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Growth on Azo Compounds and Decolorization Capacity of Some Eubacteria not Conferred by Plasmidic DNA [Version 2]

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Abstract

The bacteria are very abundant and ubiquous organisms, inhabiting under many conditions, even severely contaminated environments. The azo dyes (recalcitrant synthetic chemicals) are common pollutants, used industrially and discharged into wastewater, where they becoming a risk for human health. Fortunately, the bacteria are capable of mineralizing toxic chemical products through gene products present in plasmidic DNA. This study confirms the capability of bacterial strains, isolated from soil exposed to industrial wastewater, to grow on liquid media supplemented with Congo-red or Methyl-red, or only these azo dyes as the sole carbon source, and shows that this ability is not provided by plasmidic DNA. Three strains isolated were able to grow in the presence of azo dyes and to decolorize them from 90 up to 96%, after 96 h. The strains were identified as *Ralstonia pickettii*, *Acidovorax anthurii* and *Cupriavidus* sp. through 16S rDNA sequence, morphological and biochemical analysis. Only *Cupriavidus* sp. showed considerable growth when azo dyes were used as sole carbon source. Plasmidic DNA was present in the three strains. After the plasmidic DNA was cured in each of the strains, they maintained their ability to grown with the azo dyes, suggesting such capacity is not conferred by its plasmids.

Keywords

Congo-red; Methyl-red; Decolorization; *Ralstonia*; *Acidovorax*; *Cupriavidus*; Plasmids

Introduction

According to ancient and recent estimates, the bacteria are the most abundant group of organisms on Earth. Early from 1800, their ubiquity had been proved; they occupy several metabolic niches, which make them extremely successful organisms. The biodiversity of the bacteria, on the other hand, is an issue that is currently under study, because their simple morphological aspect encloses an incredible amount of peculiar biochemical properties. The use of genomic tools, have allowed great advances in the knowledge of bacterial genomes of several microbial communities across the world [1], and the discovery of more and novel properties from these organisms. The capability of the bacteria to colonize any habitat, has led to several research groups to explore natural environments subjected to extreme conditions in terms of temperature, pH, and salinity, among others. Particularly interesting are being the surveys of environments disturbed by human activities, in order to find species that survive and develop under hostile conditions. Bacteria inhabit sites disturbed by the presence of several contaminants, such as radioactive wastes [2], heavy metals and polycyclic aromatic hydrocarbons [3], for these reasons they have been considered as one of the main forces of biodegradation of environmental toxic compounds [4, 5]. Within the environmental contaminants existing on earth, the azo dyes are widely produced because of their importance in textile, pharmaceutical, leather, cosmetics and paper industries [6]. The azo dyes are compounds having an R-N = N-R functional group, where R and R' may be an aryl or alkyl radicals and usually the recalcitrant nature of these compounds is attributed to the azo groups (–N=N–) which are not produced in a biological way in nature [7]. During the use of azo dyes, 10-20% of industrial effluent wastes are discharged into the rivers, becoming a source of exposure to these xenobiotics for organisms inhabiting these ecosystems [8], and for those whom are in contact or even consuming the river water. Azo dyes produce mutagenic effects on bacteria [9], chromosomic aberrations on plants [10, 11], and cytotoxic and genotoxic effect on mouse germ cells [12]; in the same way, the aromatic amines produced from the cleavage of the azo dyes, are carcinogenic, mutagenic, and allergenic, causing various human maladies [13]. It is clear that the azo dyes represent a major health problem, making important their degradation in the environment, in order to achieve their transformation into much less or even nontoxic chemical compounds. Microorganisms such as bacteria decolorize azo dyes due to their azo reductase and oxidase enzymes production [14, 15]. Recent studies focused on finding bacterial strains able to decolorize or to degrade azo dyes, directed their efforts to obtain isolates of native bacteria from contaminated sites. *Shewanella*, *Bacillus*, *Pseudomonas*, *Aeromonas*, *Massilia*, *Brevibacillus*, and *Stenotrophomonas*, are examples of bacterial genera found under such conditions, and able to decolorize and in some cases, under specific conditions, to degrade the azo dyes [16, 17, 18, 19]. These studies described the potentiality of different genera of bacteria and the enzymatic complex responsible of capability to degrade azo compounds, but little have been explored regarding the participation of plasmid DNA into the production of factors for the decolorization or degradation mechanisms. Plasmids are DNA molecules capable of autonomous replication [20], which provide adaptive traits to bacteria through advantages such as survival under local selective pressures, expression of antibiotic resistance and the ability to use novel carbon sources [21], among others, so, plasmids could provide resistance to bacteria making them capable to inhabiting contaminated environments. The capability of several species of *Lactobacillus* to decolorize azo dyes has been related with a 3 kb plasmid; the genes encoding textile azo dye degradation enzymes were located on such plasmids, because the cured strain lost the decolorizing ability [22]. In *Pseudomonas* sp. capable of decolorizing azo dyes, plasmids were also detected; the cured strain gradually lost its ability to decolorize them [23]. To confirm that the decolorization genes exist in the plasmids, these ones were transformed into competent *Escherichia coli* DH5α, a strain unable to decolorize azo dyes, which acquiring this ability after the incorporation of those plasmids [23]. The aim of this work was to confirm the azo dyes decolorization capacity of Eubacteria strains isolated from soil exposed to industrial wastewater, and to confirm the role of plasmids on it.

