Safe Biodegradation of Textile Azo Dyes by Newly Isolated Lactic Acid Bacteria and Detection of Plasmids Associated With Degradation

Khaled Elbanna[1]*, Gamal Hassan[2], Manal Khider[3] and Raafat Mandour[4]

[1]Department of Microbiology,Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt
[2]Department of Genetics,Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt
[3]Department of Dairy Science,Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt
[4]Toxicology Lab, Emergency Hospital, Mansoura Universit, Egypt

Abstract

Since azo dyes are recalcitrant to complete biodegradation due to their complex structure, lactic acid bacteria under an anaerobic/aerobic sequential system was used in an attempt to achieve complete mineralization of textile azo dyes for safe degradation products. A total of 120 lactic acid bacteria (LAB) were screened for decolorization of the textile azo dyes; Reactive Lanasol Black B (RLB), Eriochrome Red B (RN) and 1, 2 metal complexes I. Yellow (SGL). The screening results showed that a total of 80 out of 120 LAB isolates were able to decolorize the dyes, in 4 h ranging from 75 to 100%. Based on API 50 CHL and 16S rDNA sequences, *Lactobacillus casei* and *L.paracasei* were the nearest phylogenetic neighbour for both strains Lab11 and Lab13, with an identity of 99 %, while *L.rhamnosus* was the nearest phylogenetic neighbour for isolate Lab2 with an identity of 99 %. The biodegradation products of RLB (as a model of textile azo dyes) by Lab2 formed during anaerobic and sequential anarobic/aerobic treatments were analyzed by HPLC. Peaks at different retention times were observed in the anaerobic stage, and these peaks completely disappeared at the end of anarobic/aerobic incubation. This result clearly indicates that the dye had been catabolized and utilized by Lab2 isolate. Among the different plasmid curing treatments, SDS at 42°C was found to be an effective treatment for curing of these isolates. Plasmid profiles of wild-type strains and their cured derivatives indicates that the loss of the ability to decolorize azo dyes correlated to loss of a 3 kb plasmid, suggesting that the genes required for textile azo dye degradation were located on this plasmid. Azo dye degradation products were less toxic to growing *Sorghum bicolor* than the original azo dyes.

Keywords: Azo dyes; Safe biodegradation; Lactic acid bacteria; Plasmid curing; 16 S rDNA, HPLC analysis

Introduction

Azo dyes are the largest group of synthetic chemicals that are widely used by the textile, leather, cosmetics, food coloring and paper production industries. The chemical structure of these compounds features substituted aromatic rings that are joined by one or more azo groups (–N=N–). The annual world production of azo dyes is estimated to be around one million tons [1] and more than 2000 structurally different azo dyes are currently in use [2]. During the dyeing process, approximately 10-15% of the used dye is released into wastewater [3]. Moreover, many azo dyes and their degradation intermediates such aromatic amines are mutagenic and carcinogenic, and discharge of them into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into bodies of water, hence affecting aquatic life [4]. The treatment of textile wastewater is essential before discharging the wastewater into a receiving water body [5]. During the past two decades, several physicochemical decolorization techniques have been reported; however, few have been accepted by the textile industries [6]. Biological methods are generally considered environmentally friendly as they can lead to complete mineralization of organic pollutants at low cost [1]. Azo dyes are generally recalcitrant to biodegradation due to their complex structures and xenobiotic nature, and typically require an anaerobic–aerobic process to achieve complete mineralization. To overcome this problem, studies included either using microbial consortia or combinations of anaerobic and aerobic steps in an attempt to achieve not only dye decolorization, but also degradation of the aromatic amines [7]. However, few studies reported the use of a single adaptable microorganism in a sequential anaerobic/aerobic treatment [8]. Moreover, the available literature on

sequential anaerobic/aerobic treatment with a single microorganism is extremely limited [9]. Apparently there is a need to develop novel biological decolorization processes leading to the more effective clean up of azo dyes using a single and adaptable microorganism that is efficient under both anaerobic and aerobic conditions. Although degradation of azo dyes by microorganisms has been extensively documented, little is known about the biodegradation of azo dyes by lactic acid bacteria [10,11]. Thus, this work aims to (I) screen and identify azo dye degrading lactic acid bacteria, (II) study the potential of these isolates in azo dye degradation, (III) detection of plasmids associated with degradation. (IV) determination of phytotoxicity of the azo dye degradation products.

