

Preliminary analysis on biodegrading strains of *Pseudomonas stutzeri*

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Abstract

The goal of our work was to study two bacterial strains, isolated from oil polluted soil. Morphophysiological analysis and preliminary molecular analysis allowed us to identify those strains as *Pseudomonas stutzeri*. Molecular analysis aimed to determine optimal conditions for the isolation and purification of chromosomal DNA and estimation of molar percentage of guanine+cytosine, and confirmed our preliminary identification.

Keywords: biodegradation, oil polluted soil, *Pseudomonas stutzeri*, taxonomy, biochemical tests, molar percentage G+C

Introduction

Human activities are heavily reliant on petroleum and synthetic oil products for transportation, energy and heating. Although fuels are toxic, many of their components are not as persistent in the environment as other oils. Petroleum products consist mostly of hydrocarbons, such as long chained alkanes and aromatic compounds, for which there is extensive evidence of microbial degradation (Atlas, 1981). Bioremediation relies on the biodegradable capabilities of microorganisms, especially of those found in polluted natural environments, to use hydrocarbons as sole carbon and energy source. As a result, monitoring microbial communities involved in biodegradation processes and metabolic abilities of dominant strains of these communities provides most valuable information and significantly improves bioremediation techniques.

We investigated the morphological and physiological characters in order to establish their affiliation, using a polyphasic approach that includes biochemical tests and preliminary molecular analysis as molar percentage guanine + cytosine in chromosomal DNA.

Materials and Methods

Sampling and strains isolation: Enrichment cultures were performed on liquid MSM (*Mineral Salts Medium*: potassium hydrogenphosphate 1g, potassium dihydrogenphosphate 0.5g, magnesium sulphate 0.2g, sodium chloride 1g, ammonium sulphate 1g, distilled water 1000ml) supplemented with 0,03%(v/v) of 98% pure quinoline (SIGMA) as unique carbon source. Cultures were incubated for 3 weeks at 28°C and orbital shaking at 250 rpm. Thirteen strains were isolated from these enrichment cultures when plated on solid LB (peptone 10g,

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yeast extract 5g, sodium chloride 10g, agar 20g, pH 7-7,5) in Petri dishes. All isolates and enrichments were preserved in liquid LB medium supplemented with 20% glycerol at -70°C in *Microbial Collection of the Laboratory of Microbial Genetics and Biotechnology* from the Faculty of Biology, University of Bucharest. In this study, we focused on two strains, designated SQ2a and SQ2b.

Reference strains used in this work were *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* GMQ-1, *Agrobacterium tumefaciens*, *Bacillus globigii* amy⁺, *Bacillus subtilis*, *S. cerevisiae* S288C, *E. coli* K12 NM522, all from the same microbial collection.

Morphological studies: Strains used in this study were plated on solid LB, in order to examine some colony traits. We performed Gram staining and studied the microscopic appearance of the cells grown in liquid LB for 18h at 28°C, 250 rpm. Pictures was taken with photo camera Taxicab MRC Zeiss of an Axioplan Zeiss Germany optic microscope, and visualized with AxioVision Zeiss 3.1.Program.

Physiological and Biochemical Tests:

Assessing the ability to reduce nitrate

Liquid 18h cultures were inoculated on IM-1 (potassium nitrate 1.5 g, nutrient broth 1.2 g, distilled water 150 ml pH 7.4, using Durham tubes). Results were read at 24, 48, 72 h using Ilesvay – Griess solution I (sulfanilic acid 0.8 g / acetic acid 5N 100 ml) and solution II (α -naphthil-amina 0.5 g / acetic acid 5N 100 ml), and also powder Zn by evaluation for several times and also NO₂⁻ Analytical Test Stripes (Merck). *Pseudomonas aeruginosa* GMQ-1 was used as positive control strain.

Assessing the ability of casein hydrolysis

Solid 18h cultures were inoculated on IM-2 (fat free milk 150 ml, agar 5 g, distilled water 200 ml) plates using the scratching method. After incubation at 28°C from 2 to 14 days, formation of a clear zone around the colonies indicated a positive reaction. *Pseudomonas aeruginosa* GMQ-1 was used as positive control strain and *Agrobacterium tumefaciens* as negative control.