Material and Methods

Isolation and Selection of Bacterial Strains

The studied bacteria strains were isolated from surface soil samples (15 cm deep) from banks of the Zahuapan River, Santa María Texcalac, Apizaco Municipality, State of Tlaxcala, Mexico, 3 Km downstream from the Xicotencatl II Industrial City, which includes textile companies that constantly dump dyes wastes into the river.

Each soil sample was diluted (1g in 9 mL of distilled water); dilutions were prepared from this solution (1:10, 1:100, and 1:1000). Each dilution (100 µL) was inoculated at Petri plates containing 30 mL of water-agar media supplemented with soil extract to 3.5% v/v (ASE), using the procedure described by Liebeke et al. [24], (mixing 400 g of soil with 1 L of distilled water for 30 min). The mixture was filtered through Whatman No. 1 and sterilized to 121°C and 15 Psi for 15 min, then stored at 4 °C until using. The ASE media was added with 10 mg/L, either Congo-red or Methyl-red, per triplicated, incubated at 32°C and observed at 24, 48 and 72 h. Bacterial strains selected were those that grew on Petri plates with culture media supplemented with the model azo dyes, Congo-red or Methyl-red. The colonies selected were purified through sequential transferring to new ASE plates. The strains selected were maintained on Petri plates containing nutritive agar supplemented with soil extract (3.5% v/v of soil extract). Contamination from other bacteria was checked periodically by streaking on nutritive agar plates. Bacterial pellets in Luria Bertani (LB) media with glycerol at 20% were prepared for long time conservation at -20 °C.

Identification of Eubacteria

Biochemical Properties

Each pure culture of the isolated strains was subjected to the following tests: Urease test, Triple Sugar Iron (TSI), Motility Indole Ornithine (MIO), Citrate test, Lysine Iron agar test (LIA) and Catalase test, in order to explore different biochemical properties for taxonomic proposals, according to Bergey´s Manual of Determinative Bacteriology [25].

Colonial Morphology and Gram Staining

Each strain was characterized recording colony shape, texture, color, brightness and type of edge, every 12 hours for 4 days [25]. Gram staining for each bacterial strain was performed following instructions from the Manual of Clinical Microbiology Procedures [26]. The discrimination of gram+ and gram- bacteria was made through observations using an optical microscope (Zeiss Axioscope II Plus).

Molecular Identification

The molecular identification of bacterial strains was performed using 16S rRNA gene sequences. The DNA was extracted using the Power Soil DNA Isolation Kit (MoBio®), following the supplier instructions. The primers selected for amplification through PCR were those reported by Lane [27]: 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1391R (5´- GACGGGCGGTGWGTRCA-3´); they were synthetized by Uniparts Company (Uniparts, S.A. de C.V., Mexico City, Mexico). Components for the reactions were: 4 µL of total DNA (10 ng), 5 µL of 10 x Taq Buffer with KCl (100mM Tris-HCl, 500 mM KCl, nonidet P40), 1.5 µL of $MgCl_2$ 25 mM, 1 µL of dNTP´s 10 mM, 1 µL of the primer 27F 20 mM, 1 µL of the primer 1392R 20 mM, 36 µL of bidistilled water, and 2.5 units of Taq Polymerase (Fermentas®). The PCR amplification was performed in a thermocycler, My Cycler (Bio-Rad®), with 30 cycles comprising the following stages: initial denaturalization at 95°C for 5 min, 30 cycles for denaturalization at 95°C during one min, annealing temperature at 52°C for one min, and polymerization at 72°C for 1.5 min, finishing with 7 min of polymerization at 72°C. Controls without DNA were ran to assure there was no contamination in the reagents. The amplifications obtained were confirmed and analyzed by electrophoresis in 0.7% of agarose gel with ethidium bromide (EtBr) 0.5 µg/L, during a 15 min period [28].