Material and Methods

Azo dyes

The commercial textile azo dyes, RLB, RN and SGL were purchased from the local market in Egypt. These dyes were selected

on the basis of their structural diversity and frequent use in local textile industries. The chemical structures of used dyes and other information were shown in (Table 1). The stock solution of dyes (1g/100 ml) was prepared by dissolving in distilled water and filtration through Whatmann No. 5 filter paper.

Phenotypic characterization of azo dye degrading bacteria

A total of 120 lactic acid bacterial isolates (LAB) isolated from dairy products using MRS medium were screened for azo dye degradation. Isolates capable of degrading azo dyes were further characterized by using the API 50 CHL System identification kit (Bio Mérieux, France) and the species were preliminarily identified from the API database.

DNA isolation, PCR amplification and sequence analysis of 16S rDNA gene

Genomic DNA was isolated according to the method described by Azcarate-Peril and Raya [12]. The 16S rDNA gene was amplified by PCR using universal eubacterial primers [13]. The 16S rDNA fragments (~1540 bp) were sequenced using an ABI 3730xl automated DNA sequencer (Applied biosystems USA) through Lab Biotechnology Company, Egypt. The 16S rDNA sequences were initially analyzed by using the program BLAST (National Center Biotechnology Information, http//:www.ncbi.nml.nih.gov). The sequencing data obtained from different primers were assembled using the CAP program (Contig Assembly and Genomic Expression programs). The sequence from the isolates and sequences of strains belonging to the same phylogenetic group and other representatives of *Lactobacillus* strains (retrieved from the NCBI database) were aligned using the computer-program ClustalX [14]. The resulting trees were displayed with Tree View [15]. The phylogenetic reconstruction was done using the neighbour joining method [16] using *Acinetobacter calcoaceticus* as an outgroup.

Culture conditions

Isolates of lactic acid bacteria were grown anaerobically at 35°C in MRS broth for 48 h for use as starters. A loopful of each starter was cultured in Hungate tubes (16 ml vol) containing 14 ml of MRS medium at 35°C for 48 h [17]. Individual azo dye stock solutions were added to the cultures at final concentration of 100 mg l^{-1}, and the cultures were incubated at 35°C under anaerobic conditions for 48 h.

Assay for the decolorization of azo dyes by LAB isolates

For measurement of decolorization of the three tested dyes RLB, RN and SGL, aliquots (1ml) of the cultures were withdrawn at respective time intervals and centrifuged at 12,000 rpm for 15 min. The absorbance of centrifuged supernatant samples was read at 598, 437 and 475 nm for RLB, RN and SGL, respectively using a JASCO V-530 UV-VIS spectrophotometer. All assays were performed in triplicate and compared with an uninoculated control. The decolorization efficiency (%) of different isolates was expressed in terms of percentage and was calculated as following:

Decolorization (%) = Initial absorbance-Final absorbance/ initial absorbance x 100.

Plasmids curing

Plasmid curing from the wild-type isolates was carried out as described by Trevors [18] using ethidium bromide (EtBr) and sodium dodecyl sulphate (SDS) at their highest sublethal concentrations, i.e. 10μg/ml and 0.8 mg/ml, respectively. Also, plasmids were cured using SDS or (EtBr) at 42°C [19]. Tetracycline (30μg/ml), Chloramphenicol (30μg/ml), Ampicillin (20μg/ml), Streptomycin (10μg/ml), Kanamycin (30μg/ml) and Gentamycin (10μg/ml) were separately added to MRS agar and were used as markers in curing experiments. Colonies that failed to grow on the selective plates (MRS agar amended with marker antibiotic) were considered as putatively cured derivatives, picked from the master MRS agar plates and checked for of the loss of plasmids.
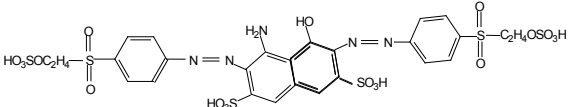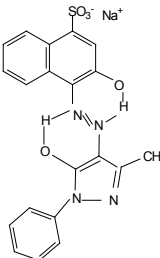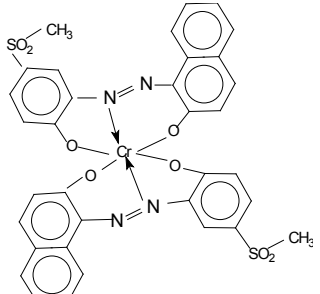
Dye name	Structure formula	Color index (CI)	λ_{max} (nm)	MW
Lanasol black B		Reactive Black B	598	991.82
Eriochrom red B		Acid Mordant Red B	437	446.41
1, 2 metal complex 1. Yellow		Acid Yellow M$_2$GLN	475	620

Table 1: Chemical structures and some information of textile azo dyes used in this study.