Assessing the ability of starch hydrolysis

Liquid 18h cultures were inoculated by scratching on IM – 3 (soluble starch 2.5 g, nutrient broth 2 g, agar 5 g, distilled water 250 ml) plates. After incubation at 28°C for 48h, formation of a clear zone around the colonies in the presence of Lugol solution (Iodine in potassium iodinate) indicated a positive reaction. *Bacillus globigii* amy⁺ was used as positive control strain and *Pseudomonas aeruginosa* GMQ-1 as negative control.

Assessing the ability of gelatin hydrolysis

Solid 18h cultures were inoculated on IM – 4 (gelatin (Merck) 1.2 g, nutrient broth 2.4 g, agar 6 g, distilled water 300 ml, pH 7 – 7.2). After incubation at 28°C for 2-14 days, readings were made by incubation for few hours at 4°C: the reaction was considered to be positive if the culture medium remained liquid. *Bacillus subtilis* was the positive control strain and *E. coli* K12 NM522 the negative control.

Assessing the ability of lipase production

Solid 18h cultures were inoculated on IM – 5 (peptone 15 g, yeast extract 5 g, sodium chloride 5g, tributyrine 5 g, distilled water 1000 ml). After incubation at 28°C for 2-14 days tributyrine hydrolysis determined formation of a clear zone around the colonies. Positive control strain was *Pseudomonas aeruginosa* GMQ-1 and negative control was *Sacharomyces cerevisiae* S288C.

Assessing the ability of L-lysine -decarboxylase production

Liquid 18h cultures were inoculated in IM – 6 (peptone 1.25g, glucose 0.25g, yeast extract 0.75g, brom cresol purple 5mg, distilled water 250ml and L-lysine 0.5%, pH 6.5). An L-lysine free negative control sample was made for each strain. After 4 days incubation at 28°C, in case of lysine decarboxilation, media turned purple because of increased pH values. Negative control strain was *Pseudomonas aeruginosa* GMQ-1.

Assessing the ability of indol production

Liquid 18h cultures were inoculated in IM – 7 (tryptone 2.5 g, sodium chloride 1.25 g, distilled water 250ml, pH 7.2). Readings were done after incubation at 28°C for 24-48h. Ehrlich – Bohme solution (di-methyl-amino-benzaldehyde 4 g, ethanol 380 ml and hydrochloric acid 80ml), few drops of xylol were used. Appearance of a red circle indicated the presence of indol in the culture medium. Negative control strain was *Pseudomonas aeruginosa* GMQ-1.

Assessing the ability of reducing hydrogen sulphite

Liquid 18h cultures were inoculated in IM – 8 (iron sulfate 50 mg, sodium tiosulfate 75 mg, nutrient broth 2 g, distilled water 200 ml pH 7.2). After incubation for 1-2 days, formation of iron sulfite followed by back color appearance medium, was interpreted as positive reaction. Positive control strain was *Pseudomonas aeruginosa* GMQ-1.

Assessing the ability of using citrate as carbon source

Solid 18h cultures were inoculated on MI – 9 tubes (Simmons-citrate: ammonium dihydrogen phosphate 0.25g, potassium dihydrogen phosphate 0.25 g, sodium citrate 1.25 g, magnesium sulphate 50 mg, sodium chloride 1.25g, agar 5g, distilled water 250 ml pH 7, bromthymol blue 8 ml from 1% solution). After few days' incubation at 28°C, appearance of green-bluish color was a positive reaction. Positive control strain was *Pseudomonas aeruginosa* GMQ-1 and negative control strain was *E. coli* K12 NM522.