Molecular sizes of the bands were determined by a Gene Ruler™ 100 bp DNA Ladder (Fermentas®), the bands were visualized with UV light under diffused light conditions (Ultra•Lūm®) and finally photographed with a Gel Doc XRTM 170-8170 (BioRad®). Amplicons were purified with the Wizard SV Gel Kit and PCR Clean-Up System (Promega®) as indicated by the manufacturer. The amplicon sequencing was performed in an automatic sequencer, ABI-Prism 3100 (Applied Biosystems®), at the Divisional Laboratory of the Molecular Biology, Universidad Autónoma Metropolitana-Iztapalapa. The nucleotide sequence was assembled using DNA Baser V. 4.2.8 [29] and compared with sequences included in the GenBank database and The Greengenes database using the software Basic Local Alignment Search Tool (BLAST). The sequences were deposited at GenBank.

Growth and Decolorization in Liquid Culture Medium Supplemented with Azo Dyes

The capacity of the strains to grow in presence of azo dyes was evaluated on microcultures. Microtubes of 2.0 mL containing 1.5 mL of LB medium added with either Congo-red or Methyl-red at 10 mg/L were inoculated with densities of approximately 10^6 cell (uniform cell density: $OD_{0.6}$) and incubated at 30°C for 96 h. The growth of the bacterial strains was determined by measuring the OD in a SmartSpec™ Plus spectrophotometer (Bio-Rad®) to 600 nm. The OD of the strains was converted from absorbance units to cellular concentration using conversion factor of 5 X 10^8 cell/mL included in the equipment software. Decolorization was determined by centrifugation of the medium at 10,000 rpm for 15 min to remove the cells. The dyes disappearance percent was evaluated into the supernatants by measuring absorbance at 580 nm using a spectrophotometer SmartSpec™Plus (Bio-Rad®).

Growth and Decolorization in Liquid Culture Medium Using Azo Dyes as the Sole Carbon Source

The ability of the strains to grow in media with azo dyes as the sole carbon source was tested by culturing the strains in microtubes of 2 mL with 1.5 mL of the mineral medium reported by Bischoff and Bold [30] containing (in grams per liter): NaCl, 24; KCl, 0.7; KH_2PO_4, 2.0; $MgSO_4 \bullet 7H_2O$, 1.0; Na_2HPO_4, 3.0; NH_4NO_3, 1.0, pH 7.0, supplemented with either Congo-red or Methyl-red at 10 mg/L. Each microtube was inoculated with approximately 10^6 cell/mL of the corresponding strain, and incubated at 30 °C for 96 h. The growth and decolorization measurements were done such as it is described above.

Detection of Plasmidic DNA

The presence of plasmidic DNA in the isolates was detected by the extraction of plasmidic DNA by the alkaline lysis procedure, using Gen Jet Plasmid Miniprep Kit (Fermentas®) as indicated by the manufacturer. The extractions obtained were confirmed and analyzed by electrophoresis in 0.7% of agarose gel with EtBr 0.5 µg/L during a 15 min period [28].

Additionally, the plasmidic DNA of each strain was digested with restriction enzymes *Pst*I, *Eco*RI, *Kspa*I, *Nco*I, *Hind*III, *Bam*HI, *Pvu*II, as a confirmation test.

Plasmids Curing

The plasmids curing from the isolates was carried out using EtBr as describe by Trevors [31] and Kojic et al. [32]. Four replicates of the strains were grown in Falcon tubes of 50 mL containing 40 mL of LB medium supplemented with EtBr at sublethal concentrations for bacteria (10, 20, 30, 40 and 80 mg/mL) and incubated for 2 weeks at 30 °C. In order to confirm the curing of plasmids, each 24 h the content of plasmidic DNA was determined in a sample of the tubes from each strain. Plasmidic DNA was extracted using alkaline lysis and confirmed by electrophoresis at the conditions already described. To confirm the viability of bacterial cells without plasmids, a sample of each strain was cultured in Petri plates containing 10 mL of nutritive agar and incubated at 30 °C for 24h. Of each strain, a bacterial colony was chosen to culture it in LB liquid medium, in order to verify the absence of plasmid DNA and to use its cell line in the tests for growth and decolorization of azo dyes.

Growth of Cured Strains on Liquid Medium Supplemented with Azo Dyes

A sample from each cured strain was inoculated on Petri plates containing ASE medium added with 10 mg/L, either Congo-red or Methyl-red, per triplicated, incubated at 32 °C and observed at 24, 48 and 72 h for detection of cell grow, to prove if the bacterial strains without plasmids lost their capability to grow on culture media supplemented with azo dyes. Cultures with cured strains in LB liquid medium added with azo dyes and in minimal liquid medium added with azo dyes were performed, in order to determine the ability of the strains to use these compounds as the sole carbon source, under the same conditions described for the test with the native strains.

Statistical Analysis

The Pearson correlation between the bacterial growth and percent of decolorization was realized in order to determinate the relation between these two variables.

Results

Isolation and Identification of Bacterial Strains

Three bacterial strains were isolated from soils constantly exposed to textile dyes wastewaters. One of them (S2RM2) was able to grow in presence of methyl-red while the two other (S2RC1 and SNRC1) grown in presence of Congo-red. None strain grew with both dyes. The morphological features of each strain are showed in Table 1. The results of the biochemical tests proved on each strain are showed in Table 2.