Curing agents treatments	concentration	No. of colonies tested from isolates			Colonies lost antibiotic resistance		
		Lab2	Lab11	Lab13	Lab2	Lab11	Lab13
(1) EtBr	10 µg/ml	50	50	50	0	0	0
(2) SDS	0.8 mg/ml	50	50	50	0	0	0
(3) EtBr at. 42°C	10 µg/ml	50	50	50	0	0	0
(4) SDS at 42°C	0.8 mg /ml	50	50	50	3	2	2

Table 2: Effect of different curing agents on cured plasmid conferring resistance to antibiotics in *lactobacillus* isolates.
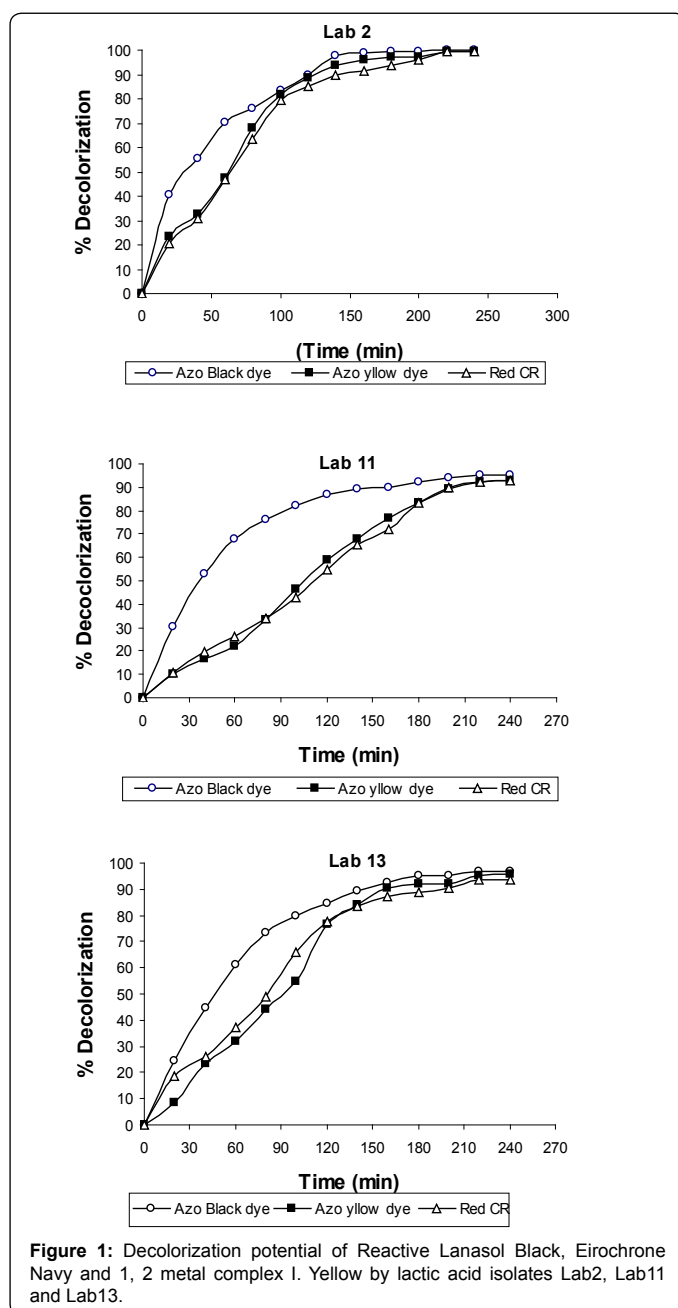

Figure 1: Decolorization potential of Reactive Lanasol Black, Eirochrone Navy and 1, 2 metal complex I. Yellow by lactic acid isolates Lab2, Lab11 and Lab13.

