment. Our results show that the majority of the selected *Pseudomonas* strains have a good colonization and survival potential in the rhizosphere environment.

Keywords: biofilm production, exopolysaccharide matrix, bacterial motility, Pseudomonas strains

INTRODUCTION

In order to develop high efficiency bacterial biopreparates, the appreciation of colonization and survival potential of the selected bacterial strains is indispensable. Colonization of the root system is a required ability of the soil bacteria with potential applications in agriculture as biocontrol, biofertiliser or phytostimulator biopreparate. In the present study we analyzed the bacterial motility that have role in the colonization of the rhizosphere, and also the biofilm forming capacity. The exopolysaccharide matrix of the biofilm serves as a shield in the protection of the bacteria from the environmental stress. There are several types of bacterial motility like sliding, gliding, twitching, darting, swimming and swarming [1]. The most studied motility types are the swimming and swarming motility, already studied in *Proteus sp.*, *Vibrio sp.*,

[a] *Politehnica" University of Bucharest, Faculty of Applied Chemistry and Material Science, Spl. Independenței, 313, Sector 6, Cod 77206, Bucharest, Romania, Tel: 40 21 402 91 00, Fax: 40 21 318 10 05, e-mail: tamaseva@sapientia.siculorum.ro*

[b] *„Sapientia" University, Cluj-Napoca, Faculty of Sciences, Miercurea-Ciuc, Bioengineering Department, Piața Libertății, 1, Cod 530104, Miercurea-Ciuc, Romania, Tel.: +40 266 317 121, Fax: +40 266 372 099, e-mail: maragyongyver@sapientia.siculorum.ro*

Serratia sp., *Chromobacterium sp.*, *Clostridium sp.*, *Bacillus sp.* and *Thiovolum sp.* strains. Motility types basically depend on the medium composition (gel strength) [1, 2, 16]. In order to swarm, cells first differentiate into swarmer cells characterized by an increase in flagellum number and the elongation of cells, and then move as multicellular groups across solid surfaces [3, 4]. This is in contrast with swimming motility that represents individual cell motility in an aqueous environment. In addition to the morphological changes, the swarmer cells produce extracellular materials, such as surfactants and exopolysaccharides to increase surface wetness and thus facilitate movement [2, 5, 6]. The concentration of agar is the factor that determine whether the cells swim or swarm or form regular colonies. On media containing low agar concentration (0.4%), the bacterial cells exhibit swimming motility, while on media solidified with 0.4–1.2% agar, the organisms swarm on the top of agar surface [1]. The increase of the agar concentration of the culture media result the inhibition of migration of microorganisms and consequently normal-sized colonies are formed.

Microorganisms can attach to surfaces due to a transition from a planktonic life form to a sessile life form, developing biofilm. The biofilm is the totality of surface-associated microbial cells enclosed in an extracellular polymeric substance (EPS) matrix [7]. The analysis of the biofilm production can be realized by spectrofluorometric assay, with fluorescent wheat germ agglutinin (WGA), crystal violet or malachite green coloration [8, 9, 10, 11].

Many *Pseudomonas sp.* strains are known as plant growth promoting bacteria due to the phosphate solubilization ability, siderophore or biocontrol agent production, as example *P. fluorescens, P. putida, P. aeruginosa, P. mendocina* [14, 15, and 18].

The main aim of the present study was to determine the biofilm production and motility of bacterial isolates in order to select potential strains for biopreparate production. The studied strains were isolated from Borsáros Raised bog, situated in the Ciuc basin, approximately 7 km to south from Miercurea Ciuc, in Harghita County, Romania. This is a nutrient poor acidophilic habitat, with *Sphagnum sp.* mosses that were reported to be associated with *Pseudomonas sp*. in Baltic Sea Coast [20].

The isolated bacteria were cultured on specific King's B media and classified as *Pseudomonas sp.* strains due to the phenotypic, cultural and taxonomically important biochemical properties (gelatine-, glucose-, lactose degradation, nitrate reduction, oxidase test etc.).

RESULTS AND DISCUSSION

The swimming and swarming mobility of 37 *Pseudomonas* strains was analyzed. The swimming ability was determined on Luria Bertani broth with 0,3% agar concentration, respectively the swarming ability was determined on Luria Bertani broth with 0,5% agar concentration [12].

