BIODEGRADATION OF SYNTHETIC DYES BY SOME BACTERIAL STRAINS ISOLATED FROM SOIL

BIODEGRADAREA COLORANȚILOR SINTETICI DE CĂTRE TULPINI BACTERIENE IZOLATE DIN SOL

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Abstract: *In present study, 19 bacterial strains were isolated from different type of soil (Danube – Delta Biosphere Reserve), molecularly identified (16S rDNA sequencing), and tested for their textile dye biodegradation potential and tolerance level to heavy metals and high salinity. The strains Pseudomonas sp., Bacillus sp. and Thalassospira sp. were found to degrade 72.64% RO16 dye, but only Pseudomonas putida removed (56% decolorization) the RB4 dye. Also, was found a good tolerance to salinity (8% NaCl) in case of Pseudoarthrobacter sp., Enterobacter sp., Thalassospira sp., Bacillus sp., and Pseudomonas sp. strains. Most of the bacterial strains tolerated 70 ppm of cromium (Cr^{6}); only two strains, Cupriavidus respiraculi and Pseudomonas putida showed maximum tolerance to 70 ppm cadmium (Cd^{2+}); all strains of Pseudomonas sp. showed tolerance to 100 ppm lead (Pb^{2+}). The selected strains could be used in bioremediation process of industrial dye waste waters.*

Key words: bacterial strains, Danube – Delta Biosphere Reserve, bioremediation, textile dyes, heavy metals, salinity

Rezumat. *În studiul de față au fost izolate 19 tulpini bacteriene din tipuri variate de sol provenite din Rezervația Biosferei Delta – Dunării, identificate molecular (secvențiere 16S rDNA) şi testate din punctul de vedere al capacității de bioremediere a coloranților textili şi a nivelului de toleranță la metale grele şi salinitate crescută. Tulpinile de Pseudomonas sp., Bacillus sp. şi Thalassospira sp. pot degrada 72.64% colorant RO16, iar Pseudomonas putida poate recuperara 56% din colorantul RB4. S-a constatat o bună toleranță la salinitate (8% NaCl) a tulpinilor de Pseudoarthrobacter sp., Enterobacter sp., Thalassospira sp., Bacillus sp., şi Pseudomonas sp.. Majoritatea tulpinilor au tolerat cromul in concentrație de 70 ppm (Cr^{6}); tulpinile de Cupriavidus respiraculi şi Pseudomonas putida au tolerat 70 ppm cadmiu (Cd^{2+}); toate tulpinile de Pseudomonas sp. au prezentat toleranță la 100 ppm plumb (Pb^{2+}). Tulpinile bacteriene selectate pot fi utilizate in procesul de bioremediere al apelor uzate din industria coloranților.*

Cuvinte cheie: tulpini bacteriene, Rezervația Biosferei Delta – Dunării, bioremediere, coloranți textili, metale grele, salinitate

INTRODUCTION

Bioremediation of contaminated soil and waste waters is a cheap alternative to physico-chemical treatments. Industrial waters are coming from various industries producing or using synthetic pigments and dyes. Due to the complexity

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of the composition of these wastewater (heavy metals, high salinity, synthetic dyes recalcitrant to biological degradation, etc.) and for the success of the biotechnological process, some preliminary studies regarding the biodegradation capacity of the dyes and, also for the tolerance of the microorganisms subjected to the new abiotic stress conditions are needed (Ali, 2010; Gadd, 2010). Biological treatments have a high efficiency in detoxifying of diluted industrial effluents and have low operational costs (Allam, 2017).

The present research aim to identify the biodiversity of the microflora isolated from various terrestrial ecosystems (Danube – Delta Biosphere Reserve) in order to select the microorganisms with native bioremediation capacity of the textile dyes and their tolerance to various toxic compounds, especially heavy metals (as components of the dye structure or from various additives used in the dyeing process).