Plasmid DNA isolation

Plasmid DNA was isolated from wild type and cured isolates based on the alkaline lysis method described by Azcarate-Peril and Raya [12]. DNA preparations were separated by electrophoresis on 0.8% agarose gels at 50 V for 3 h. The gels were then stained with ethidium bromide and bands were visualized on an UV transilluminator.

Molecular sizes of plasmids were determined by comparison with a 1-kb DNA ladder.

Toxicity study

For phytotoxicity study, degradation metabolites of the RLB were extracted in ethyl acetate, dried and then dissolved in water to a final concentration of 1000 ppm. The phytotoxicity study was carried out at room temperature (35±2°C) with *Sorghum bicolor*, in three sets each plate contained ten seeds, by adding 5 ml of the untreated azo dyes (1000 ppm), its degradation products (1000 ppm) per day and water (as control). Seeds germination (%) was recorded after 7 days.

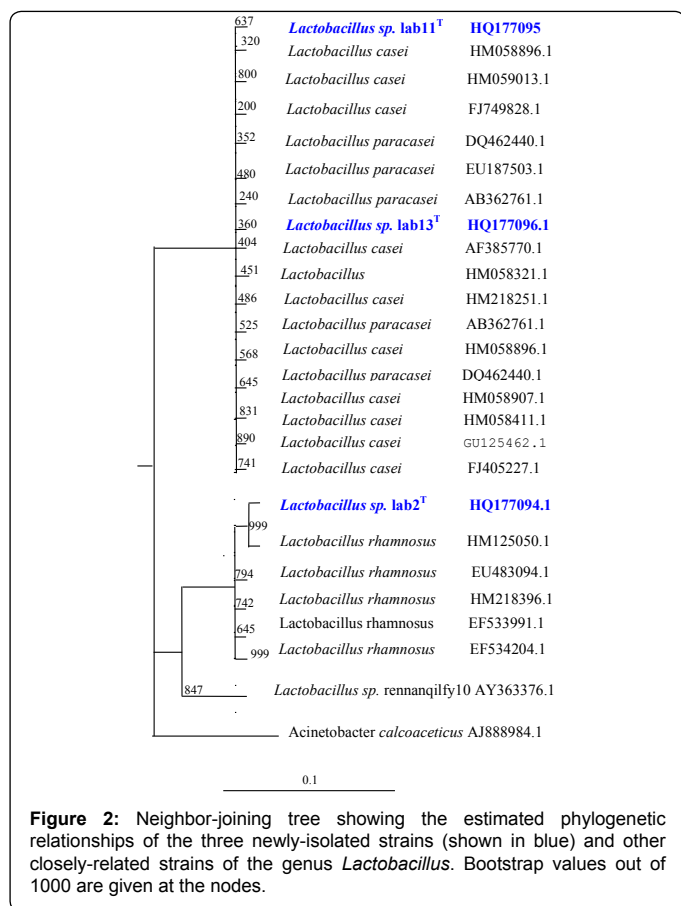## HPLC analysis of decolorization metabolites

For HPLC analysis, isolate Lab 2 was grown anaerobically at 35°C for 48 h in Hungate tube containing 14 ml MRS broth, and then RLB was added to the culture at final concentration of 100 mg l^{-1}. The culture was incubated anaerobically at 35°C for 10 h, and subsequentially incubated aerobically for 4h (anaerobic/aerobic sequential system). Ten ml culture were withdrawned after 2 and 10 hours of anaerobic incubation and after 4 hours of aerobic incubation which followed the 10 hours of anaerobic incubation and centrifuged at 12,000 rpm for 15 min. The supernatants were clarified by passing through a 0.45µm membrane filter. Degradation products were extracted thrice with diethyl ether, then extracts were pooled and evaporated to dryness, and the final residue was dissolved in methanol and analyzed using HPLC according to Zhao and Hardin [20].

