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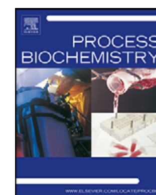

Microaerophilic–aerobic sequential decolourization/biodegradation of textile azo dyes by a facultative *Klebsiella* sp. strain VN-31

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ABSTRACT

Four different azo dyes were decolourized and biodegraded in a sequential microaerophilic–aerobic treatment by a facultative *Klebsiella* sp. strain VN-31, a bacterium isolated from activated sludge process of the textile industry. Dye decolourization was performed under microaerophilic conditions until no colour was observed (decolourization percentage >94%). The medium was then aerated to promote the biodegradation of the amines produced. The presence of aromatic amine in the microaerophilic stage and its absence in the aerobic stage demonstrate azo bond reduction and an oxidative biodegradation process, respectively. Total Organic Carbon (TOC) reduction for the growth medium plus dyes was ~50% in the microaerophilic stage and ~80% in the aerobic stage. The degradation products were also characterized by FT-IR and UV–vis techniques and their toxicity measured using *Daphnia magna*. The results provide evidence that the successive microaerophilic/aerobic stages, using a single *Klebsiella* sp. strain VN-31 in the same bioreactor, were able to form aromatic amines by the reductive break down of the azo bond and to oxidize them into non-toxic metabolites.

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1. Introduction

Pollution problems due to textile industry effluents have increased in recent years. From the available literature it can be estimated that approximately 75% of the dyes discharged by textile-processing industries belong to the classes of reactive (~36%), acid (~25%) and direct (~15%) dyes [1]. In these classes, the azo dyes (aromatic moieties linked together by azo (–N=N–) chromophores) are the most important chemical class of synthetic dyes and pigments, representing between 60% and 80% of the organic dyes used in industries such as the textile, leather, plastic, cosmetic and food industries [2]. Recent studies have shown that azo dyes contribute to the mutagenic activity of ground and surface waters polluted by textile effluents [3]. Furthermore, their discharge into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into bodies of water, hence affecting aquatic life [4]. Moreover, it is very difficult to treat textile industry effluents because of their high BOD, COD, heat, colour, pH and the presence of metal ions [5]. In recent years, new processes for dye degradation and wastewater reutilization have been developed [6]. In particular, systems based on biological processes using a large variety of bacterial strains, allow for degradation and mineralization with a low environmental impact and without the use of potentially toxic chemical substances, under mild pH and temperature conditions [7–10]. Amongst these systems, several facultative anaerobic bacterial strains including *Sphingomonas* sp., *Pseudomonas luteola*, *Streptococcus faecales* and *Klebisiella pneumonae* have been described as being capable of reducing azo dyes [11–14].

Reductive azo dye decolourization by microorganisms usually starts with the cleavage reduction of the azo bond under anaerobic or microaerophilic conditions, and leads to the accumulation of toxic aromatic amines [4]. To overcome this problem, recent studies included combinations of anaerobic and aerobic steps in an attempt to achieve not only dye decolourization but also degradation of the aromatic amines [15–17]. However, very few studies have been performed using sequential microaerophilic/aerobic conditions with the same microorganism, preferring the use of consortia or different microorganisms, used separately under anaerobic, microaerophilic and aerobic conditions [18,19].

In this study, degradation of four azo dyes was carried out under microaerophilic conditions (O_2 limited environments) until no colour was observed using a facultative *Klebsiella* sp. strain VN-31. The medium was then aerated by stirring to promote oxidation of the aromatic amines formed by reductive break down of the azo

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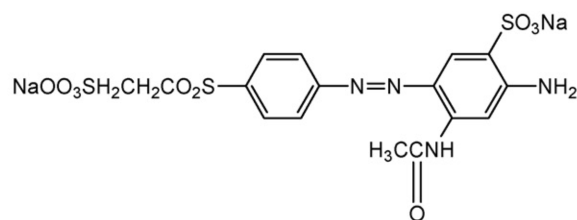