After 24 h incubation on 28ºC the strains with swimming and swarming ability were selected. The strains were grouped in three groups: 18 strains gave positive result for both swimming and swarming (figure 1, A and B); 10 strains gave positive result for swimming and negative result for swarming (figure 1, C and D); and 9 strains gave negative results for both types of mobility (Figure 1, E). After one week incubation on the surface of the agarized media the formation of concentric zones were observed (figure 1, F - I). This is the result of the differentiation and dedifferentiation cycles of the selected strains from normal flagellated vegetative cell to hyper-flagellated elongated swarm cell and reverse [1, 13].

The biofilm production ability of the *Pseudomonas sp.* strains was realized by crystal violet staining by spectrofluorometric method.

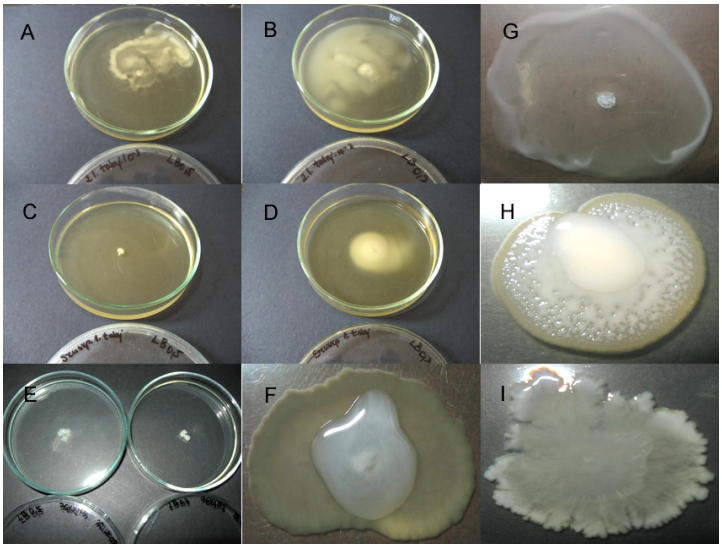

Figure 1. Images of the motility assay.
A, B: strain with swimming-swarming ability; C, D: strain with swimming ability;
E: no swimming swarming motility; F, I: one week cultures with
concentric zones are showed on.

For the determination of the biofilm production, we obtained 24 hours culture in King's B broth, containing the same glicerol amount in case of every strain. For each strain, 150 µl of 24 h culture suspension was loaded into 96-well polystyrene microtiter plate (Falcon). As a control, 150 µl of sterile King's B medium was used. To determine the cell growth, before and after the incubation we measured the optical density of the cell suspension on 620 nm. After 24 h of incubation at 28ºC, the medium was removed from

the plate and the wells were washed twice with 300 µl sterile demineralised water to remove the non- and low-adherent bacteria. An amount of 200 µl per well of 1% (v/v) crystal violet solution in water were added for 45 min to stain the biofilm. The stain was removed and the wells were washed three times with 300 µl sterile demineralised water to remove the excess stain. The stained biofilm is shown on Figure 2, A-C. The dye bounded to the exopolysaccharide matrix was dissolved in 250 µl of absolute ethanol (figure 2, D). A volume of 200µl of the stained ethanol was transferred to a new microtiter plate. The optical density (OD) of each well was measured at 492 nm using a microtiter plate reader (Fluorostar Optima, BMG Labtech), to determine the quantity of the bounded dye.

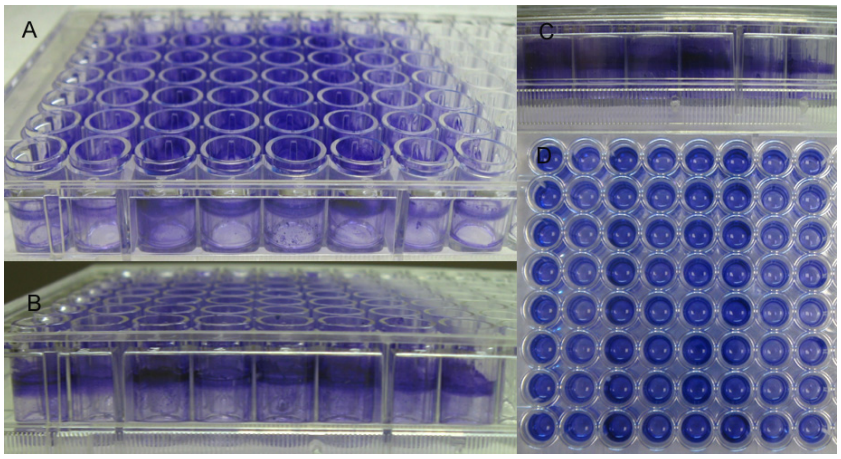

Figure 2. Biofilm stained with crystal violet, A-C: the stained biofilm on the surface of the microtiter plate; D: the bounded crystal violet dissolved in ethanol.