Results and Discussion

Decolorization of azo dyes by LAB isolates

Although, several bacteria and fungi are capable of catabolizing and mineralizing azo dyes, information on the ability of safe food microorganisms such as lactic acid bacteria (LAB) to degrade azo dyes is scant [21]. In this work, the first screening experiments indicated that 80 out of 120 LAB isolates were able to decolorize the three tested dyes RLB, RN and SGL. These isolates showed decolorization percentages ranging from 75 to 100 % within 4 h, indicating that degradation of azo dyes seems to be widespread among lactic acid bacteria. Based on the screening results, the three most effective isolates (Lab2, Lab11, and Lab13), which showed the highest decolorization potential for all tested azo dyes in anaerobic condition were selected for further studies. The results in (Figure 1) indicate that all the three *Lactobacillus* isolates exhibited high efficiency of decolorization ranging from 92.6 to 100 % within 4 h. Isolate Lab2 exhibited complete decolorization of 100, 99.7 and 99.4 %, for RLB, RN and SGL, respectively. The decolorization efficiency of isolate Lab11 were 95.2, 93 and 92.6 %, and of Lab13 96.8, 95.5 and 93.7% for RLB, RN and SGL, respectively. Hu [22] reported; 37, 88, 92.4 and 93.2 % decolorization of reactive azo dyes Red G, V2RP, RP2B and RBB, respectively by *Pseudomonas luteola* after 42 h incubation under static conditions. However, compared to degradation rates previously reported, decolorization of the textile azo dyes was lower than what obtained in the present study. It was mentioned that, most azo dyes are reduced anaerobically to the corresponding amines with cleavage of azo bonds by bacterial azoreductase, but they are difficult to degrade aerobically [23]. In general microbial degradation of azo dyes involves the reductive cleavage of azo bonds with the help of an azoreductase enzyme under anaerobic conditions, and this involves a transfer of four electrons, which acts as a final electron acceptor, resulting in dye decolorization and the formation of colorless solutions [24]. In the present study, complete decolorization was achieved in a

Figure 2: Neighbor-joining tree showing the estimated phylogenetic relationships of the three newly-isolated strains (shown in blue) and other closely-related strains of the genus *Lactobacillus*. Bootstrap values out of 1000 are given at the nodes.

short period of time by Lab2 indicates the effectiveness of lactic acid bacteria in textile azo dye bioremediation. It is worth mentioning that rapid initial decolorization (80%) of all the three tested textile azo dyes was achieved by Lab2 within the first two hours. This suggest that Lab 2 isolate may have an efficient enzymatic system for the cleavage of azo bonds, which caused rapid decolorization, and consequently, could successfully be employed in the treatment of wastewaters contaminated with azo dyes.

Identification and characterization of azo dye-degrading strains

Based on their superior decolorization potential, the three selected isolates were chosen for further phenotypic and genotypic characterization. All the three isolates were Gram-positive, facultatively aerobic, non-motile, non-spore-forming regular single rods, pairs or rarely short chains. The optimal temperature and pH values for growth in MRS medium were around 37°C and 6.4, respectively. The strains were oxidase and catalase -negative. Based on the results of the API 50 CHL test, isolates Lab11and Lab13 were identified as strains of *Lactobacillus casei*, while isolate Lab2 could not be identified by this method. However, it was reported that the identification of *Lactobacillus spp.* by biochemical methods alone is not always reliable [25,26]. Therefore, the phylogenetic relationships among the lactobacilli in this study were confirmed by comparing the 16S rDNA genes. The sequences of these isolates are available in GenBank under the accession numbers HQ177094, HQ177095 and HQ177096 for Lab2, Lab11 and Lab13 respectively. The 16S rDNA sequence analysis (Figure 2) showed that *Lactobacillus casei* and *Lactobacillus paracasei* were the nearest phylogenetic neighbours for

strains Lab11 and Lab13 with an identity of 99 %, while *Lactobacillus rhamnosus* was the nearest phylogenetic neighbour for isolate Lab2, with an identity of 99 %.

Analysis of Degradation Products

Biodegradation of azo dyes under anaerobic conditions results in colorless but toxic and carcinogenic aromatic amines, of which some are readily metabolized under aerobic conditions [27]. In the present work, the degradation products of RLB by Lab2 which resulted from anaerobic treatment or sequential anaerobic/aerobic treatment were analyzed by HPLC (Figure 3). Analysis of RLB at the beginning of the anaerobic incubation showed a peak at a retention time of 10.22 minutes, which corresponded to that of unmetabolized RLB azo dye. This peak disappeared completely at the end of the aerobic treatment. Also, unidentified small peaks at retention times of 12.8 and 15.19 minutes were detected. The intensity of these peaks increased towards the end of anaerobic stage. Furthermore new peaks at retention times of 19.49, 21.65, 22.13, 22.57 and 25.16 minutes were detected. Interestingly, these peaks completely disappeared completely at the end of the aerobic treatment. These results clearly indicate that the dyes could have been catabolized and utilized by Lab2 and that the anaerobic/aerobic process drastically improves the complete mineralization of azo dyes. Tan et al. [28] and Sponsa and Isik [29] reported that the prerequisite for the complete mineralization of azo dyes is a combination of reductive and oxidative steps. They also reported that decolorization of azo dyes normally begins with an initial reduction or cleavage of azo bond by azoreductase anaerobically, which results into colorless compounds. This is followed by complete degradation of aromatic amines strictly under aerobic conditions. Therefore, anaerobic/aerobic processes are crucial for the complete mineralization of azo dyes.