Table 1: Morphologic colonial features and micromorphology of the bacterial strains isolated in Petri plates containing culture medium and azo dyes.

Strain Code	Azo dye	Colonia shape in frontal view	Colonia shape in lateral view	Edge	Color	Rigid-ness	Texture	Bright-ness	Decolori-zation halo	Micro morphology
S2RM2	Methyl-red	Circular	Convex	Smooth	Beige	Soft	Viscose	Shiny	Present	Coccobacillar
S2RC1	Congo-red	Circular	Convex	Smooth	Pink	Soft	Viscose	Shiny	Absent	Coccobacillar
SNRC1	Congo-red	Circular	Flat	Smooth	Red	Rigid	Dry	Opaque	Absent	Coccobacillar

The morphological features and biochemical properties of the strains shown that they belong to different genera. The strain S2RM2 belongs to *Ralstonia* (which was recently segregated from *Pseudomonas*), the strain S2RC1 to *Acidovorax*, and the strain SNRC1 to *Cupriavidus*. Complementary, the analysis of the sequence of the 16S region of the bacterial strain S2RM2 (1287 bp) showed 99% similarity with *Ralstonia pickettii* (NCBI reference number: NC_022514), a strain isolated from trichlorophenol contaminated soil in Okayama, Japan [33]. For the strains S2RC1 and SNRC1, the analysis of the sequences of the 16S region (1281 and 1283 bp respectively) showed 99% similarity of both, the former with *Acidovorax anthurii* (GenBank reference number: KP641171) and the latter with *Cupriavidus* sp. (GenBank reference number: KT321704), isolated from activated sludge in China and from a copolymer sample from field soil in Russia, respectively [34, 35]. The sequences obtained for the studied strains were deposited at the GenBank database with reference numbers: KX834415 for S2RM2 strain; KX834414 for S2RC1 strain; and KX834416 for SNRC1 strain.

Table 2: Biochemical features and Gram staining of the bacterial strains.

Strain	Catalase	MIO	Urease	Citrate	LIA	TSI	Gram
S2RM2	+	-	-	-	-	+	-
S2RC1	+	-	+	+	-	-	-
SNRC1	+	-	-	-	-	-	-

Growth Capacity on Liquid Medium Supplemented with Azo Dyes

The selected bacterial strains grew well in medium with azo dyes. These first results shown that the strains decolorized azo dyes percentages, range from 91 to 93% of Congo-red (Figure 1a and 1b), and 96% of methyl-red (Figure 1c) at 96 h incubation.

For all strains, a directly proportional linear relationship between bacterial growth and decolorization of the dyes was observed ($r = 0.8667$ to 0.94; $p<0.001$), meaning that the correlation model explains 75.12 to 88.36% of the found variability, i.e., the decolorization rate is strongly correlated with the bacterial growth. S2RC1 strain had the shortest lag phase, but did not reach the stationary growth phase at 96 h. S2RM2 and SNRC1 strains showed a lag phase of approximately 36 h. S2RM2 strain did not reach the stationary phase during the culture time. The linear correlation explains 75.12% of the found variability. In the case of the SNRC1 strain, linear correlation explains 88.36% of the variability.

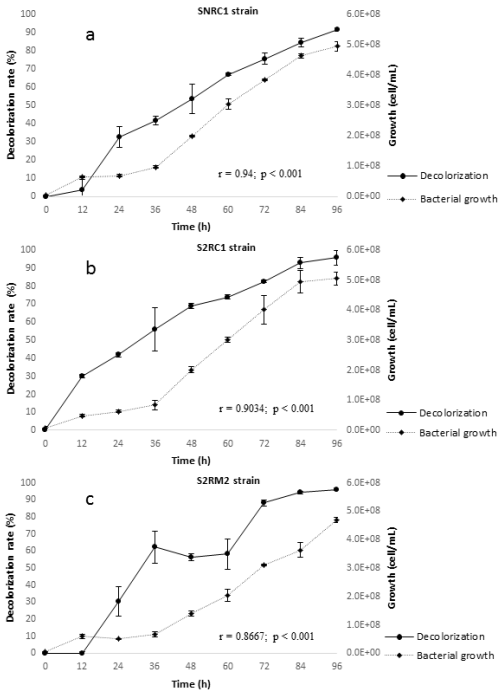

Figure 1: Growth kinetics (rhombus, right axis) and decolorization capability (circles, left axis) of strains culture in LB medium added with azo dyes. a) SNRC 1 strain grown in Congo-red. b) S2RC1 strain grown in Congo-red. c) S2RM2 strain grown in methyl-red. The error bars represent the standard deviation of three seperated replicates of each experiment.