MATERIAL AND METHOD

Sample collection. Bacteria capable of degrading the textile dyes are originated from Danube – Delta Biosphere Reserve (DDBR). Soil samples were collected from various sites and soil type and diluted 1:50 in distilled water. The slurry was plated on yeast – mannitol-agar (YMA) supplemented with Reactive Orange 16 by conventional spread plate method. Plates were incubated at 30°C for 72 hours and colonies with dye adsorbtion capabilities were picked, isolated and purified. Finally, 19 bacterial strains were isolated, identified and maintained at - 80°C in glycerol.

Phylogenetic analysis of 16S rDNA sequences. Bacterial strains were grown in liquid YMB medium and incubated for two days at 28ºC on a rotary shaker. Equal aliquots of bacterial cultures were collected by centrifugation and total genomic DNA was isolated using Bacteria DNA Preparation kit (Jena Bioscience, Germany) according to manufacturer instruction. The conserved region of 16S rDNA was amplify using the fD1 and rD1 universal primers (Weisburg *et al*.,1991) as previously described by Efrose *et al*., 2018. PCR amplification products were purified and directly sequenced on both strands using the same primers as for PCR (CEMIA, Greece). The sequences obtained from the newly isolated strains were corrected and assembled using DNA Baser v. 3.5.4 program and used in the phylogenetic analysis together with the sequences of the reference bacteria retrieved from the NCBI/GenBank database. All sequences were aligned using the CLUSTAL W software and the phylogenetic tree was built with the Neighbor-Joining method based on Kimura's two-parameter model as implemented by *MEGA7* v.*7.0*.26 software package (Kumar *et al*., 2016). Bootstrap confidence levels were calculated for 1000 replicates. The acquired sequences for the selected bacterial strains, were deposited in GenBank/NCBI database using Sequin Application (v. 13.05).

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rDNA sequences obtained from the seven selected bacterial strains, which exhibited multiple biotechnological potential are: MH456790 (CR-B4); MH456792 (CR-B16); MH456794 (CR-B20); MH456795 (CR-B421); MH456796 (CR-B32); MH456797 (CR-B33); MH456798 (CR-B34). Accession numbers for the 16S rDNA sequences of the related reference strains are specified in the corresponding phylogenetic tree.

Screening of dye degrading bacteria. The dye biodegradation potential of newly isolated bacteria has been assessed by using various liquid culture media (mineral medium V1 and nutritive medium (TY) V2), as previous described (Stedel *et al*., 2019). Two chemically different synthetic dyes has been tested, as follows: azo-dye Reactive Orange16 (λ_{max}= 495 nm) and antraquinonic dye Reactive Blue 4 (λ_{max}= 595 nm).The stock solutions of dyes (1000 mg L^{-1}) were prepared and filter sterilized (Millipore filter, 0.22µm, Millipore Corp., Bedford, USA). The decolorization assay has been performed in 250-ml flasks contained 100 ml of culture media, supplemented with 20 ppm textile dyes. Bacterial inoculum (2%) was added and the cultures were incubated at 30°C in stationary conditions. The control flasks without inoculums and respectively, dyes were also kept as controls. The spectrophotometric readings (supernatants) were performed after six days of incubation, and the decolorization efficiency was determined as follow:

$$\text{Decolorization (\%)} = \frac{\text{initial absorbance-final absorbance}}{\text{initial absorbance}} x100$$

Stress tolerance. Heavy metal and high salinity tolerance were determined on agarized culture medium (TY) supplemented with heavy metals, at various concentrations: Cr^{6+} ($K_2Cr_2O_7$) and Cd^{2+} ($CdCl_2$) (0.1 - 70 ppm), Pb^{2+} ($Pb(NO_3)_2$) (15 - 600 ppm) and also, NaCl (w/v) (0.1; 0.5; 2.0; 4.0, and 8.0 %). The readings were made after three days of incubation at 30°C. The highest concentration of NaCl and heavy metal salts supporting bacterial growth on agarized plates was defined as the maximum tolerance level.