Plasmids curing

The bacterial resistance to antibiotics is a common phenotypic character that is often plasmid-encoded [30]. In the present study, cells did not lose their resistance towards antibiotics such as kanamycin, streptomycin and gentamycin after treatment with the highest sublethal doses of both curing agents; EtBr and SDS. The highest concentrations of EtBr and SDS that allowed the growth of the strains was determined (Table 2). No growth was observed at concentrations above 12µg/ml and 1mg/ml for EtBr and SDS, respectively. Concentrations of 10µg/ml and 0.8 mg/ml were therefore chosen as the highest sublethal concentrations for EtBr and SDS, respectively. This indicate that EtBr and SDS alone are not capable of curing plasmid from the selected isolates, while some of the cells grown in the presence of the highest sublethal concentration of SDS at 42°C were unable to grow on MRS agar supplemented with kanamycin, streptomycin and gentamycin. This shows that SDS at 42°C effectively cured the selected isolates of plasmids. It was previously reported that curing with SDS at elevated temperature was effective in a wide variety of bacteria [31].

Plasmid profiles

To determine the plasmid harboring the catabolic genes involved in azo dye degradation, electrophoretic profiles of plasmids isolated from the cured isolates were compared with those of the non-cured strains. (Figure 2) showed that all cured derivative strains exhibited plasmid profiles different from their corresponding wild-type. Isolates Lab2 and Lab11 were similar in their plasmid profiles, containing four different plasmids with molecular weights of 16, 8, 3 and 2.5 kb (Figure 2 lane 1, 3), while Lab13 contains three plasmids with
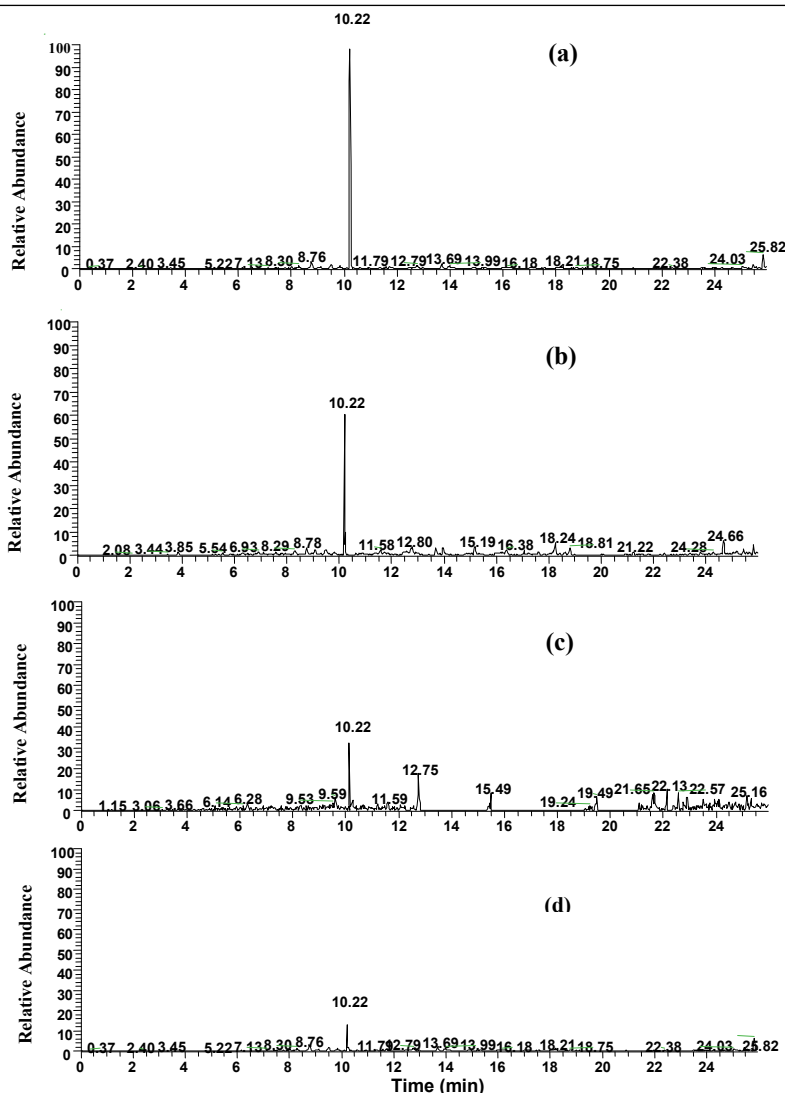
Figure 3: HPLC analysis of Reactive Ianasol Black and its degradation metabolites resulting under anaerobic–aerobic conditions: (a) Untreated dye, (b) at the beginning of the anaerobic incubation (after 2 h); (c) after anaerobic incubation for 10 h; and (d) after anaerobic (10 h)-subsequently aerobic incubation for 4 h.
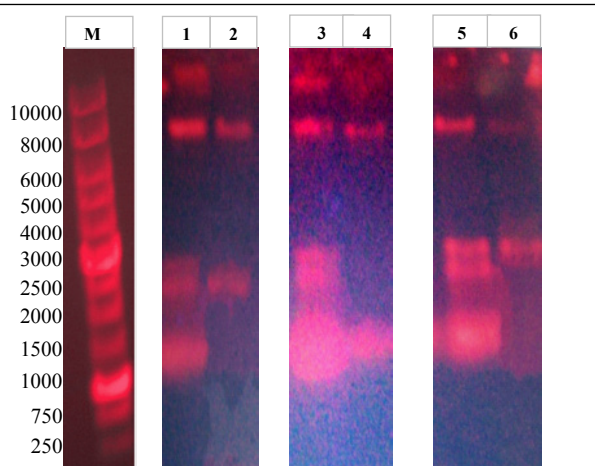