Pseudomonadaceae-specific pigments production (pyocyanine, pyoverdine and fluoresceine)

Solid 18h cultures were inoculated on *Pseudomonas Agar P Base* (peptone 20 g, magnesium chloride 1,4 g, potassium sulfate 10 g, glycerol 10 ml, agar 12,6 g, distilled water 1000ml, pH 7,1) plates and *Pseudomonas Agar F Base* (peptone from casein 10 g, peptone from meat 10 g, magnesium sulfate 1,5 g, di-potassium hydrogen phosphate 1,5 g, glycerol 10 ml, agar 12 g, distilled water 1000 ml, pH 7,1) plates. After incubation at 28°C for one week, appearance of blue to green surrounding zone was due to pyocyanin formation, red to dark brown zone was due to pyorubin production by cultivation on *Pseudomonas Agar P Base* but yellow to greenish-yellow zone was due to fluorescein production on *Pseudomonas Agar F Base*.

Plasmid DNA analysis: Plasmid DNA was isolated and purified using a modified alkaline-lysis technique (*Birboim-Dolly technique*). The lysozyme concentration in TEG buffer (Tris

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25mM, EDTA 10mM, glucose 50mM, pH 8.0) was 30mg/ml for the strains studied in this paper and 10mg/ml for *E. coli* V517, used as molecular marker strain. Additionally, a kit extraction was performed using a Promega –Wizard Plus Minipreps DNA purification System.

Isolation and purification of chromosomal DNA: was performed after a CTAB protocol (Ausubel, 1994) with some modifications. Genomic DNA was isolated from 3 ml of overnight culture (28°C in liquid LB, 250 rpm). After harvesting, cells were resuspended in 700 µl TEG pH 8,0 (Tris 25 mM, EDTA 10mM, Glucose 50mM). Cellular walls were broken using thermal shock between -70°C and +42°C for 20 min, repeated for 3 times. Cell lysis was performed with SDS 20%. Protein precipitation was performed with NaCl 5M and CTAB/NaCl 10%/ 0,7% (v/v) by incubation at 65°C for 15 minutes. The deproteinization was done with isoamyl alcohol/ chloroform 24:1 (v/v) and fenol/cloroform 1:1 (v/v).

Nucleic Acids Electrophoresis: Electrophoretic analysis of DNA was performed using horizontal submerge agarose gel 1%(wt/vol) in TBE buffer (Tris 0,089M, acid boric 0,089M, EDTA 0,002M, pH 8,5). Electroforesis was run at 2,5V/cm and DNA stained with ethidium bromide 0,5 µg/ml.

Spectrophotometric Analysis of DNA: Spectrophotometric analysis of chromosomal DNA was carried out with an UV-VIS ULTROSPEC 3000 (Pharmacia-LKB) spectrophotometer. Absorption spectra were obtained for wavelength ranging between 200 and 350 nm. DNA purity was estimated from A_{260} , A_{280} and A_{230} values. Contamination was considered to be minimum for A_{260}/A_{280} values ranging between 1,8 and 2,0 and, respectively, greater than 2,0 for A_{260}/A_{230} .

Determination of Molar Percentage of Guanine Plus Cytosine: In order to determined the T_m values of chromosomal DNA, we used a UV-VIS ULTROSPEC 3000 (Pharmacia-LKB) equipped with a Peltier unit and SWIFT- T_m v1.05 program. DNA denaturation was performed in a temperature range between 20-100°C with a rate of 1°C/min and the DNA absorbance values at $\lambda = 260$ nm have been continuously monitored. Molar percentage guanine plus cytosine (mol % GC) was calculated using Owen's formula: % mol GC = $2,08 T_m - 106,4$.

Results and Discussion

Morphological characterization: The two strains presented distinct colony morphology on LB plates (Tab.1.). Strain SQ2a formed dark-yellow almost circular smooth-surface colonies with a diameter between 0.5-2.0 mm. Strain SQ2b formed dark-yellow rough-surface colonies with a star shape and a diameter between 0.5-2.0 mm.

Table 1. Macroscopic and microscopic aspect of SQ2a and SQ2b

Morphological traits	SQ2a	SQ2b
Colonies morphology	Circular, dark-yellow, smooth-surface	Star-shape, dark-yellow, rough-surface
Cell shape	Short rods, single cells, or in pairs	Short rods, single cells, or in pairs
Gram stain	Negative	Negative

Microscopically, cells of both strains were very similar consisting of short Gram-negative rods that appear as single cells, or in pairs.