RESULTS AND DISCUSSIONS

1. Isolation and molecular identification of bacterial strains

A number of seven bacterial strains (CR-B2, CR- B3, CR-B4, CR-B7, CR-B8, CR-B14, CR-B16,) were isolated from agro ecosystems (harvesting sites: Pardina- Chilia Veche and Ostrovu Tataru - cultivated with corn, wheat, barley, autumn barley and rapeseed); also, twelve bacterial strains were isolated from the natural ecosystems (soils from the Murighiol - Dunavățu de Jos area, Sf. Gheorghe, Lake Saraturi, from solonceacs or alluvial soils) (CR-B20, CR-B21, CR-B23, CR-B24, CR-B25, CR-B27, CR-B28, CR-B29, CR-B31, CR-B32, CR-B33, CR-B34).

The isolates relatedness to the previously classified bacterial strains and their taxonomic assignation was assessed by the sequence analysis of 16S rDNA. Nearly complete 16S rDNA sequences were determined by sequencing and used to construct the respective dendrogram (fig. 1). Phylogenetic analysis showed a high diversity of the native bacterial strains and grouped them in ten well supported phyletic groups together with their closely related bacteria. Pair-wise analysis of the acquired sequences revealed that the native bacterial isolates exhibited 98.1 to 100% sequence identity to their closest affiliated bacterial strains comprised in the 16S rDNA phylogeny which belong to various taxonomic groups: *Variovorax* sp., *Cupriavidus* sp., *Pseudomonas* sp.; *Enterobacter* sp., *Thallasopsira* sp., *Starkeya* sp., *Paenibacillus* sp.; *Bacillus* sp.; *Rhodococcus* sp., and *Arthrobacter* sp.

