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Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process

Franciscon Elisangela [a,*], Zille Andrea [b], Dias Guimaro Fabio [a], Ragagnin de Menezes Cristiano [a], Durrant Lucia Regina [a], Cavaco-Paulo Artur [c]

[a] Campinas State University, Department of Food Science, 13083-970 Campinas, São Paulo, Brazil
[b] IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal
[c] University of Minho, Department of Textile Engineering, 4800-058 Guimarães, Portugal

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ABSTRACT

A facultative *Staphylococcus arlettae* bacterium, isolated from an activated sludge process in a textile industry, was able to successfully decolourize four different azo dyes under microaerophilic conditions (decolourization percentage >97%). Further aeration of the decolourized effluent was performed to promote oxidation of the degradation products. The degradation products were characterized by FT-IR and UV–vis techniques and their toxicity with respect to *Daphnia magna* was measured. The amine concentrations as well as the total organic carbon (TOC) levels were monitored during the biodegradation process. The presence of aromatic amine in the microaerophilic stage and its absence in the aerobic stage indicated the presence of azoreductase activity and an oxidative biodegradation process, respectively. TOC reduction was ~15% in the microaerophilic stage and ~70% in the aerobic stage. The results provided evidence that, using a single *Staphylococcus arlettae* strain in the same bioreactor, the sequential microaerophilic/aerobic stages were able to form aromatic amines by reductive break-down of the azo bond and to oxidize them into non-toxic metabolites.

1. Introduction

Azo dyes, which are aromatic compounds with one or more (–N=N–) groups, are the most important and largest class of synthetic dyes used in commercial applications (Vandevivere et al., 1998). They are considered as xenobiotic compounds that are very recalcitrant to biodegradation processes (Zollinger, 1991; Stolz, 2001). The textile industry accounts for two-thirds of the total dyestuff market. During the dyeing process, approximately 10–15% of the dyes used are released into the wastewater. The presences of these dyes in the aqueous ecosystem are the cause of serious environmental and health concerns (Fang et al., 2004; Asad et al., 2007). Several methods are used to treat textile effluents to achieve decolourization. These include physicochemical methods such as filtration, coagulation, carbon activated and chemical flocculation (Gogate and Pandit, 2004a, b). These methods are effective but they are expensive and involve the formation of a concentrated sludge that creates a secondary disposal problem (Maier et al., 2004). In recent years, new biological processes, including aerobic and anaerobic bacteria and fungi, for dye degradation and wastewater reutilization have been developed.

The most widely researched fungi in regard to dye degradation are the ligninolytic fungi (Bumpus, 2004). White-rot fungi in particular produce lignin peroxidase, manganese-peroxidase and laccase that degrade many aromatic compounds due to their non-specific enzyme systems (Stolz, 2001; Robinson et al., 2001; McMullan et al., 2001; Wesenberg et al., 2003; Forgacs et al., 2004; Harazono and Nakamura, 2005; Pazarlioglu et al., 2005; Toh et al., 2003; Mohorcic et al., 2006; Madhavi et al., 2007). Large literature exists regarding the potential of these fungi to oxidize phenolic, non-phenolic, soluble and non-soluble dyes (Field et al., 1993; Pasti-Grigsby et al., 1992; Bumpus, 1995; Conneely et al., 1999; Kapdan et al., 2000; Borchert and Libra, 2001; Tekere et al., 2001; Kapdan and Kargi, 2002; Martins et al., 2002; Libra et al., 2003). Laccase preparations obtained from *Pleurotus ostreatus*, *Schizophyllum commune*, *Sclerotium rolfsii* and *Neurospora crassa*, increased up to 25% the decolourization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes (Abadulla et al., 2000). In contrast, manganese peroxidase was reported as the main enzyme involved in dye decolourization by *Phanerochaete chrysosporium* (Chagas and Durrant, 2001) and lignin peroxidase for *Bjerkandera adusta* (Robinson et al., 2001). Some non-white-rot fungi that can successfully decolourize dyes have also been reported (Kim and Shoda, 1999; Cha et al., 2001; Ambrósio and Campos-Takaki, 2004; Bumpus, 2004; Tetsch et al., 2005). However, the long growth cycle

* Corresponding author. Tel.: +55 19 3521 2173; fax: +55 19 35212153.
E-mail address: elisfran@fea.unicamp.br (F. Elisangela).