The biofilm production ability of the *Pseudomonas sp.* strains is presented in figure 3. The grey column represents the optical density of the crystal violet bounded to the exopolysaccharide matrix, and the black column represents the ratio of the optical densities: OD_{492}/OD_{620}. This ratio represents the real value of the biofilm production, scaled with the optical density of bacterial cells. A number of 11 strains (S.2T, S.1.2.G, $1.2.T.10^{-1}$, $1.2.T.10^{-2}$, $1.2.T.10^{-3}$, $1.3.T.10^{-1}$, $1.3.T.10^{-2}$, $1.3.T.10^{-3}$, $1.4.T.10^{-3}$, $2.2.T.10^{-3}$, $2.5.T.10^{-2}$) were selected having good biofilm forming capacity.

The results obtained from the analysis of the motility and biofilm production was evaluated using principal component analysis (PCA), represented on figure 4. The essence of the principal component analysis is to reduce the dimensionality of a data set consisting of a large number of

interrelated variables, while retaining as much as possible of the variation present in the data set, by transforming to a new set of variables, the principal components (PCs), which are uncorrelated.

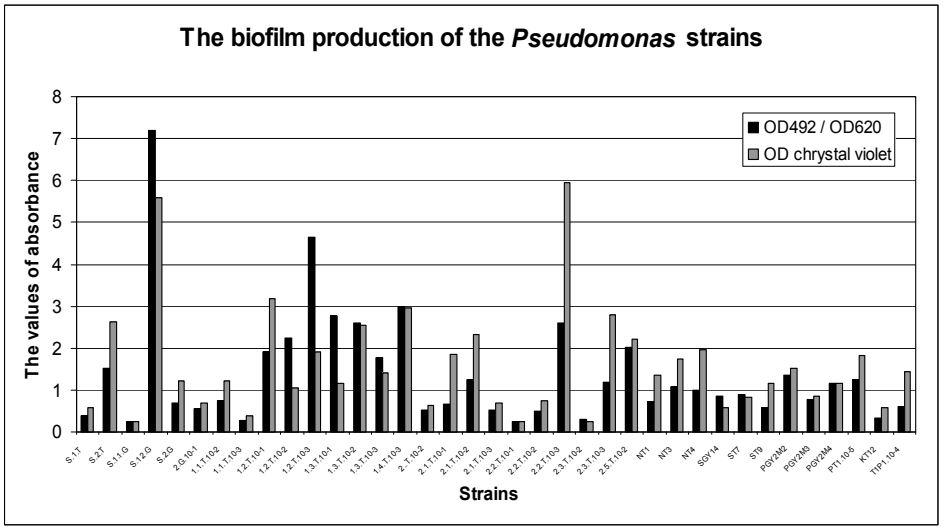

Figure 3. The biofilm production of the *Pseudomonas* strains.

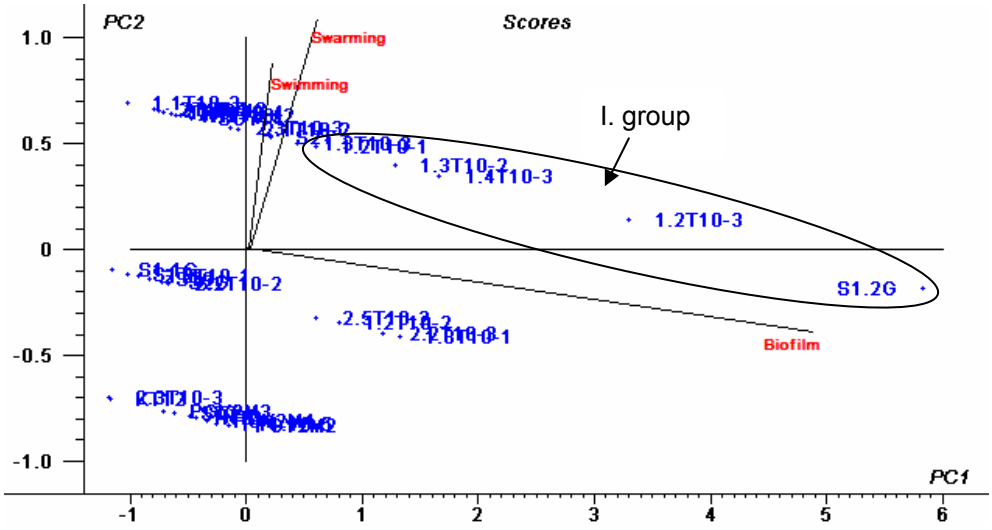

Figure 4. Principal component analysis of the studied characters.