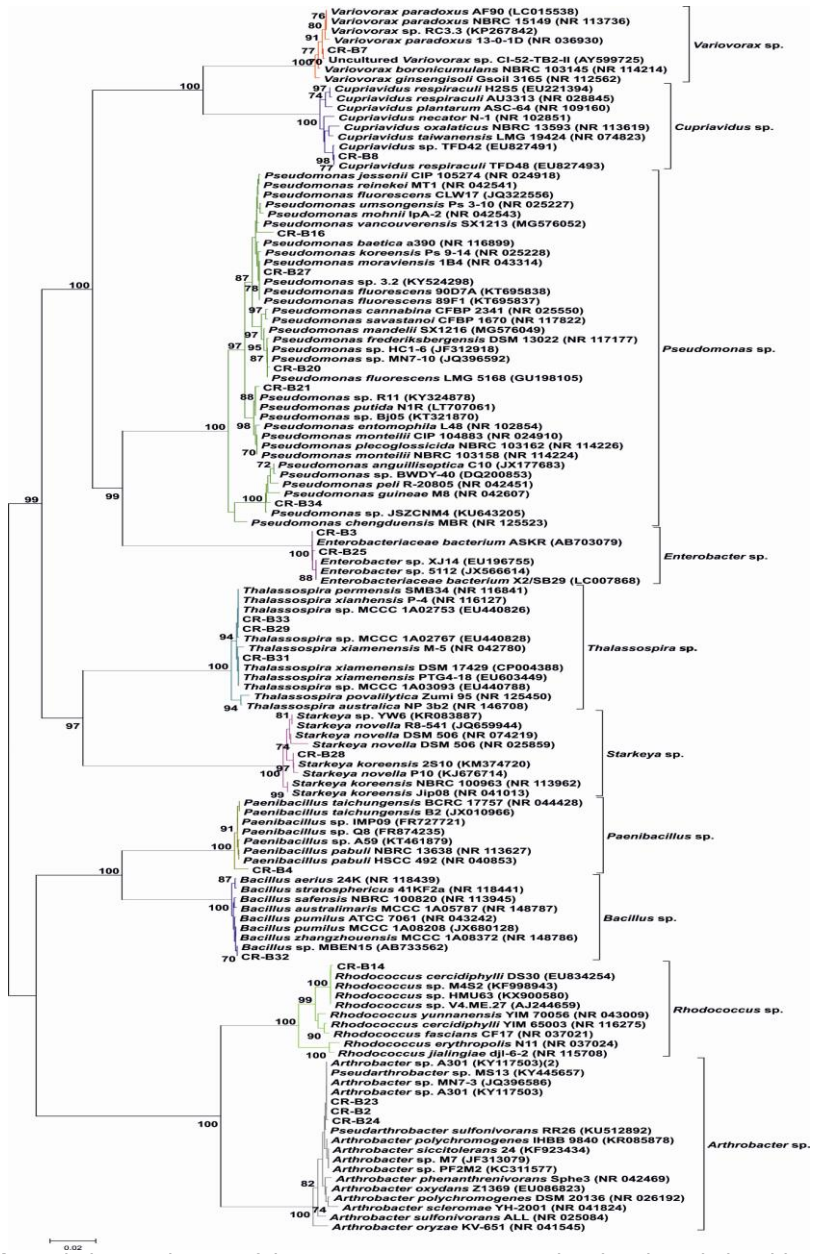

Fig.1 Phylogenetic tree of the 16S rDNA sequences showing the relationships of the newly isolated bacterial strains obtained from various habitats from DDBR with selected reference strains for recognized bacterial species. The Neighbor-Joining dendrogram was constructed using the Kimura 2-parameter model. Bootstrap values (based on 1000 replicates) below 70% are not shown. The scale bar represents 0.02 nucleotide substitutions.

2. Evaluation of the textile dyes biodegradation abilities

The native bioremediation performances of the newly bacterial strains were different. Thus, in the experimental conditions, nine of the newly bacterial strains were able to degrade the Reactive Orange 16 azo dye. Of these, six strains showed a natural decolorization capacity of over 50%, as follows: 96.56% (CR-B34 - *Pseudomonas* sp .); 91.46% - (CR-B20 - *Pseudomonas* sp.); 91% - (CR-B16 - *Pseudomonas* sp.); 91.57% - (CR-B32 - *Bacillus* sp.); 75.23% - (CR-B21- *Pseudomonas putida*) and 72.64% (CR-B33 - *Thalassospira sp.*) in stationary conditions, at 30°C, depending on the establisment of reductive conditions necessary for the degradation of the azo-linkage (after 6 days incubation). Only one strain (CR- B21 - *Pseudomonas putida*) was able to decolorize the antraquinonic RB 4 dye (tab. 1).

Table 1

Screening of soil bacteria isolates for textile dye biodegradation potential

Bacterial strains	Decolorization (%)	
	V1	V2
	Reactive Orange 16	
CR-B2 (*Pseudarthrobacter sp.*)	14.45	16.98
CR-B4 (*Paenibacillus sp.*)	36.42	67.22
CR-B16 (*Pseudomonas sp.*)	89.90	91.05
CR- B20 (*Pseudomonas sp.*)	91.46	37.87
CR- B21 (*Pseudomonas putida*)	47.76	75.23
CR-B24 (*Pseudarthrobacter sp.*)	17.98	19.23
CR-B32 (*Bacillus sp.*)	26.43	91.57
CR-B33 (*Thalassospira sp.*)	16.64	72.64
CR-B34 (*Pseudomonas sp.*)	91.98	96.56
	Reactive Blue 4	
CR- B21 (*Pseudomonas putida*)	56.73	17.46
CR-B34 (*Pseudomonas sp.)*	12.45	5.67

Reactive dyes, such as RB4 and RO16, are synthetic compounds with high water solubility and non-degradable under typical aerobic conditions, in biological treatment systems. Also, the dyes are very poorly adsorbed on the surface of biological solids, which results in a high residual dye load in effluents. The reactive dyes released into the environment represent an important pollution problem due to the coloring of surface waters (rivers), absorbing and reflecting light, which interferes with aquatic ecosystems. They also, have acute and/or chronic toxic effect on living organisms. The bioremediation efficiency of the synthetic dyes by the newly isolated strains can be further increased by optimizing the process parameters (especially carbon and nitrogen sources, pH, aerobic or anaerobic conditions, temperature).