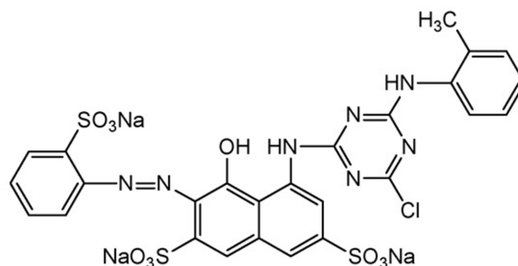

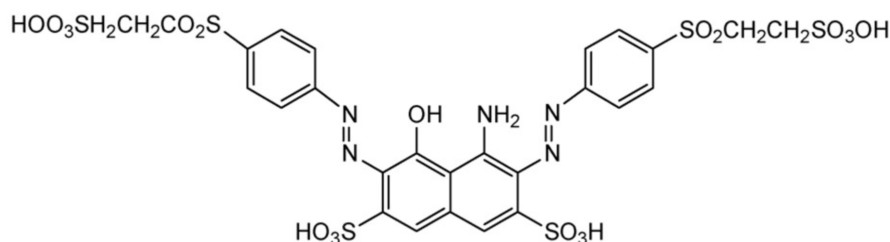

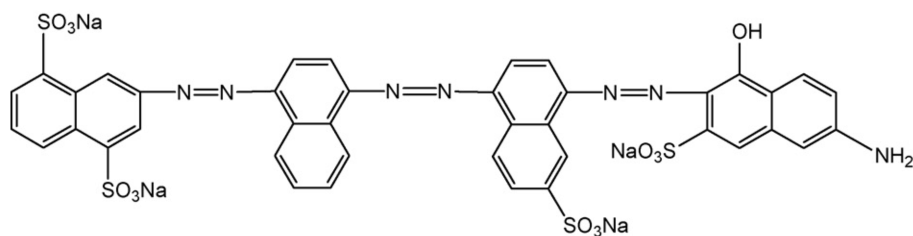

Fig. 1. Chemical dye structures.

bond, into non-toxic metabolites. The degradation products were characterized by FT-IR and UV–vis techniques and their toxicity and Total Organic Carbon (TOC) measured. Thus, the main achievement of this work was to prove that the degradation of azo dyes in a successive microaerophilic/aerobic process using, exclusively, a facultative anaerobic *Klebsiella* sp. bacterium isolated from textile dye effluents 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.

2. Material and methods

2.1. Chemicals and medium

The azo dyes Reactive Yellow 107 (RY107), Reactive Black 5 (RB5), Reactive Red 198 (RR198) and Direct Blue 71 (DB71) were kindly provided by the textile company Vicunha, 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), KH_2PO_4 (0.2 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $FeSO_4{\cdot}7H_2O$ (0.01 g/L), NaCl (0.1 g/L) and $CaCl_2{\cdot}2H_2O$ (0.02 g/L). The medium was supplemented with 100 mg/L of dye, 3 g/L of glucose and 1 g/L of sodium pyruvate and was described as mineral medium rich (MMR).

2.2. Strain isolation and characterization

The microorganisms were isolated from the activated sludge produced by the Vicunha textile company, Itatiba, Brazil. Serial dilutions (10^{-1} to 10^{-6}) of the samples collected were inoculated into Nutrient Agar Medium by the spread plate technique. Isolated strains were inoculated into MMR with the azo dyes (100 mg L^{-1}/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 rRNA gene sequence analysis. Genomic DNA was obtained using guanidium thyocianate method according to Pitcher et al. [20]. Cultures were harvested at the end of the exponential growth phase by centrifugation at 18.600 × *g* for 3 min. Cells were resuspended in 100 μL of fresh lysozyme (50 mg/mL) in TE buffer (10 mmol Tris–HC1; 1 mmol/L EDTA, pH 8) and were incubated at 37 °C for 30 min. Cells were lysed with 0.5 mL of guanidium thyocianate (5 mol/L guanidium thiocyanate (Sigma), 100 mmol/L EDTA and 0.5%, v/v, sarkosyl) and vortexed briefly. The lysates were cooled on ice, 0.25 mL cold 7.5 mol/L ammonium acetate added with mixing, held on ice for a further 10 min and then 0.5 mL chloroform and isoamilic alcohol (24:1) mixture added. The phases were mixed thoroughly, transferred to a 1.5 mL Eppendorf tube and centrifuged (18.600 × *g*) for 10 min. Supernatant fluids were transferred to Eppendorf tubes and 0.54 volumes of cold 2-propanol added. The tubes were inverted for 1 min to mix the solutions and the fibrous DNA precipitate was deposited by centrifugation at 10.000 × *g* for 20 s. Pellets of DNA were washed in 70% ethanol and dried under vacuum heated at 65 °C with mixing until dissolved. DNA samples were redissolved overnight at 4 °C in a 50 μL of sterile, deionized water.