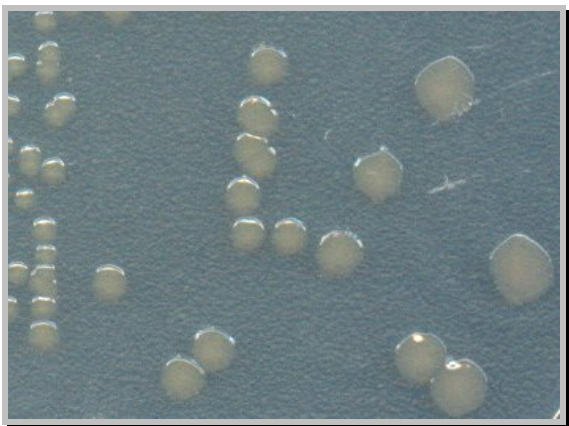


Figure 1. Colonies appearance of SQ2a.



Figure 2. Colonies appearance of SQ2b.

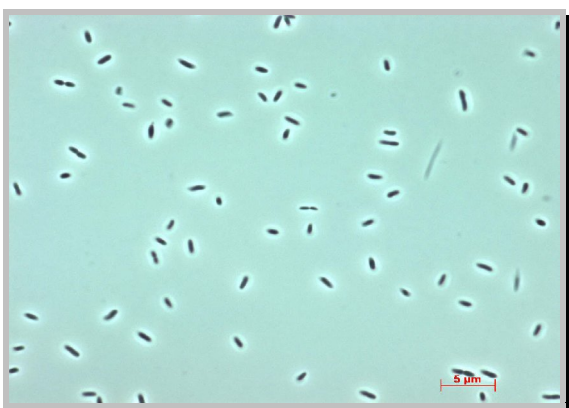


Figure 3. Microscopic morphology of SQ2a cells.

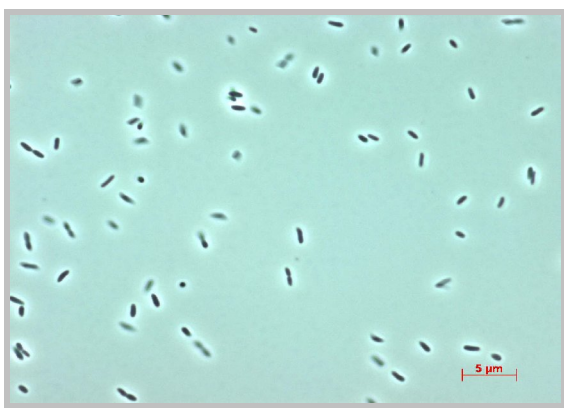


Figure 4. Microscopic morphology for SQ2b cells.

Physiological and Biochemical Tests: All the tests over the ability of use or production of different compounds were similar (Tab. 2). Our results allow us to consider as a preliminary determination of the phylogenetic affiliation in genus *Pseudomonadales*.

Table 2. Results for biochemical tests for strains SQ2a and SQ2b.

Strain	IM-1	IM-2	IM-3	IM-4	IM-5	IM-6	IM-7	IM-8	IM-9
SQ2a	NO ₂ N ₂	—	+	+	+		—	—	+
SQ2b	NO ₂ N ₂	—	+	+	+		—	—	+

Plasmid DNA analysis: Even if the protocol we used was suitable for natural plasmid detection, yet no plasmid was detected in none of the two isolates.

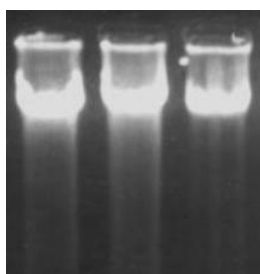
Isolation and purification of chromosomal DNA: Spectrophotometric and electrophoretic analysis revealed that chromosomal DNA was highly concentrated (Tab.3) and a high level of

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molecular integrity (Fig. 5), demonstrated that our protocol is suitable for these bacterial strains, isolated from oil-contaminated environment, well known having a strong wall resistance. Further more, not using lysozim, means that proteic contamination of DNA extract is much lower comparing with initial protocol and also the method of thermal shock is much chipper (Fig. 6-8).