3. Stress tolerance

Related to the high salinity tolerance, many bacterial strains manifested tolerance to high salinity (4% NaCl). The *Pseudoarthrobacter sp*. (CR-B2) and *Enterobacter sp*. (CR-B3) (among isolates from agroecosystems in the Chilia Veche area), as well as the strains of *Thalassospira sp*. (CR-B29, CR-B31, and CR-B33), *Bacillus sp*. (CR-B32)

and *Pseudomonas sp*. (CR-B34) (among isolates from solonceacs type soil, collected from the protected areas in the Murighiol area), showed a good development at the maximum concentration tested of NaCl (8%) (tab. 2).

Table 2

High salinity tolerance of the soil bacteria isolated from RBDD

No.	Geographical origin	Bacterial strains	NaCl (%)				
			0.1	0.5	2.0	4.0	8.0
1.	Chilia Veche – Pardina	CR-B2 (*Pseudarthrobacter sp*.)	+	+	±	±	±
2.	Ostrovu Tataru	CR-B3 (*Enterobacter sp.)*	+	+	+	+	±
3.	Ostrovu Tataru	CR-B4 (*Paenibacillus sp.)*	+	+	+	+	-
4.	Ostrovu Tataru	CR-B7 (*Variovorax paradoxus*)	+	+	-	-	-
5.	Ostrovu Tataru	CR-B8 (*Cupriavidus respiraculi*)	+	+	-	-	-
6.	Stationarul Pardina	CR-B14 (*Rhodococcus sp*.)	+	+	+	+	-
7.	Chilia Veche – Pardina	CR-B16 (*Pseudomonas sp.)*	+	+	+	-	-
8.	Dunavatu de Jos	CR- B20 (*Pseudomonas sp.)*	+	+	+	-	-
9.	Dunavatu de Jos	CR- B21 (*Pseudomonas putida*)	+	+	+	±	-
10.	Dunavatu de Jos	CR-B23 (*Arthrobacter polychromogenes)*	+	+	+	±	-
11	Murighiol – Sf. Gheorghe	CR-B24 (*Pseudarthrobacter sp*.)	+	+	+	±	-
12.	Murighiol – Sf. Gheorghe	CR-B25(*Enterobacter sp*.)	+	+	+	±	-
13.	Murighiol – Sf. Gheorghe	CR-B27 (*Pseudomonas fluorescens*)	+	+	++	-	-
14.	Murighiol – Saraturi Lake	CR-B28 (*Starkeya sp*.)	+	±	±	±	-
15.	Murighiol – Saraturi Lake	CR-B29 (*Thalassospira sp*.)	+	+	+	+	+
16.	Murighiol – Saraturi Lake	CR-B31 (*Thalassospira sp.)*	+	+	+	+	+
17.	Murighiol – Saraturi Lake	CR-B32 (*Bacillus sp*.)	+	+	+	+	+
18.	Murighiol – Saraturi Lake	CR-B33 (*Thalassospira sp*.)	+	+	+	+	+
19.	Murighiol – Saraturi Lake	CR-B34 (*Pseudomonas sp.)*	+	+	+	+	+