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and the complexity of the textile effluents, which are extremely variable in composition, limit the performance of these fungi. Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved, application of white-rot fungi for the removal of dyes from textile wastewaters faces many problems such as large volumes produced, the nature of synthetic dyes, and control of biomass (Palma et al., 1999; Nigam et al., 2000; Zhang and Yu, 2000; Robinson et al., 2001; Mielgo et al., 2001; Stolz, 2001). In contrast, the bacterial reduction of the azo bond is usually nonspecific and bacterial decolourization is normally faster. Microbial decolourization and degradation has appeared as an environmentally friendly and cost-competitive alternative to chemical decomposition processes (Verma and Madamwar, 2003; Moosvi et al., 2005; Pandey et al., 2007; Whiteley, 2007; Khalid et al., 2008; Dhanve et al., 2008; Ozdemir et al., 2008). Research on bacterial strains that are able to decolourize azo dyes under aerobic (*Xenphylus azovorans* KF46F, *Bacillus* strain, *Kerstersia* sp. strain VKY1 and *Staphylococcus* sp.) and anaerobic conditions (*Sphingomonas xenophaga* BN6, *Eubacterium* sp., *Clostridium* sp., *Butyrvibrio* sp. or *Bacteroides* sp.) have been extensively reported (Rafii et al., 1990; Kudlich et al., 1997; Suzuki et al., 2001; Blumel et al., 2002; Olukanni et al., 2006; Dos Santos et al., 2007; Vijaykumar et al., 2007; Hsueh and Chen, 2008; Lin and Leu, 2008). Based on the available literature, it can be concluded that the microbial decolourization of azo dyes is more effective under anaerobic conditions. On the other hand, these conditions lead to aromatic amine formation, and these are mutagenic and toxic to humans (Chung and Stevens, 1993; Do et al., 2002; Pinheiro et al., 2004) requiring a subsequent oxidative (aerobic) stage for their degradation. In this context, the combined anaerobic/aerobic biological treatments of textile dye effluents using microbial consortia are common in the literature (Chang and Lin, 2000; Van der Zee and Villaverde, 2005; Lodato et al., 2007). However, few commercial or industrial applications have been developed using a single adaptable microorganism in a sequential anaerobic/aerobic treatment (Isik and Sponza, 2003; Isik and Sponza, 2004; Supaka et al., 2004). Moreover, the available literature on the sequential microaerophilic/aerobic treatment with a single microorganism is extremely limited (Sandhya et al., 2004; Xu et al., 2007). Apparently there is a need to develop novel biological decolourization processes leading to the more effective clean up of azo dyes using a single microorganism that is efficient under both anaerobic/microaerophilic and aerobic conditions. Thus the main objective of this study was to observe the degradation of four azo dyes in a successive microaerophilic/aerobic process using exclusively a facultative anaerobic *Staphylococcus arlettae* bacterium isolated from textile dye effluents. Dye degradation was performed under microaerophilic conditions until no residual colour was observed. The medium was subsequently aerated by stirring to promote oxidation of the aromatic amines formed by the reductive break-down of the azo bond into non-toxic metabolites. The effects of sodium pyruvate and yeast extract as carbon sources on the decolourization were also investigated, since it has been reported that the type of carbon source could affect dye decolourization and its subsequent reduction (Nigam et al., 1996; Brás et al., 2001; Kim et al., 2008). The amine concentrations and TOC were monitored during the biodegradation process. The degradation products were also characterized using FT-IR and UV–vis techniques, and their toxicity measured.

2. Materials and methods

2.1. Chemicals and media

The azo dyes (100 mg L^{-1}) CI Reactive Yellow 107 (RY107, 0.18 mM), CI Reactive Black 5 (RB5, 0.11 mM), CI Reactive Red 198 (RR198, 0.13 mM) and CI Direct Blue 71 (DB71, 0.1 mM) were kindly provided by the Vicunha Textile Company, Itatiba, Brazil. The structures of the dyes are shown in Fig. 1. All other analytical grade reagents were purchased from Sigma and used without further purification. The mineral salts medium (MM) at pH 7 used in all the batch experiments contained: K_2HPO_4 (1.6 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), $(NH_4)_2SO_4$ (1.0 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.2 g L^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g L^{-1}), NaCl (0.1 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.02 g L^{-1}). The rich mineral medium (MMR) consisted of MM supplemented with 100 mg L^{-1} of each dye, 3 g L^{-1} of glucose and 1 g L^{-1} of yeast extract and was autoclaved at 121 °C for 15 min.

2.2. Strain isolation and characterization

The microorganisms were isolated from different stages of the activated sludge produced by the Vicunha Textile Company, Itatiba, Brazil. Serial dilutions (10^{-1}–10^{-6}) of the samples collected were inoculated into nutrient agar medium by the spread plate technique. Isolated strains were inoculated into MM with a low glucose concentration (0.1 g L^{-1}) and mixed with azo dyes (100 mg L^{-1} per dye) and incubated under microaerophilic conditions at 30 °C for 7 days. The strain that achieved the best decolourization was selected for this study.

Identification of the isolated strain was performed by 16S rDNA sequence analysis. Genomic DNA was obtained according to Pitcher et al. (1989). The 16SrRNA gene was amplified by PCR using the specific primers, 27f and 1401r for the universal bacteria domain (Lane, 1991).

Fifty-microlitre reaction mixtures were prepared containing 100 ng of total DNA, 2 U of *Taq* polymerase (Invitrogen®), 0.2 mM of deoxynucleoside triphosphates and 0.4 μM of each primer. The PCR amplifications were done using an initial denaturation step of 2 min at 94 °C, followed by 10 cycles of 1 min each at 94 °C, 30 s at 69 °C, decreasing 0.5 °C each cycle, and 3 min at 72 °C, followed by another 10 cycles of 1 min each at 94 °C, 30 s at 63 °C and 3 min at 72 °C, in an Eppendorf thermal cycler.