The 16S rRNA gene was amplified by PCR using the specific primers, 27f and 1401r for the universal Bacteria Domain. Fifty microliter reaction mixtures were used contained 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 carried out using an initial denaturation step of 2 min at 94 °C, followed by 10 cycles of 1 min 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 at 94 °C, 30 s at 63 °C and 3 min at 72 °C, in an Eppendorf thermal cycler (Eppendorf Mastercycler Gradient). The PCR product was purified on GFX™ PCR DNA Kit 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 the isolates were assembled in a contig using the phred/Phrap/CONSED program [21].

Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data obtained from reference and type strains available in the public databases GenBank and RDP (Ribosomal Database Project II Release 9, Michigan State University, USA) using the BLASTn and Seqmatch, respectively. The sequences were aligned using the CLUSTAL X program and analyzed with MEGA software [22,23]. Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA using Kimura's DNA substitution model [24]. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software [25]. The 16S rRNA partial sequence determined in this study were deposited at the Genbank database under the accession number FJ468444.

2.3. Aromatic amines detection

The aromatic amines in the solid phase were determined by the modified method of Marik et al. [26]. Samples were taken after incubation under 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 at 100 °C for 5 min. The absorption was measured in a Hexios α Unicam UV–vis spectrophotometer at 560 nm. A calibration curve was prepared using aniline-2-sulfonic acid as a model product of azo dye reduction, and the sample amine concentration was calculated in mM. The value of the control was subtracted from that of the biodegraded samples. The use of a single aromatic amine as model substrate introduces a very low error because the chloranil reaction is very specific to primary aromatic amines. The colour intensity could be slightly affected by the position of amino group due to steric hindrance. However, the presences of others ring substituents interfere weakly with the colorimetric reaction between the primary aromatic amine and the chloranil. Moreover, secondary and tertiary aromatic amines, as well as pyridine and pyrimidine moieties, all tested negative under these conditions [26].

2.4. Dye decolourization

Decolourization assays under microaerophilic conditions were performed in cultures containing 350 mL of MMR (pH 7) supplemented with 100 mg/L^{-1} of dyes. The TOC of this medium was around 2000 mg/L (dyes TOC ∼ 60 mg/L). Samples were incubated under microaerophilic conditions at 30 °C for 168 h or until no colour was observed. Microaerophilic conditions were achieved by placing culture flasks in sealed jars containing microaerobac gas generators envelopes (Probac-Brazil), reducing the oxygen level to 15–5% and generating an enriched carbon dioxide environment within the incubator jars after the system was properly activated according to the manufacture's instructions.

The culture was then aerated by stirring without any further supplementation of the medium. Dye decolourization was measured in a UV–vis spectrophotometer (Shimadzu 2101) for the microaerophilic and aerobic stages and the percentage of effluent decolourization calculated.

2.5. UV–vis analysis

The dye degradation products produced during biodegradation after incubation 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 plus KBr to a final weight of 0.4 g). The samples were then ground, desorbed at 60 °C for 24 h and compression molded in a uniaxial hydraulic press under a load of 0.9 MPa to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using a FT-IR Spectrum 2000 PerkinElmer spectrometer with a resolution of 4 cm^{-1} and averaged over 32 scans. 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, the experimental sample then scanned. The FT-IR spectrum of the control was finally subtracted from the spectra of the dye and dye degraded samples.

2.7. TOC measurement

The existence of organic carbon in the dye containing samples was monitored by measuring the TOC under microaerophilic conditions and after agitation using a TOC analyzer (Shimadzu 5000A) with direct injection of the samples after centrifugation (20.000 × *g* for 15 min) and filtration through a 0.45 μm pore size.