Table.3 Spectrophotometric analysis of chromosomal DNA samples.

Strain	A ₂₃₀	A ₂₆₀	A ₂₈₀	A ₂₆₀ / A ₂₃₀	A ₂₆₀ / A ₂₈₀	µg DNA/µl
<i>Ps. aeruginosa</i> ATCC 27853	0.45	1.15	0.55	2.55	2.1	11.5
SQ2a	0.25	0.52	0.25	2.08	2.08	5.2
SQ2b	0.5	1.35	0.40	2.70	3.37	13.5



1. 2. 3.

Figure 5. Agarose gel electrophoresis of genomic DNA. Lanes:
1. SQ2b; 2. SQ2a; 3. *Ps. aeruginosa* ATCC 27853

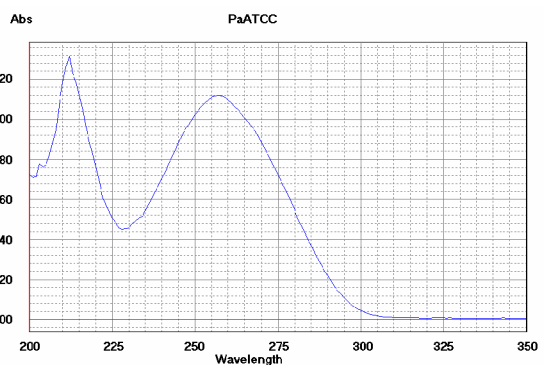


Figure 6. Wavelength scan spectrum of chromosomal DNA of strain *Pseudomonas aeruginosa* ATCC 27853

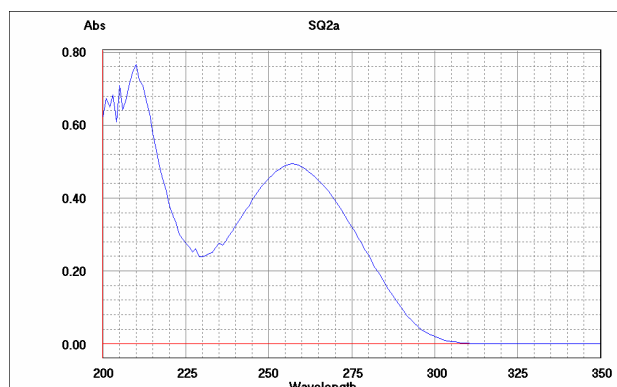


Figure 7. Wavelength scan spectrum of chromosomal DNA of strain SQ2a

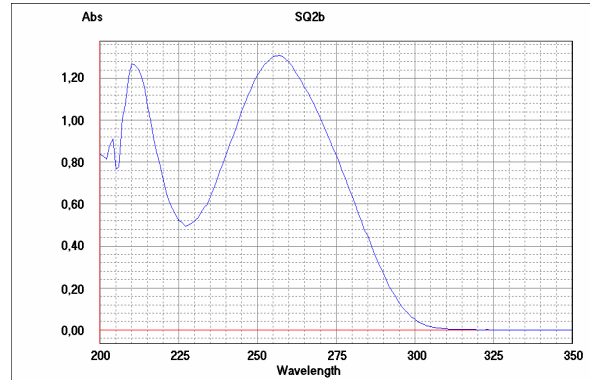


Figure 8. Wavelength spectrum of chromosomal DNA of strain SQ2b

All this data allow us to consider that modification that we made of the original protocol for isolation and purification of chromosomal DNA is providing samples with high level of molecular integrity and minimal contamination with polysaccharide, proteins and ARN molecules, that was suitable for determination of guanine+cytosine content.