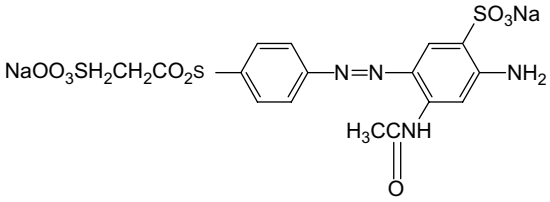
The PCR product was purified on GFX™ PCR DNA and a Gel Band Purification kit (GE HealthCare) for automated sequencing in the MegaBace DNA Analysis System 1000. The sequencing was carried out using the 10f (5′GAG TTT GAT CCT GGC TCA G3′); 765f (5′ATT AGA TAC CCT GGT AG3′); 782r (5′ACC AGG GTA TCT AAT CCT GT3′) and 1100r (5′AGG GTT GGG GTG GTT G 3′) primers and the DYEnamic ET Dye Terminator Cycle Sequencing Kit for the automated MegaBace 500 system (GE Healthcare), according to the manufacturer's instructions.

Partial 16S rRNA sequences obtained from isolates were assembled in a contig using the phred/Phrap/CONSED program (Godon et al., 1997; Ewing et al., 1998). Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data from the reference and type strains available in the public databases GenBank and RDP (Ribosomal Database Project, Wisconsin, USA) using the BLASTn and RDP sequence match routines, respectively. The sequences were aligned using the CLUSTAL X program and analyzed with the MEGA software 2001 (Thompson et al., 1994; Kumar et al., 2004). Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA, using Kimura's DNA substitution model (Kimura, 1980).

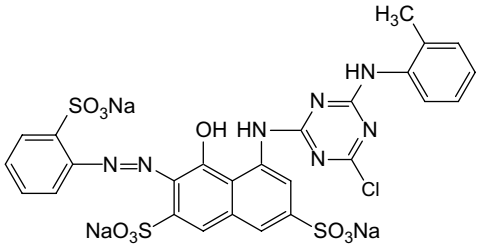
The phylogenetic reconstruction was done using the neighbour-joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software (Saitou and Nei, 1987).

2.3. Aromatic amine detection

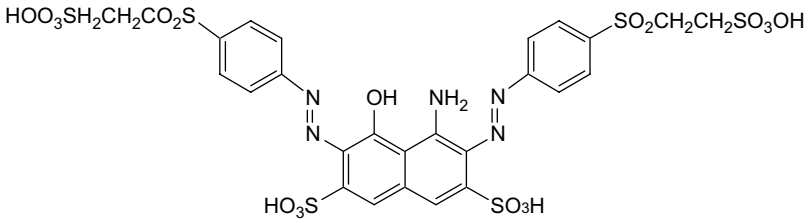
The aromatic amines in the solid phase were determined by the modified Marik and Lam method (2003). Samples were taken after



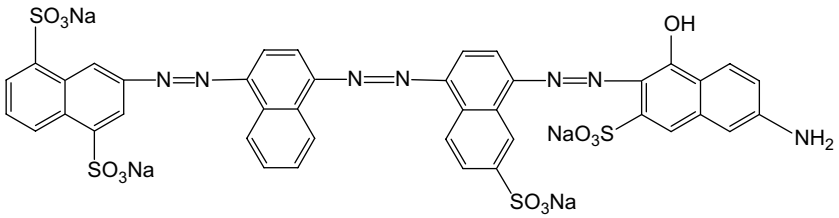
C.I. Reactive Yellow 107 (RY107)



C.I. Reactive Red 198 (RR198)



C.I. Reactive Black 5 (RB5)



C.I. Direct Blue 71 (DB71)

Fig. 1. Chemical dye structures.

incubation under the microaerophilic and aerobic conditions, frozen and freeze-dried (FTS system model Dura-Dry MP). The samples (5 mg) were dissolved in 5 mL of a 0.4% solution of chloranil in dimethylformamide (DMF) and heated to 100 °C for 5 min. The absorption was determined in a UV–vis Heliosα Unicam spectrophotometer at 560 nm. A calibration curve was prepared using aniline-2-sulfonic acid as a model product of the reduction of azo dyes, and the sample amine concentration was calculated in millimoles L^{-1}. The control was the MMR medium without the dye, treated with the bacterium under microaerophilic and aerobic conditions.

2.4. Dye decolourization

Decolourization assays were carried out under static conditions with 350 mL cultures of MMR (pH 7) supplemented with 100 mg L^{-1} of the dyes. The samples were incubated under microaerophilic conditions at 30 °C for 168 h or until no colour was observed. Further aeration was carried out in a shaker at 150 rpm to promote oxidation of the degradation products. Dye decolourization was measured in a UV–visible spectrophotometer (Shimadzu 2101) for the microaerophilic and aerobic stages, and the percentage of effluent decolourization was calculated.

2.5. UV–vis analysis

The dye degradation products produced during biodegradation under microaerophilic and aerobic conditions were studied by following the change in the UV–vis spectra (from 200 to 800 nm) using a UV–vis spectrophotometer (Agilent 8453).

2.6. Infrared spectrum analysis

The controls and samples were dried and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g). The samples were then ground, desorbed at 60 °C for 24 h and pressed to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using an FT-IR Spectrum 2000 Perkin–Elmer spectrometer. The spectra were collected within a scanning range of 400–4000 cm^{-1}. The FT-IR was first calibrated for background signal scanning with a control sample of pure KBr, and then the experimental sample was scanned. The FT-IR spectrum of the

control was finally subtracted from the spectra of the non-degraded and degraded dyes.