2.8. Toxicity test

The samples taken after treatment with *Klebsiella* sp. strain VN-31 were centrifuged at 20.000 × *g* for 20 min and filtered through a 0.45 μm pore size filter. Acute toxicity tests with D*aphnia magna* (Crustacea, Cladocera) were carried out according to the ABNT norms (Associação Brasileira de Normas Técnicas NBR 12713) [27]. The sensitivity tests were carried out with neonates (6–24 h of life). For each concentration (1%, 25%, 50%, 75%, 100%), 5 organisms were used in 5 mL flasks. The tests and the control in distilled 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 numbers of immobile organisms were counted after 20 s of light exposure.

3. Results

3.1. Strain isolation and identification

The phylogenetic tree of the partial sequences based on the 16S rRNA gene of the *Klebsiella* sp. strain VN-31 was constructed by the neighbor-joining method on the program Mega 2.0. The bootstrap and values higher than 70% were indicated on the tree (Fig. 2). The evolutive distance was based on the Kimura 2p model [24]. The numbers of the GenBank access are in parenthesis. *Sulfobacillus acidophilus* DSM 10332^{T} was used as the outgroup. The nucleotide alignment of strain VN-31 supported values of the boot strap of 99% similarity to *Klebsiella pneumoniae* subsp *pneumoniae* and other *Klebsiella* sp. The phylogenetic tree showed the grouping of VN-31 within the *Klebsiella* sp., biochemical tests being required to confirm the subspecies.

3.2. Decolourization

The strain *Klebsiella* sp. strain VN-31 was tested to separately decolourize four azo dyes (Reactive Yellow 107, Reactive Red 198, Reactive Black 5 and Direct Blue 71) in a microaerophilic/aerated sequential process. Complete decolourization (>94%; Table 1) of the azo dyes was achieved in the microaerophilic stage and no significant colour changes were detected in the following aerobic stage. *Klebsiella* sp. strain VN-31 could only decolourize the dyes effectively when the medium was supplemented with glucose and pyruvate. In the absence of glucose and pyruvate, the culture was unable to grow and decolourize, thus indicating an obligate requirement for a supplementary carbon source for growth and

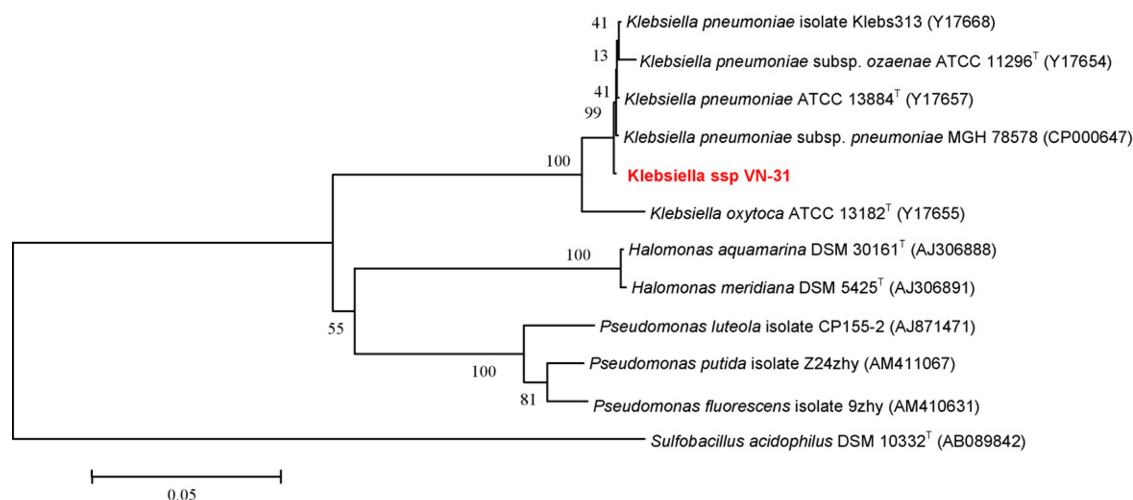

Fig. 2. Phylogenetic tree of the *Klebsiella* sp. strain VN-31 for the partial sequences based on 16S rDNA.