+++ very good growth ; ++ week growth; - no growth

Table 3

Heavy metals tolerance of the soil bacteria isolated from RBDD

No.	Bacterial strains	Cd^{2+}					Cr^{6+}	Pb^{2+}			
		5.0	15	30	50	70	70	70	100	200	300
1.	CR-B2 (*Pseudarthrobacter sp*.)	+	-	-	-	-	+	-			
2.	CR-B3 (*Enterobacter sp.)*	+	-	-	-	-	+	+	+		
3.	CR-B4 (*Paenibacillus sp.)*	+	-	-	-	-	+	-			
4.	CR-B7 (*Variovorax paradoxus*)	+	+	+	-	-	+	±			
5.	CR-B8 (*Cupriavidus respiraculi*)	+	+	+	+	+	+	±			
6.	CR-B14 (*Rhodococcus sp*.)	+	-	-	-	-	+	±	-	-	-
7.	CR-B16 (*Pseudomonas sp.)*	+	+	-	-	-	+	+	+	-	-
8.	CR- B20 (*Pseudomonas sp.)*	+	+	+	-	-	+	+	+	+	
9.	CR- B21 (*Pseudomonas putida*)	+	+	+	+	+	+	+	+	+	+
10.	CR-B23 (*Arthrobacter polychromogenes)*	+	-	-	-	-	±	-			
11	CR-B24 (*Pseudarthrobacter sp*.)	+	-	-	-	-	±	-			
12.	CR-B25(*Enterobacter sp*.)	+	+	-	-	-	+	±	±	-	-
13.	CR-B27 (*Pseudomonas fluorescens*)	+	+	-	-	-	+	+	+	-	-
14.	CR-B28 (*Starkeya sp*.)	+	+	-	-	-	+	±			
15.	CR-B29 (*Thalassospira sp*.)	+	+	-	-	-	+	+			
16.	CR-B31 (*Thalassospira sp.)*	+	+	-	-	-	+	+			
17.	CR-B32 (*Bacillus sp*.)	+	+	-	-	-	+	±	-	-	-
18.	CR-B33 (*Thalassospira sp*.)	+	+	-	-	-	+	±			
19.	CR-B34 (*Pseudomonas sp.)*	+	-	-	-	-	+	+	+	+	-

+++ very good growth ; ++ week growth; - no growth

Most of the bacterial strains tolerated concentrations up to 15 ppm Cd^{2+}. Two strains (CR-B7 - *Variovorax sp*. and CR-B20 - *Pseudomonas sp*.) could tolerate up to 30 ppm Cd^{2+}. More, CR-B8 (*Cupriavidus respiraculi*) and CR-B21 (*Pseudomonas putida*) strains tolerated the maximum tested concentration of 70 ppm Cd^{2+}. Related to the tolerance of bacterial isolates to Cr^{6+} additioned to the culture medium, all of them had a good development at the maximum tested concentration (70 ppm). Lower tolerance was observed in the case of *Arthrobacter sp*. (CR-B23) and *Pseudoarthrobacter sp.* (CR-B24) that were sensitive to all tested concentrations. Also, most of the strains manifested a maximum tolerance to 70 ppm Pb^{2+} while *Pseudomonas sp*. (CR-B20; CR-B21, and CR-B34) strains tolerated 100 ppm Pb^{2+}(tab. 3).

CONCLUSIONS

1. In the present work we have reported on the isolation, molecular identification and characterization of the indigenous bacterial strains with textile dye biodegradation potential.

2. The newly isolated bacterial strains obtained from various unfavorable environments from Danube Delta Biosphere Reserve were clustered, based on 16S rDNA phylogeny, in ten distinct lineages, highly affiliated to recognized bacterial species which belong to different taxonomic groups.

3. The GenBank (http://www.ncbi.nlm. nih.gov/GenBank) accession numbers for the sequences acquired from the selected bacterial strains are: MH456790 (CR-B4); MH456792 (CR-B16); MH456794 (CR-B20); MH456795 (CR-B421); MH456796 (CR-B32); MH456797 (CR-B33); MH456798 (CR-B34).

4. A significant result of this study was the isolation of bacterial strains with multiple tolerance to heavy metals, salinity and synthetic dyes, at concentrations ussualy meet in textile dye waste waters.

5. Based on the native potential of bioremediation, the newly isolated bacterial strains can be further exploited to develop a new biotechnology – based bioremediation of industrial waste waters.

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