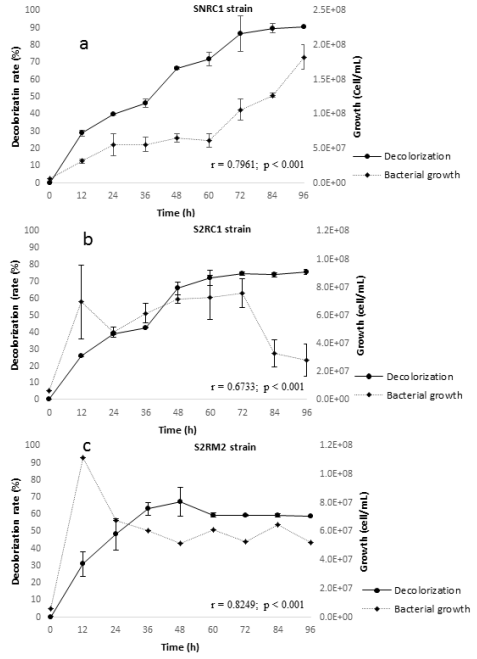

Figure 2: Growth kinetics (rhombus, right axis) and decolorization capability (circles, left axis) of strains culture in mineral medium added with azo dyes. a) SNRC 1 strain grown in Congo-red. b) S2RC1 strain grown in Congo-red. c) S2RM2 strain grown in methyl-red. The error bars represent the standard deviation of three seperated replicates of each experiment.

Growth Capacity on Culture Medium Using Azo Dyes as the Sole Carbon Source

In media using Congo-red or methyl-red as the sole carbon source, the strains showed growth only after 12 h of culture, however, it was lower in contrast with the growth levels obtained when an additional carbon source was used. A slow-growth of the SNRC1 strain was observed up to 72 h, increasing until 37% of the cell/mL at 84 h, in relation to the growth obtained in cultures using an additional carbon source at 96 h. The 90.7% of Congo-red was decolorized at 96 h by this strain, showing strong positive correlation with bacterial growth ($r = 0.7961$; $p<0.001$) (Figure 2a). The S2RC1 strain showed a slow growth from 12 h until the 72 h of the culture, reaching 16% of cell/mL regarding the growth obtained in cultures using an additional carbon source. Despite of this, the cells were able to decolorize 95% of Congo-red at 96 h (Figure 2b). The bacterial density started to decrease at 84 h, while the decolorization percentage remained without change. This strain showed also a strong positive linear correlation only considering the phase of active growth (from 0 to 72 h) ($r=0.6733$; $p<0.001$). A different behavior was observed with the strain S2RM2, with growth started from12 h but reaching only 14% of cell/mL comparing with the growth obtained in the culture using an additional carbon source. This cellular concentration was constant until the end of the culture time. The strain was able to decolorize only 58.5% of methyl-red, but a positive linear correlation with bacterial growth was also observed ($r = 0.8249$; $p<0.001$) (Figure 2c).

For SNRC1 and S2RM2 strains around 63 to 65% of the biomass and Congo-red decolorization percentage correlation was explained by the model. Meanwhile, only 45% of this correlation was explained for the S2RC1 strain. Strictly, only the SNRC1 strain shows capability for use Congo-red as sole carbon source. However, the data obtained for the S2RM2 strain, suggest the action of other factors on this phenomenon, because despite their apparent inability to grow using azo dyes as the only carbon source, discoloration of these is observed. It is possible that for these strains, the use of azo dyes as the sole carbon source implies an excessive energetic cost or even intoxication, stopping the production of biomass while for S2RC1, it is observed cellular dead despite of shown Congo-red decolorization.

Detection of Plasmidic DNA

All strains had plasmidic DNA resolved by electrophoresis in agarose gel as show in figure 3. The profile of plasmidic DNA shows this is native plasmidic DNA of high molecular weight, greater than the last band of molecular weight marker λ*Pst*I (11,501 bp).

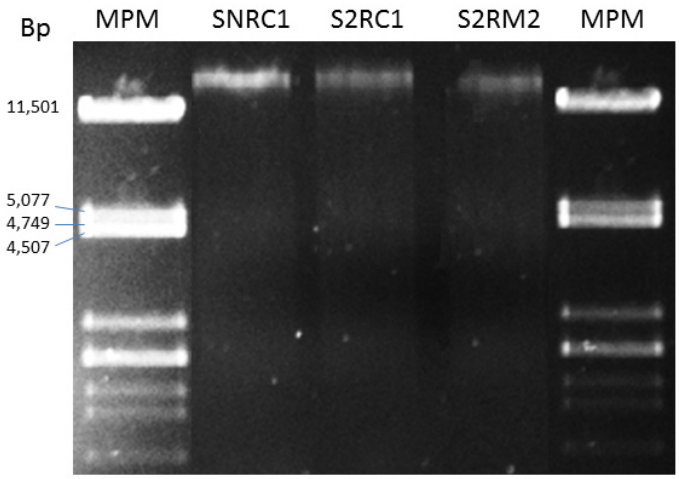

Figure 3: Electrophoretic separation of plasmidic DNA from strains isolates. Line 1 and 5: Molecular weight marker λPstI. Lines 2: Plasmidic DNA from *Cupriavidus* sp strain; Line 3: Plasmidic DNA from *Acidovorax anthurii* strain; Line 4: Plasmidic DNA from *Ralstonia pickettii* strain.