Figure 4: Electrophoretic separation profiles of plasmid DNA's from wild-type isolates and their cured derivatives. M: 1-kb DNA ladder; Lane 1: Lab2 WT; Lane2: Lab2CD, Lane 3: L11WT; Lane 4: L11CD, lane 5: L13WT, lane 7 L13CD. WT= wild-type, CD= cured derivative.

molecular weights of 8, 3 and 2.5 kb, (Figure 2 lane 5). Compared to the plasmid profiles of the wild-type strains, it was noticed that the cured derivatives of Lab2 lost plasmids with molecular weights of 3 and 16 kb, and that the cured derivatives of Lab11 lost plasmids with molecular weights of 16, 3 and 2.5 kb. The cured derivatives of Lab13 lost only one plasmid with molecular weight of 3 kb. These results indicate that loss of azo dyes decolorization ability may be correlated with a loss of the 3 kb plasmid, and that the genes encoding textile azo dye degradation were located on plasmids. Pouwel and Leer [32] reported that most *Lactobacillus* species have at least one indigenous plasmid functionally correlated with phenotypical properties, including antibiotic resistance and azo dye degradation.

Phytotoxicity of Dyes and their Biodegradation Products

It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. Seed germination bioassay is the most common technique used to evaluate the phytotoxicity. This can be achieved by phytotoxicity tests with the original dyes and their biodegradation products. Seed germination (%) of *Sorghum bicolor* was 35.3, 92.3 and 94 % after treatment with untreated

RLB, dye metabolites and a water control, respectively. These results indicate that the azo dye degradation products formed after biodegradation by lactic acid bacteria were less toxic compounds compared to the original azo dyes. These results are in agreement with result of Kalyani et al. [33] and Mane et al. [34] who found that the metabolites products after biodegradation of Reactive Red 2 and Reactive Blue 59 were less toxic compared to the original dye. In this study, Lab2 was capable to detoxify the RLB to an extent allowed the germination to reach the percentage above mentioned.

Conclusion

The results obtained in this study are very promising for the treatment of wastewater streams since lactic acid bacterial isolates used could able to achieve complete mineralization of textile azo dyes for safe degradation products in rapid and short time under sequential anaerobic/aerobic system. This methodology using a single microorganism in azo dyes decolorization was shown to be very effective. However, further work is needed to identify the gene(s) responsible for this kind of textile azo dyes decolorization.

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