2.7. TOC measurement

The presence of organic carbon in the samples containing dyes was monitored by measuring the total organic carbon (TOC) under microaerophilic conditions and after agitation, using a TOC analyzer (Shimadzu 5000A) with automatic injection of the samples (500–2000 μL) (Shimadzu Corporation, 1997). Samples were centrifuged (20,000 × *g* for 15 min) and filtered through a 0.45-μm pore size filter. The TOC measurement is based on the combustion of the total carbon (TC) on a sample filled tube with a catalyst and heated to 680 °C. The standard deviation is less than 2% for full scale ranging from 2000 ppm to 4000 ppm. All the samples were analysed using a five-point calibration curve.

2.8. Toxicity test

After treatment with *Staphylococcus arlettae*, the samples were centrifuged at 20,000 × *g* for 20 min and filtered through a 0.45-μm pore size filter. The acute toxicity tests with *Daphnia magna* (Crustacea, Cladocera) were carried out according to the ABNT NBR 12713 methodology (1993). The sensitivity tests were carried out with neonates (6–24 h old). For each sample concentration (1, 25, 50, 75, 100%), 5 organisms were used in a 5 mL flask. The tests under the microaerophilic and aerobic conditions and the control in water were carried out in triplicate for each concentration. The flasks containing the samples were maintained at 20 ° C for 48 h in the absence of light. The number of immobile organisms was then counted after 20 s of light exposure.

3. Results

3.1. Strain isolation and identification

The *Staphylococcus arlettae* strain VN-11 is a gram positive coccus, non-motile and facultative anaerobic bacterium. Heterotrophic bacteria, such as *Staphylococcus* sp., are usually dominant in the activated sludge system, degrading and eventually mineralizing organic compounds to carbon dioxide and water (Sidat et al., 1999; Ajibola et al., 2005; Olukanni et al., 2006).The VN-11 strain was isolated from raw effluents from a textile industry in Campinas, Brazil, and selected based on its ability to discolour a mixture of four azo dyes (100 mg L^{-1} each) in a study in MM supplemented with a low glucose concentration (0.1 g L^{-1}). The 16S rRNA gene sequence of the VN-11 strain was determined and compared with available 16S rRNA gene sequences from organisms in the GenBank and RDP databases. The VN-11 strain was phylogenetically positioned in the genus *Staphylococcus*. The nucleotide alignment of the strain VN-11 supported bootstrap values of 99% similarity to *Staphylococcus arlettae* and other strains including the sequence of the strain types *S. gallinarum* ATCC 35539^T, *S. cohnii*, ATCC 29974^T (D83361). The evolutive distance was based on the Kimura 2p model (Kimura, 1980). The GenBank accession numbers are represented in parentheses. *Sulfobacillus acidophilus* DSM 10332^T was used as the outgroup. The phylogenetic tree (Fig. 2) showed the grouping of VN-11 within *Staphylococcus arlettae*, confirming its identification due to the high bootstrap value.

3.2. Decolourization

The strain *Staphylococcus arlettae* was tested to separately decolourize four azo dyes (CI Reactive Yellow 107, CI Reactive Red 198, CI Reactive Black 5 and CI Direct Blue 71) in a microaerophilic/aerated sequential process. Complete decolourization (>97%, Table 1) of the azo dyes was achieved in the microaerophilic stage, even if the bacteria showed little growth (data not shown), and no significant changes were detected in the following aerobic stage. *Staphylococcus arlettae* could only decolourize the dyes effectively when the medium was supplemented with yeast extract. The decolourization time showed a relationship with the chemical structure of the dyes. The monoazo RY107 and RR198 were decolourized in 12 and 10 h, respectively. The diazo RB5 and the triazo DB71 were decolourized after 24 and 48 h, respectively (Table1).

3.3. Aromatic amine determination

All the decolourized dye media showed the presence of aromatic amines after the microaerophilic stage, with the exception of DB71. In this latter case the measurement could not be carried out, due to interference by the chemical structure of this dye with the methodology used (Table 1). The monoazos RY107 and RR198 showed amine concentrations of0.21 and 0.13 mM, respectively. The diazo RB5 showed the highest amine concentration (0.28 mM). After the aerobic stage a significant reduction in the amine concentrations was observed (Table 1). The mass balance of the azo dyes (%), with the exception of DB71, was estimated from the amine concentrations after the microaerophilic dye degradation, assuming that that 1 mole of RY107 and RR198 should produce 2 moles of amines and that 1 mole of RB5 should produce 3 moles of amines (Nam and Tratnyek, 2000). The monoazo RY107 and RR198 achieved a 58% and 100% of mass balance, respectively. The