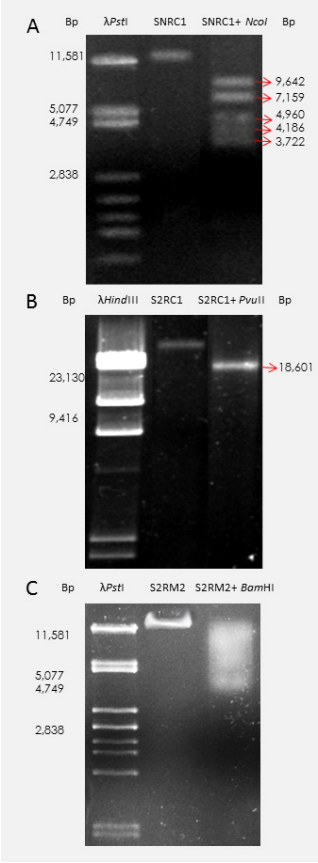

Figure 4: Electrophoretic separation of plasmidic DNA from strains isolates digested with restriction enzymes. A) Lines 1: Molecular weight marker *λPst*I. Lines 2: Plasmidic DNA from *Cupriavidus* sp strain; Line 3: Fragments of plasmidic DNA obtained with the enzyme *Nco*I. B) Lines 1: Molecular weight marker *λHind*III. Lines 2: Plasmidic DNA from *Acidovorax anthurii* strain; Line 3: Fragment of plasmidic DNA obtained with the enzyme *Pvu*II. C) Lines 1: Molecular weight marker *λPst*I. Lines 2: Plasmidic DNA from *Ralstonia pickettii* strain. Line 3: Fragments of plasmidic DNA obtained with the enzyme BamHI.

Figure 4 shows the plasmidic DNA fragments resulted from digestion with restriction enzymes. For plasmidic DNA of SNRC1, the best digestion was obtained by NcoI restriction enzyme, with five bands of 9.642, 7.159, 4.960, 4.186 and 3.722 bp (Figure 4 A). Plasmidic DNA of S2RC1 was digested with PuvII restriction enzyme, observing a single lower molecular weight band of 18.601 bp (Figure 4 B). Finally, for the plasmidic DNA of S2RM2 strain, only digestion with the restriction enzyme BamHI was obtained (Figure 4 C), however, no definite bands have been obtained still from the resulting DNA fragments.

Plasmid Curing

In some species of bacteria, the curing process usually required the combination of diverse curing agents. In this study, such process was carried out using only ethidium bromide (EtBr) at several concentrations to prove its action as curing agent. S2RM2 strain loses its plasmidic DNA from the 7th day, when it was exposed to 40 and 80 µg/L of EtBr. At 30 µg/L concentrations of EtBr, the loss of plasmidic DNA was observed from the 9th day. For SNRC1 and S2RC1 strains, the loss of the plasmidic DNA was observed from the 9th day using concentrations from 30 to 80 µg/L of EtBr. The electrophoresis in agarose gel confirmed the missing of all plasmidic DNA in all the three strains. This shows that EtBr at 30 °C effectively cured the plasmid of all studied strains.

Cured Strains Ability to Grow on Culture Medium Supplement with Azo Dyes

In the present study, the cured derivative strains grow and decolorize the azo compounds in the same way as the non-cured strains in presence of Congo-red or methyl-red in both liquid media cultures (Figure 5 and Figure 6). S2RC1 cured strain does not show a decrease in the biomass for the last two times of the culture as happens in S2RC1 non-cured strain; however, it does not show growth after 48 h. These results suggest that the plasmidic DNA is not related to the ability to decolorize azo dyes in these bacteria.