Determination of Guanine+Cytosine Content of the genomic DNA (mol%GC):

Estimation of molar percentage of guanine+cytosine (molar % G+C) in chromosomal DNA was performed using thermal denaturation technique, using as test strain *Pseudomonas aeruginosa* ATCC 27853. Our results obtained for the test strain confirms the accuracy of technique we used, as well as the validity of mol%GC for the SQ2a and SQ2b, values that are in ranging between 58-69 for *Ps. stutzeri* and 58-69 for *Ps. aeruginosa* ATCC 27853. This are very large limits for a species but is well known that genus *Pseudomonadales* have a high capabilities of later transfer genes.

Our results allow us to consider that the modification of the original protocol we used for isolation and purification of bacterial genomic DNA provides DNA samples with high level of molecular integrity and minimal contamination with polysaccharide, proteins and ARN molecules.

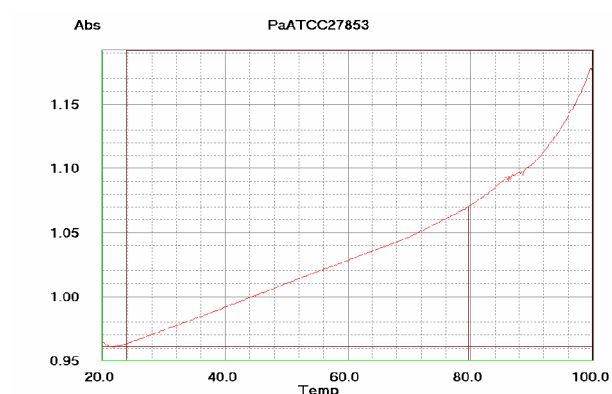


Figure 9. Hyperchromic shift curve of chromosomal DNA from *Pseudomonas aeruginosa* ATCC 27853.

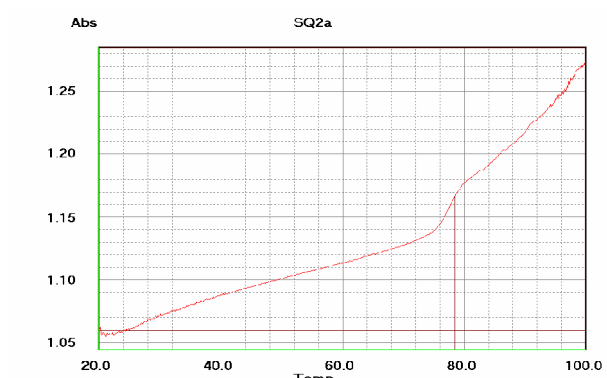


Figure 10. Hyperchromic shift curve of chromosomal DNA from SQ2a.

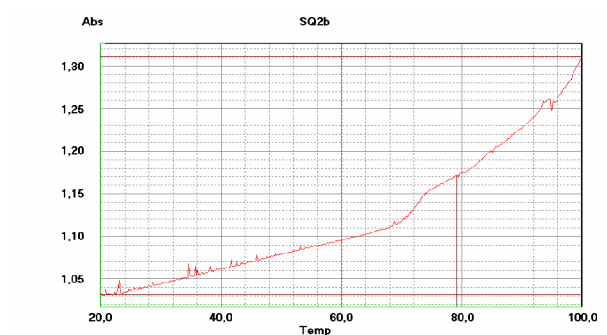


Figure 11. Hyperchromic shift curve of chromosomal DNA from SQ2a.

Tabel 4. Values of molar % G+C of the strains studied.

Strain	T _m	Molar % G+C
<i>Ps. aeruginosa</i> ATCC 27853	79,7	59,4
SQ2a	78,6	57,0
SQ2b	79,3	58,5

Our results (Fig.9-11, Tab.3), sustain our preliminary taxonomic affiliation of both strains to *Pseudomonas stutzeri* but further analysis, especially at a molecular level is necessary, for a more accurate taxonomic identification.

Conclusions

Our results underline once more the importance of polyphasic approaches in studying bacterial strains. Molar percentage of guanine + cytosine confirmed our preliminary affiliation of the two isolates to species *Pseudomonas stutzeri* but further molecular analysis on 16S ribosomal DNA genes will provide more accurate information.

Acknowledgments

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