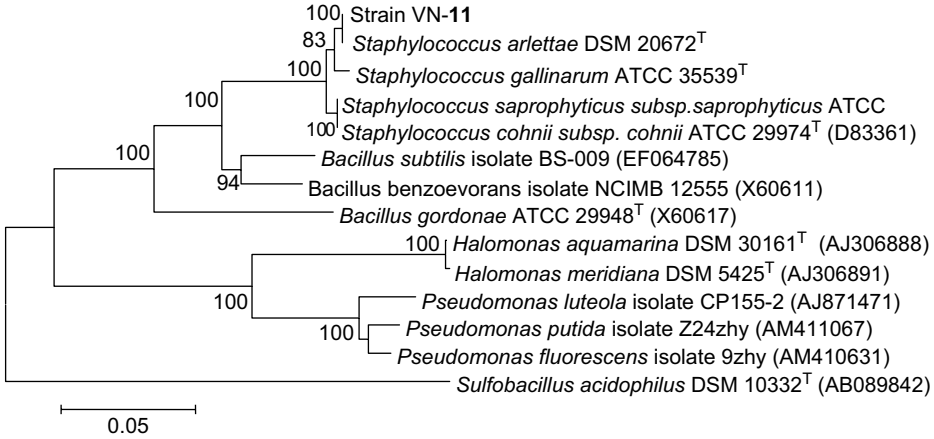


Fig. 2. Phylogenetic tree of *Staphylococcus arlettae* strain VN-11 for the partial sequences based on 16 rDNA.

Table 1
Amine concentrations (mM) ±SD, decolourization times (h) ±SD and decolourization (%) ±SD in solutions incubated with *Staphylococcus arlettae* under microaerophilic and aerobic conditions in the presence of azo dyes

Dyes	Amine concentration (mM)	Decolourization time (h)		Decolourization (%)	
	Microaerophilic	Aerobic	Microaerophilic	Microaerophilic	Aerobic
RY107	0.21 ± 0.02	0.01 ± 0.02	12 ± 2	98.5 ± 0.5	99.5 ± 0.36
RB5	0.28 ± 0.02	0.01 ± 0.03	24 ± 3	100 ± 0.11	98.9 ± 0.26
RR198	0.13 ± 0.05	0.04 ± 0.03	10 ± 2	97.7 ± 0.5	98 ± 0.2
DB71	n.d.	n.d.	48 ± 4	99 ± 0.2	96.3 ± 0.5

diazo RB5, which is known as one of the most recalcitrant dyes, achieved only a 40% of mass balance.

3.4. UV–vis characterization

The biodegradation of the four azo dyes was monitored by UV–vis analysis. For untreated dyes, as shown in Fig. 3a, RY107 presented two absorbance peaks at 301 and 550 nm. As shown in Fig. 3b, RR198 presented absorbance peaks at 513, 380, 290 and 220 nm and a shoulder at 325 nm. Fig. 3c shows that RB5 presented large peaks at 580 and 314 nm. Two additional peaks with low absorbance were observed at 220 and 390 nm. As shown in Fig. 3d, DB71 presented one large peak at 412 nm and a smaller peak at 300 nm. Wide band absorption near 220 nm was observed for all the dyes. For treated dyes, after biodegradation of the four azo dyes in the microaerophilic and aerobic treated solutions, the absorbance peaks in the visible region disappeared, indicating complete decolourization. In the UV spectra, the peaks at 285 and 320 nm disappeared and were replaced by new peaks at 245 and 260 nm (Fig. 3).

3.5. FT-IR characterization

The FT-IR spectra obtained from the untreated dye samples showed several peaks in the region where N–H and O–H stretching is normally observed (3300–3500 cm^{-1}). After the microaerophilic and aerobic treatments a significant reduction in absorption was observed in this region. Other bands located in the range from 1610–1630 cm^{-1} and at 1402 cm^{-1} disappeared in the microaerophilic stage after reductive treatment. Moreover, in the microaerophilic stage two new bands were observed in the carbonyl region at around 1680–1600 cm^{-1}, attributed to amine groups. These two bands disappeared in the aerobic stage and a new peak around 1680 cm^{-1} was observed. In the aerated samples a new broad region between 2400 and 2500 cm^{-1}, associated with carboxylic acid and NH_3^+ ions, and new peaks at 850,950 cm^{-1} and 1140 cm^{-1}, were observed (Fig. 4).

3.6. Toxicity test and TOC reduction

The results for *Daphnia magna* toxicity are presented as the percentage of death occurring in the solutions produced during the incubation of *Staphylococcus arlettae* under microaerophilic and aerobic conditions, as compared to the control composed of the dye solution and the culture medium without the bacterium. The tests were carried out in a 1:4 dilution of the original supernatant concentration, since 100% mortality occurred in the original and 1:2 supernatant concentrations. The controls showed equal mortality for all the dyes (40–47%). Under microaerophilic conditions, mortality decreased for all the dyes and when the samples were