Our strains were grouped in five groups, listed in table 1.

Table 1. Groups of the strains obtained after the PCA analysis.
(+) - positive result, (–) - negative result.

Nr.	Groups	Strains
I.	+ swarm, + swim, good biofilm producer	6 strains: S.1.2.G, 1.2.T.10^{-1}, 1.2.T.10^{-3}, 1.3.T.10^{-2}, 1.3.T.10^{-3}, 1.4.T.10^{-3}
II.	+ swarm, + swim, weak biofilm producer	12 strains: S2T, 2.G.10^{-1}, 1.1.T.10^{-2}, 1.1.T.10^{-3}, 2.T.10^{-1}, 2.1.T.10^{-1}, 2.1.T.10^{-2}, 2.1.T.10^{-3}, 2.3.T.10^{-}3, NT1, SGY14, T1P1.10^{-4}
III.	- swarm, + swim, good biofilm producer	4 strains: 1.2.T.10^{-2}, 1.3.T.10^{-1}, 2.2.T.10^{-3}, 2.5.T.10^{-2}
IV.	- swarm, + swim, weak biofilm producer	6 strains: S1T, S.1.1.G, S.2.G, 2.2.T.10^{-1}, 2.2.T.10^{-2}, ST9
V.	- swarm, - swim, weak biofilm producer	9 strains: 2.3.T.10^{-2}, NT3, NT4, ST7, PGY2M2, PGY2M3, PGY2M4, PT1. 10^{-5}, KT12

CONCLUSIONS

In this work we have established culture techniques for studying the surface behaviour of thirty seven *Pseudomonas sp.* strain. We grouped these strains by their motility, and we obtained eighteen strains, that were capable both to swim and swarm. The importance of flagelar motility in colonization of the plant roots by bacteria was clearly demonstrated in different *Pseudomonas sp.* strains [17, 19]. Chin-A-Woeng et al. [19] demonstrated that the tomato root colonization by the wild type of *Pseudomonas chlororaphis* was greater than that by the non-motile mutants in a competitive situation.

Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals,disinfectants, antibiotics and also phagocitosis better than the planktonic organisms [21]. The biofilm production ability of the *Pseudomonas sp.* strains was analysed, and eleven strains proved to be good biofilm producers.

Based on the principal component analysis we obtained six strains able to swim and swarm, and with biofilm producing ability. These strains proved a good colonizing and surviving potential in laboratory conditions. These results support the need for further investigation of the colonizing and surviving ability of selected strains.

EXPERIMENTAL SECTION

Bacterial strains and growth media

37 *Pseudomonas* strains were isolated from Borsáros Raised Bog natural reserve. Soil samples were taken from a depth of 10-15 cm and have been preserved in sterile plastic bags. Samples were processed on the sampling day, by using a soil dilution technique (serial dilutions). From each

dilution 1 ml sample was used for the inoculation of *Pseudomonas* specific King's B culture media. The selective King's B agar media contained 20g/L Proteose peptone, 10 ml/L glycerol, 1,5 g/L K_2HPO_4, 1,5 g/L $MgSO_4.7H_2O$ and 20 g/L agar. The inoculated plates were incubated at 28°C for 24 hours. The Luria Bertani (LB) agar used for motility assay contained 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and respectively 0,3% or 0,5% agar.

Motility assay

For the analysis of swarming and swimming motility we used spot inoculation method, the selected strains were inoculated on the centre of the LB media containing 0,5% (swarming) or 0,3% (swimming) agar. The plates were incubated at 28ºC. After 24 h the swarming and swimming ability of strains was determined. The assays were performed in duplicate.

Biofilm evaluation with crystal violet staining

For each strain, 150 µl of 24 h culture suspension in King's B broth was loaded into 96-well polystyrene microtiter plate (Falcon). As a control, 150 µl of sterile King's B broth was used. Before and after the incubation we measured the optical density of the cell suspension on 620 nm. After 24 h of incubation at 28ºC, the medium was removed and the wells were washed twice with 300 µl sterile demineralized water. An amount of 200 µl per well of a 1% (v/v) crystal violet solution in water were added for 45 min. After the staining the wells were washed tree times with 300 µl sterile demineralized water to remove excess stain. The dye bounded by the adherent cells was dissolved with 250 µl of absolute ethanol. Two hundred µl of the solubilised solution were transferred to a new microtiter plate. The OD (optical density) of each well was measured at 492 nm using a microtiter plate reader (Fluorostar Optima, BMG Labtech). The assay was performed in eight repeats for each strain.

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