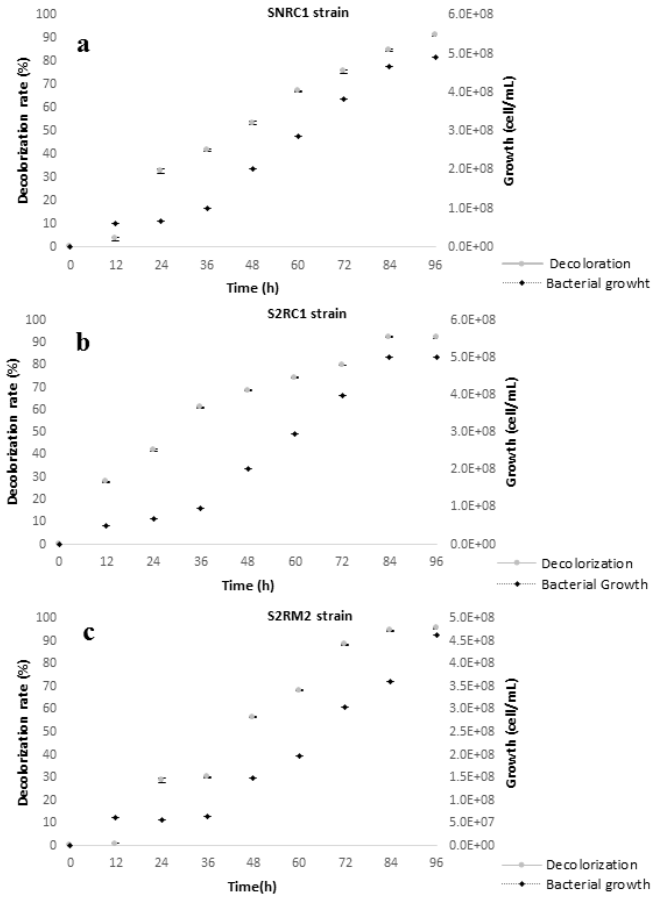

Figure 5: Growth kinetics (rhombus, right axis) and decolorization capability (circles, left axis) of cured strains culture in LB medium added with azo dyes. a) SNRC1 cured strain grown in Congo-red. b) S2RC1 cured strain grown in Congo-red. C) S2RM2 cured strain grown in methyl-red. The error bars represent the standard deviation of three separated replicates of each experiment.

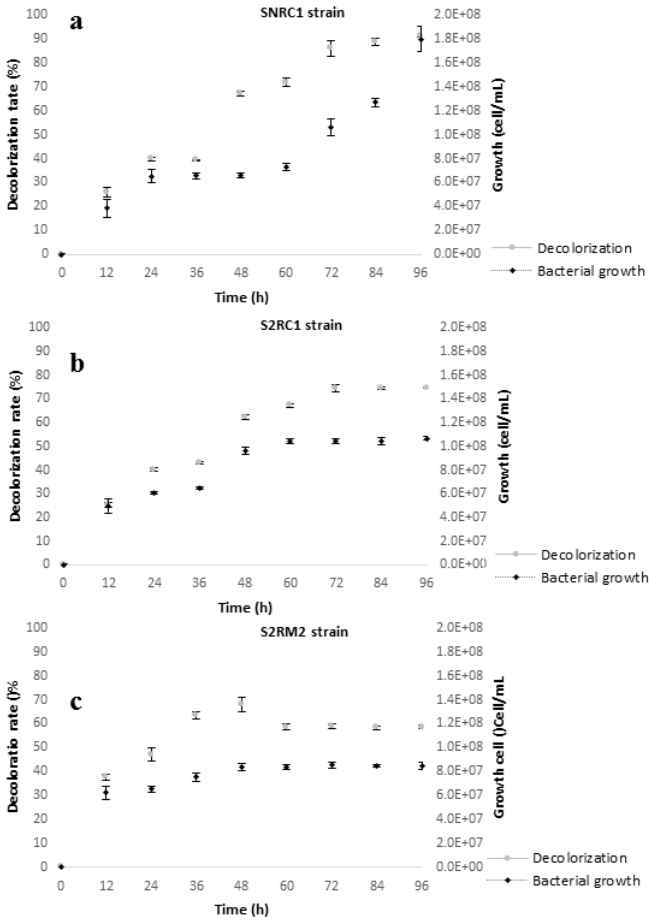

Figure 6: Growth kinetics (rhombus, right axis) and decolorization capability (circles, left axis) of cured strains culture in mineral medium added with azo dyes. a) SNRC1 cured strain grown in Congo-red. b) S2RC1 cured strain grown in Congo-red. C) S2RM2 cured strain grown in methyl-red. The error bars represent the standard deviation of three separated replicates of each experiment.

Discussion

Several bacterial genera have been isolated from azo dyes polluted sites. Azoreductases and oxidasas had been described as part of the enzymatic complexes formed by these bacteria, which apparently confer them their resistance to such xenobiotics, being *Pseudomonas*, *Bacillus* and *Lactobacillus*, the most outstanding [36, 37, 38]. *Sphingomonas*, *Aeromonas*, *Brevibacillus*, *Streptomyces*, *Lysinibacillus*, *Geobacter*, *Klebsiella*, etc., among other genera have recently been recognized as well as bacteria able to decolorize azo dyes [39-45]. In this study, three bacteria strains belong to *Cupriavidus* sp., *Acidovorax anthurii* and *Ralstonia pickettii* were isolated from soil exposed to industrial wastewater. *Cupriavidus* species have been described as resistant to heavy metals [46] and from the effects of several stressors, so they are persisting organisms in disturbed environments [47]. In this study, the *Cupriavudus* sp. strain (SNRC1) grew and decolorized 91% of the Congo-red dye added to LB liquid medium after 96 h of culture. In the same way, SNRC1

strain grew and decolorized 90.7% of azo dye, when Congo-red dye was used as the sole carbon source. The SNCR1growth was slower and there was less cell concentration during the culture time, the exponential growth phase started at 72 h, and the stationary growth phase was not reached.