Table 1
Amine concentrations (mM) ± SD, decolourization times (h) ± SD, and decolourization (%) ± SD in solutions incubated with *Klebsiella* sp 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	0.16 ± 0.04	0.01 ± 0.02	72 ± 4	100 ± 0.1	92.8 ± 0.5
RB5	0.24 ± 0.02	0.01 ± 0.03	120 ± 8	94 ± 0.6	92.8 ± 0.3
RR198	0.1 ± 0.03	0.02 ± 0.02	96 ± 5	98 ± 0.5	100 ± 0.1
DB71	n.d.	n.d.	168 ± 12	94 ± 0.4	96.6 ± 0.4

n.d., Not detected.

dye decolourization (data not shown). The decolourization time showed a relationship with the chemical structure of the dyes. The monoazo dyes RY107 and RR198 were decolourized in 72 and 96 h, respectively. The diazo RB5 and triazo DB71 were decolourized after 120 and 168 h, respectively (Table 1).

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, for which the measurement could not be made due to interference by the chemical structure of this dye with the methodology used (Table 1). The concentrations of aromatic amines determined were in accordance with the number of azo bonds in the chemical structure of the dye. The monoazo dyes RY107 and RR198 showed amine concentrations of 0.16 and 0.1 mM, respectively, and the diazo RB5 showed the highest amine concentration (0.24 mM). After the aerobic stage a significant reduction in the amine concentration was observed (Table 1).

3.4. UV–vis characterization

The biodegradation of the four azo dyes was monitored by UV–vis analysis. *Untreated dyes*: Fig. 3(A) shows that RY107 presented two absorbance peaks at 285 and 410 nm. Fig. 3(B) shows that RR198 presented absorbance peaks at 510, 380 and 285 nm and a shoulder at 320 nm. Fig. 3(C) shows that RB5 presented intense peaks at 570 and 320 nm. Two additional peaks with low absorbance were observed at 440 and 390 nm. Fig. 3(D) shows that DB71 presented an intense peak at 575 and three shoulders at 290, 300 and 320 nm. Wide band absorption near 250 nm was observed for all the dyes. *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 their complete decolourization. In the UV spectra, the peaks at 285 and 320 nm disappeared following by the formation of a new peak at 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 within the region 1610–1630 cm^{-1} and at 1402 cm^{-1} disappeared during the microaerophilic stage after the reductive treatment. Moreover, during the microaerophilic stage, two new bands appeared in the carbonyl region at around 1680–1600 cm^{-1}, attributed to the formation of amine groups. These two bands disappeared during the aerobic stage and a new peak around 1680 cm^{-1} was observed. In the aerated samples a new broad region was observed between 2300 and 2500 cm^{-1}, associated with carboxylic acids and NH_3^+ ions, and also new peaks at 850, 950 cm^{-1} and 1140 cm^{-1}.

3.6. Toxicity test and TOC reduction

The results for *D. magna* toxicity are presented as the percentage of death occurred during the incubation of *Klebsiella* sp. strain VN-31 under microaerophilic and aerobic conditions, as compared to a control composed of the dye solution and the culture medium without the bacteria. The tests were carried out in a 1:4 dilution of the original supernatant concentration, since 100% of mortality occurred in the original and 1:2 supernatant concentrations. The controls showed equal mortality for all the dyes (47%) except for DB71, which presented 53% of mortality.