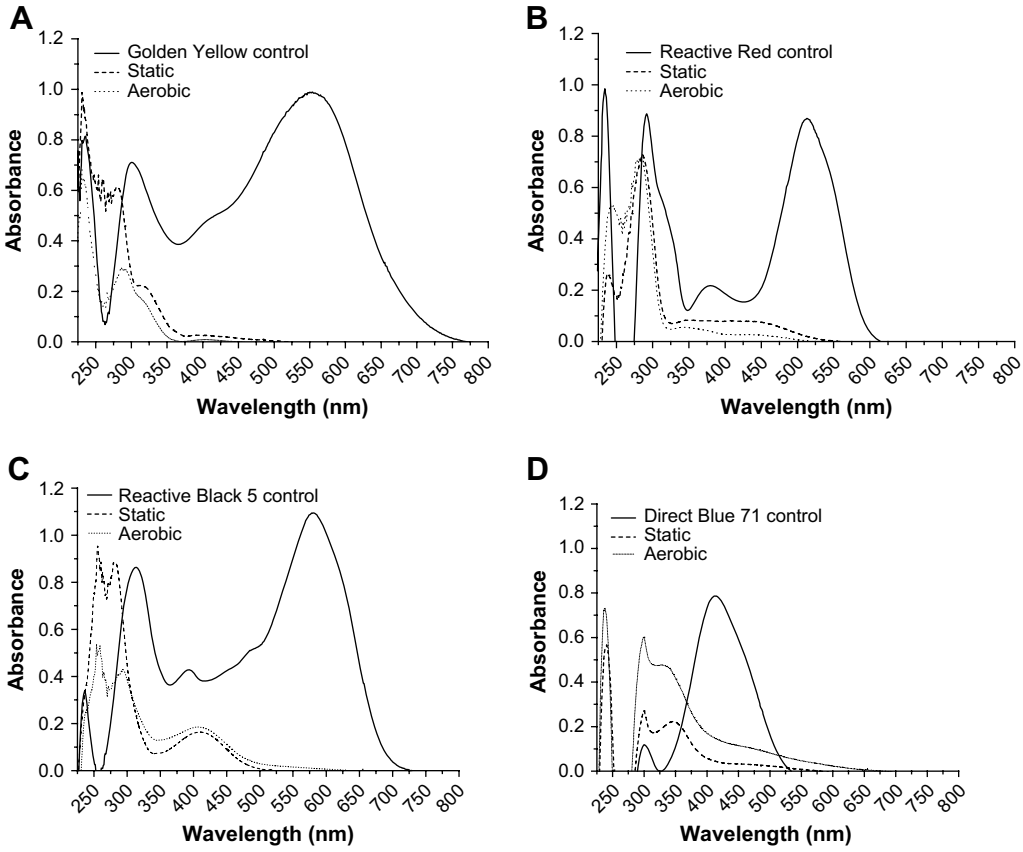


Fig. 3. UV–vis spectra of the azo dyes before (straight line) and after microaerophilic (dashed line) and aerobic (dotted line) treatments: (A) RY107, (B) RR198, (C) RB5, (D) DB71.

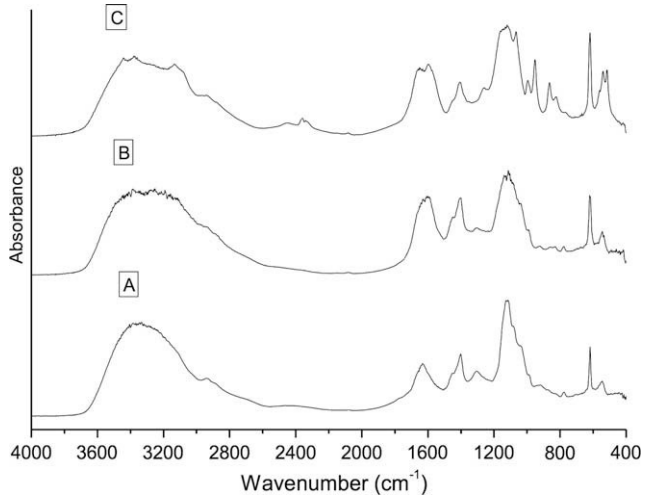


Fig. 4. FT-IR spectra of the azo dye RR198 before (A) and after microaerophilic (B) and aerobic (C) treatments.

aerated, no mortality was detected for any of the dyes. The reduction in TOC under microaerophilic conditions was only ~15%. Conversely, a significant increase in TOC reduction (up to 88%) was observed in the aerobic stage (Table 2).

4. Discussion

Staphylococcus arlettae strain VN-11 is a gram positive coccus, non-motile and facultative anaerobic bacterium. Although this bacterium has shown greater dye degradation ability as compared to other bacteria, there is no available literature on dye decolourization with *Staphylococcus arlettae*. Previous studies have shown that strains of *Staphylococcus* sp., isolated from soil in a textile-effluent treatment plant, were able to decolourize the sulfonate azo dye Congo Red (Park et al., 2005). Chen et al. (2005) isolated the gene encoding NADPH-flavin azoreductase (Azo1) from the human skin bacterium *Staphylococcus aureus* ATCC 25923, which confirmed that the enzyme responsible for dye decolourization could be an inducible flavoprotein using both NADH and NADPH as electron donors, as previously reported for other bacterial strains (Moutaouakkil et al., 2003). Azoreductase is the key enzyme responsible for the reductive azo dye degradation in bacterial species. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH, thus impeding electron transfer from NADH to the azo bonds (Chang and Lin, 2001). The advantage of the anaerobic reduction of azo dyes is that oxygen depletion is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerophilic bacteria to reduce azo dyes. The reaction takes place at neutral pH values and is extremely unspecific (Stolz, 2001). However, the precise mechanism of anaerobic azoreduction is still not totally understood. It was recently suggested that microbial anaerobic azoreduction was linked to the electron transport chain, and that dissimilatory azoreduction was a form of microbial anaerobic respiration (Hong et al., 2007). In addition, different models for the nonspecific reduction of azo dyes by bacteria, which do not require transport of the azo dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested (Maier et al., 2004). These results suggested that azo dye reduction was a strain-specific mechanism that could be performed by an azoreductase enzyme or by a more complex metabolic pathway. Thus, due to the lack of information about the metabolism of *Staphylococcus arlettae* the usual true time dependant kinetic determinations of the azoreductase activity using the azo dye as substrate were not performed, and the azo reduction mechanism in *Staphylococcus arlettae* will be the subject of a future specific study.