Acidovorax anthurii has been described as phytophatogenic bacteria [48]. They have not been studied in the context of decolorization or degradation of azo compounds. The S2RC1 strain grew and decolorized 93% of Congo-red in LB liquid medium. This bacterium, as well as the previous, slow down its growth in liquid media with azo dye as the sole carbon source, but produced a 95% of Congo-red decolorization.

Ralstonia is a genus segregated from *Pseudomonas*, which is part of the tolerant microbiota from disturbed ecosystems. The S2RM2 strain belongs to *Ralstonia pickettii*, which has been found in soils and compost from around the world. This species degrades and mineralize biaryl compounds [49] and aerobically mineralize 2, 6-dichlorophenol [50]. It can also resist the effect of various stressors [47]. The results of this study show that the S2RM2 strain at 96 h of culture decolorized 96% of methyl-red dye in LB media liquid medium, but only when glucose is supplemented as additional carbon source. This strain used more time (96 h) than other strains of *Pseudomonas* (24 h) to achieve a high-percent of decolorization [23]. However, when methyl-red was used as the sole carbon source, after 12 h of culture it was decolorized only 58%. At that time the strain reaches the stationary growth phase. These results lead to proposal that in a media with an accessible carbon source as glucose, it acts as fuel for the bacterial production of enzymes able to metabolize the azo dyes. Once this is triggered, the strain could eventually use the azo dyes as carbon source, but if these dyes are the only carbon source, the enzymatic production is difficult and the growth limited.

For some bacterial species the decolorization and degradation mechanisms have been found encoded by genes of plasmidic DNA. Plasmids and megaplasmids on *Cupriavidus metallidurans* and *Ralstonia pickettii*, are responsible for their ability to survive in heavy metals contaminated environments or in the presence of some others environmental stressors [46, 47]. Concerning the azo dyes decolorization or degradation, only for *Pseudomonas* and *Lactobacillus* the participation of plasmids has been pointed out. Elbanna et al. [22] reported the presence of plasmidic DNA with molecular weights of 2.5 to 16 Kb in three acid lactic bacteria strains, able to decolorize and degrade textile azo dye. The loss of azo dyes decolorization ability was due to loss of 3 Kb molecular weight plasmids, suggesting by the authors that genes encoding textile azo dye degradation enzymes were located in plasmids. On the same way Zeng et al. [23] found plasmids approximately 2 Kb of molecular weight in *Pseudomonas* sp. R1 related to the red X-3B decolorization. As previously pointed out, the loss of these plasmids resulted in the loss of the capability of azo dye decolorization by *Pseudomonas* sp. In order to confirm the role of plasmidic DNA on decolorization processes, the authors carried out the transformation of *E. coli* DH5α with the plasmid from *Pseudomonas* strain. The transformed bacterium exhibited a significant azo dye decolorizing capability, proving involvement of the plasmids in the decolorization mechanism. The three strains isolated in this study did not shown little plasmids, but they possess plasmidic DNA of high molecular weight which could be completely eliminated through a treatment using only EtBr. For this reason, it is suggested that the mechanism of Congo-red and methyl-red decolorization in *Cupriavidus* sp., *Acidovorax anthurii* and *Ralstonia pickettii*, SNRC1, S2RC1 and S2RM2 strains, respectively, is encoded by genomic DNA, although it is not ruled out the possibility that plasmidic DNA elements could provide benefits to tolerate other stressful situations to the studied strains.

Conclusions

Acidovorax anthurii, *Cupriavidus* sp. and *Ralstonii picketii*, the three studies strains, could decolorize Congo-red or methyl-red under the tested growth conditions, however, the decolorization time reaction of the strains is comparatively slower regarding to the *Pseudomonas* species. All the three strains can use the azo dyes as carbon source, particularly *Cupriavidus* sp., which was able use them for generation of new cells. It is necessary to confirm the enzymatic azoreductase or oxidase activities and the intermediate compounds formed, and to improve the conditions for raise a better decolorization rate. Apparently, the azo dyes decolorization on the studied strains is not conferred by encoding genes of plasmidic DNA. The study of the genic elements of the plasmids will allow to known the possible adaptive advantages conferred.

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