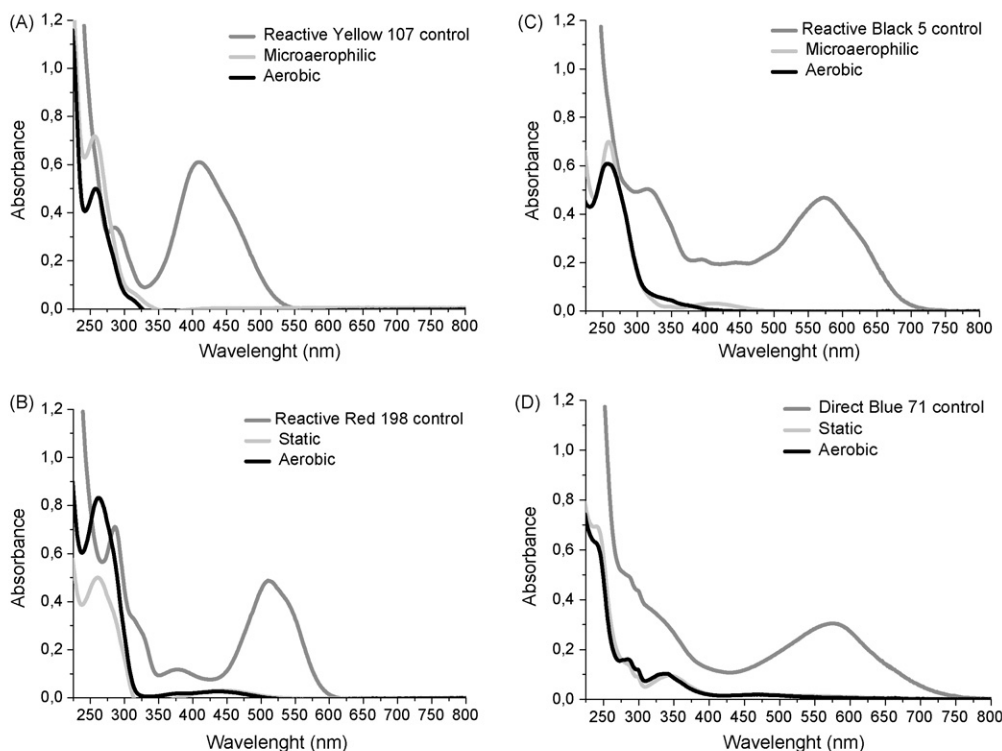

Fig. 3. UV–vis spectra of the azo dyes before (straight line) and after microaerophilic (dashed line) and aerobic (dotted line) treatments: (A) RY107; (B) RR198; (C) RB5; (D) DB71.

Under microaerophilic conditions, mortality decreased for all the dyes except for the DB71 dye, which showed an increase in the percentage mortality (60%). When the samples were aerated, no mortality was detected for any of the dyes except for that containing the triazine RR198, which maintained 10% of mortality. The TOC reduction (Table 2) are explained as the percentage of Total Organic Carbon occurred in the medium (MMR) including glucose, pyruvate and dyes. After 7 days the reduction in TOC under microaerophilic conditions was only ~50%. However, after shaking (aerobic condition), a significant increase in TOC reduction (~80%) was observed.

4. Discussion

The *Klebsiella* sp. strain VN-31 is a gram negative, facultative anaerobic bacterium of the family Enterobacteriaceae. Even though is commonly found in the normal flora intestinal, there are numerous reports about the presence this strain in contaminated soil and wastewaters indicating its ability to metabolize toxic compounds [14,28].

Although this bacterium has shown considerable dye degradation ability as compared to other bacteria, there is little literature regarding dye decolourization using *Klebsiella* sp. Previous studies have shown that strains of *Klebsiella oxytoca* isolated from cyanide-containing wastewater were able to use nitriles as the sole source of nitrogen [28]. Wong et al. isolated five bacteria from dye-contaminated sludge and found that two bacteria, identified as *Klebsiella* ssp. and *K. pneumonae*, showed decolourization ability with respect to the Methyl Red dye [14].

Azoreductase is the key enzyme responsible for reductive azo dye degradation in bacterial species. Azoreductases isolated from several bacteria have been shown to be inducible flavoproteins and to use both NADH and NADPH as electron donors [29]. 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. The advantage of the anaerobic reduction of azo dyes is that the depletion of oxygen is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerobic bacteria to reduce azo dyes. The reaction takes place at neutral pH values and is extremely unspecific [30]. However, the precise mechanism of anaerobic azo-reduction is not yet totally understood. A different model was recently suggested for the non-specific reduction of azo dyes by bacteria, which does not require transport of the azo dyes or reduced flavins through the cell membrane [12]. Earlier studies provided evidence that microbial anaerobic azo-reduction was linked to the electron transport chain, and suggested that dissimilatory azo-reduction was a form of microbial anaerobic respiration [31]. In addition, different models for the non-specific reduction of azo dyes by bacteria, which did not require transport of the azo dyes or reduced flavins through the cell membrane and that described the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested [32]. 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 scarcity of information on the metabolism of *Klebisella* sp., the usual true time dependant kinetic studies of azoreductase activity using the azo dye as substrate were not

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

Dyes	Mortality (%)[a]			TOC reduction (%)[b]	
	Control	Microaerophilic	Aerobic	Microaerophilic	Aerobic
RY107	47	33	0	56	78
RB5	47	40	0	46	74
RR198	47	27	10	54	64
DB71	53	60	0	51	87

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

performed, and the azo reduction mechanism in *Klebsiella* sp. strain VN-31 will be the subject of a further specific study.