Table 2
Mortality for *Daphnia magna* exposed to a 1:4 dilution of the supernatant containing azo dyes and incubated with *Staphylococcus arlettae*, and the % TOC removal under static and aerobic conditions

Dyes	Mortality (%)[a]		TOC reduction (%)[b]		
	Control	Microaerophilic	Aerobic	Microaerophilic	Aerobic
RY107	40	20	0	12	65
RB5	40	30	0	17	62
RR198	47	27	0	7	51
DB71	47	30	0	16	88

[a] SD ± 11% for all the data.
[b] SD ± 2% for all the data.

In the present work, the strain *Staphylococcus arlettae* was tested to separately decolourize four azo dyes (RY107, RR198, RB5 and DB71) in a sequential microaerophilic/aerated process in the presence of yeast extract as the source of the electron donors NAD and NADH. In the absence of yeast extract, a partial decolourization (<50%) was achieved after 168 h for RY107 and RR198, but there was no decolourization for any of the dyes when the yeast extract and glucose were substituted by sodium pyruvate (data not shown). It is known that the decolourization rate of azo dyes is increased by using redox mediators such as the water-soluble flavins ($FADH_2$, $FMNH_2$), NADH or NADPH, which speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk electron donors to the electron-accepting azo dyes (Dos Santos et al., 2004; Chang et al., 2001a; Chung and Stevens, 1993; Gingell and Walker, 1971). Thus *Staphylococcus arlettae* indicated the obligatory requirement of yeast extract as a redox mediator to attain efficient dye decolourization. Yeast extract, a powder supplement consisting of protein, free amino nitrogen, B vitamins, minerals, nucleotides and other yeast cell components, has been the most commonly used nitrogen source for dye decolourization processes (Robert et al., 1998). Many pure cultures like *Pseudomonas luteola*, *Klebsiella pneumoniae* and *Aeromonas hydrophila* have exhibited effective decolourization of different dyes in the presence of yeast extract (Hu, 1998; Chang et al., 2001b; Wong and Yuen, 1996; Chen et al., 2003; Khehra et al., 2005). Moreover, a recent study showed that a combination of the variables including glucose and yeast extract resulted in more than 90% decolourization of the azo dye Direct Black 22 (Mohana et al., 2008).

The chemical structures of the dyes greatly influence their decolourization rates, and the decolourization efficiency is limited to several azo dye structures (Chivukula and Renganathan, 1995). Dyes with simple structures and low molecular weights usually exhibit higher rates of colour removal, whereas colour removal is more difficult with highly substituted, high molecular weight dyes (Chen et al., 2003; Pearce et al., 2003). For this reason, RY107 and RR198, which are both monoazo, showed a short decolourization time (12 and 10 h, respectively) and the highly substituted diazo RB5 and the triazo DB71 showed longer decolourization times (24 and 48 h, respectively) (Table1). It has been reported that the turnover rate of monoazo dyes increased with increasing dye concentration, whereas the turnover rate of the diazo and triazo dyes remained constant as the dye concentration increased (Hu, 2001). Moreover, the azo compounds with a hydroxyl or amino groups were more likely to be degraded than those with methyl, methoxy, sulfo or nitro groups (Nigam et al., 1996). Usually, the presence of sulfonates in reactive dye structures results in low levels of colour removal. However, this is not applicable to direct dyes (DB71) that usually exhibit high levels of colour removal independent of the number of sulfonate groups in the dye

structure, reinforcing the idea that steric hindrance and the number of azo bonds are responsible for the different decolourization times (Hitz et al., 1978). It has also been reported that a correlation between the enzyme redox potential and its activity towards the substrates could influence the decolourization rate (Call and Mucke, 1997; Xu et al., 1996). In this context, the decolourization times obtained in the present work were in agreement with those of Zille et al. (2004), who found a linear relationship between the cathodic peak potentials and the time of maximum decolourization for several azo dyes using an ascomycete yeast *Issatchenkia occidentalis*. Thus the ability of the bio-agents to degrade azo dyes depends on the structural characteristics of the dye, the temperature and the pH of the medium, the presence of intermediates and the difference between the redox potentials of the biocatalyst and the dye. Further studies will be carried out to measure the redox potential of the dyes by cyclic voltametry in order to verify this correlation.

The biodegradation of the azo dyes was also monitored by UV–vis and FT-IR analyses (Figs. 3 and 4). Although the presence of the typical absorption peak of the hydrogenated azo bond structure (Ar—NH—NH—Ar′) at 245 nm in the spectra seems to indicate only partial azo bond disruption after biodegradation of the four azo dyes in the microaerophilic and aerobic treated solutions, the absorbance peaks in the visible region disappeared indicating complete decolourization (Qing, 1989). The presence of high concentrations of aromatic amines in the microaerophilic stage confirmed this statement (Table 1). In the UV spectra, the decrease in absorbance of the peaks at 220 and 320 nm corresponding, respectively, to the benzene and naphthalene rings (Yang, 1987; Mielgo et al., 2001), and the formation of a new peak at 260 nm, suggested that the reductive destruction of the azo conjugated structure disclosed the narrow multi-peaks of aromatic rings in the spectra (Feng et al., 2000). In the FT-IR analysis, interference by the yeast extract added to the medium restricted data interpretation, showing very similar spectra. However, some conclusions were attained, and the dye (RR198), used as a model substrate, is shown in Fig. 4. The bands located within the range 1610–1630 cm^{-1} and at 1402 cm^{-1} were due to azo linkages –N═N– on aromatic structures and of –N═N– stretching in α-substituted compounds, respectively (Parikh, 1974). These peaks decreased during the treatment and in some cases disappeared completely in the spectrum of the microaerophilic and aerobic treated dyes, confirming the previous UV–vis results about azo linkage disruption (Coates, 2000). In the microaerophilic stage, the reduction of the azo linkage peak was followed by the formation of two bands in the carbonyl region at around 1680–1600 cm^{-1}. Two bands in this region were consistent with an amide derived from ammonia or a primary amine. In the aerobic stage these two bands disappeared and a new peak around 1680 cm^{-1} was observed. The presence of this additional group, due to the conjugation of C═C and C═O groups, suggested that this peak could belong to a carbonyl group in a carboxylic acid, ketone, ester or conjugated aldehyde group attached to an aromatic ring (Gavril and Hodson, 2007). The fact that no new peaks appeared between 3300–3500 cm^{-1} (attributed to azo bonds and OH groups in position α relative to the azo linkage) and in the region between 1340 and 1250 cm^{-1} ($-NH_2$) after the aerobic treatment, suggested that the azo linkage could be transformed into N_2 or NH_3 or incorporated into the biomass (Spadaro and Renganathan, 1994; Goszczynski et al., 1994; Shaw and Freeman, 2004). Moreover, the presence of a new broad region between 2400 and 2500 cm^{-1} in the aerobically treated samples, could indicate the presence of carboxylic acid and NH_3^+ ions (symmetric stretching mode), suggesting partial mineralization. Also the presence of new peaks at 850 and 950 cm^{-1} (associated with the out-of-plane bending vibration of substituted benzenes) and the peak at 1140 cm^{-1} that could belong to acetate, formates, propionates, benzoates, etc., suggested that the products were undergoing irreversible chemical changes, probably due to concomitant biodegradation and autoxidation reactions of the products formed during the reductive dye degradation (Skoog et al., 1998). A large fraction of the aromatic amines from azo dyes are susceptible to autoxidation, producing water-soluble, highly coloured dimers, oligomers and eventually dark-coloured polymers with low solubility (Kudlich et al., 1999). Remarkably, in contrast to the expectation that bio-recalcitrant aromatic amines would tend to autoxidise forming coloured products, in the present experiment no increase in colour in the visible region was observed in the aerobic stage, suggesting that the aromatic amines were effectively biodegraded. However, although in some cases biodegradation of the dye's cleavage products was demonstrated (Coughlin et al., 2003), it is difficult to predict the fate of the aromatic amines during the anaerobic/aerobic treatment of azo dyes, because it is not clear whether their removal was due to biodegradation, adsorption or chemical reactions (Van Der Zee and Villaverde, 2005). The toxicity results for the controls (40–47%), shown in Table 2, are in agreement with the findings reported by Hunger and Jung (1991) that the reactive dyes and hydrolyzed reactive dyes had a low toxic potential towards aquatic organisms as compared to the basic, acid and dispersed dye.

The *Daphnia magna* toxicity test demonstrated that the degradation under microaerophilic conditions was not sufficient to remove the toxicity of the samples (~30%). Therefore, oxidation of the aromatic amines, as confirmed by the absence of amines in the aerobic stage (Table 1) was necessary to diminish the toxicity in the medium. As shown in Table 2, when the medium was incubated under microaerophilic conditions, the reduction in TOC was only ~15%, even after 7 days of incubation. Conversely, a significant increase in TOC reduction (~70%) was observed in the aerobic stage. It was concluded that even if the microorganisms were able to decolourize the dyes under microaerophilic conditions, the aerobic microorganisms needed aeration not only for amine removal but also for TOC stabilization (Sponza and Isik, 2005).

5. Conclusions

The strain VN-11 isolated from the dye effluent was identified by 16S rDNA as *Staphylococcus arlettae*. All the dyes tested were totally and rapidly decolourized under microaerophilic conditions, with some differences in decolourization times depending on the dye structure, as confirmed by the UV–vis analysis. Decolourization was strongly dependent on the presence of yeast extract in the medium, indicating the need for additional vitamin and nitrogen sources. The formation of amines in the microaerophilic stage and their disappearance in the aerobic stage was confirmed by the direct measurement and by FT-IR analysis. In the aerobic stage the partial mineralization of the dye degradation products and of the medium metabolites, was confirmed by the FT-IR, toxicity and TOC measurements. Moreover, high decolourization efficiency was attained in the presence of only slight growth of the bacterium, which would result in very low amounts of sludge formation, thus avoiding high disposal costs. This methodology using a single microorganism in a sequential microaerophilic/aerobic process was shown to be very effective in azo dye decolourization. In a single reactor with a single bacterium, only changing the agitation conditions, it was possible not only to decolourize the dyes, but also to achieve a good degree of mineralization and low toxicity, with low running and maintenance costs.

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