In the present work the strain of *Klebsiella* sp. strain VN-31 was tested to separately decolourize four azo dyes (RY107, RR198, RB5 and DB71) in a sequential microaerophilic/aerated process. RY107 and RR198 are both monoazo dyes and showed relatively short decolourization times (72 and 96 h respectively). The increase in degradation time (24 h) for RR198 was probably due to the triazine group, whose degradation is more recalcitrant than that of the benzene and naphthalene rings. The chemical structures of the dyes greatly influence their decolourization rates and the decolourization efficiency was limited to several azo dye structures [33].

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 [34]. For this reason, the highly substituted diazo RB5 and the triazo DB71 showed longer decolourization times (120 and 168 h respectively). It has been reported that the azo compounds with hydroxyl or amino groups are more likely to be degraded than those with methyl, methoxy, sulfo or nitro groups [35]. 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 [36]. It has also been reported that a correlation between the enzyme redox potential and its activity towards the substrates could influence their decolourization rates [37]. In this context, the present decolourization times are in agreement with those of Zille et al., who found a linear relationship between the cathodic peak potentials and the time of maximum decolourization for several azo dyes using the ascomycete yeast *Issatchenkia occidentalis* [38]. Thus, the ability of the bio-agents to degrade azo-dyes depends on the structural characteristics of the dye, the temperature and pH of the treatment, 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 potentials of the dyes by cyclic voltammetry in order to verify this correlation.

Biodegradation of the azo dyes was also monitored by UV–vis (Fig. 3) and FT-IR analyses. 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. Moreover, the absence of the typical absorption peak of the hydrogenated azo bond structure (Ar···NH···NH···Ar′) at 245 nm in all the dyes indicated complete disruption of the azo bonds [39]. 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 285 and 320 nm, related to the benzene and naphthalene rings, respectively, and the formation of a new peak at 260 nm, suggested that the reductive destruction of the conjugated azo structure uncovered the fine multi-peaks of aromatic rings in the spectra [39]. In the FT-IR analysis, 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 [40]. These peaks diminished during the treatment and in some cases disappeared completely from the spectrum of the microaerophilic and aerobic treated dyes, confirming the previous UV–vis results about disruption of the azo linkage. In the microaerophilic stage, the reduction in 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. During 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 the peak at 1680 cm^{-1} could belong to a carbonyl group in a carboxylic acid, ketone, ester or conjugated aldehyde group attached to an aromatic ring [40]. The fact that no new peaks appeared between 3300 and 3500 cm^{-1} (attributed to azo bonds and an OH group in the α-position 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 have been transformed into N_2 or NH_3 or incorporated into the biomass [41]. Moreover, the presence of a new broad region between 2300 and 2500 cm^{-1} in the aerobically treated samples, could indicate the presence of carboxylic acid and NH_3^+ ions (symmetric stretching mode), suggesting a 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 acetates, formates, propionates, benzoates, 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 [41]. 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 [42]. Remarkably, contrary to expectations that biorecalcitrant aromatic amines would tend to autoxidize, forming coloured products, in the present experiment, no increase in colour was observed during the aerobic stage, suggesting that the aromatic amines were effectively biodegraded. However, although in some cases biodegradation of the dye cleavage products was demonstrated [43], it is difficult to predict the fate of aromatic amines during the anaerobic–aerobic treatment of azo dyes, because it is not clear whether their removal is due to biodegradation, adsorption or chemical reactions [17].

The toxicity results shown in Table 2 are in agreement with the findings reported by Hunger and Jung that the reactive dyes and hydrolyzed reactive dyes had a low toxic potential in aquatic organisms as compared to basic, acid and disperse dyes [44]. The increase in the mortality percentage of the DB71 dye under microaerophilic conditions could be attributed to the triazo bonds binding four aromatic rings, thus generating more toxic amines than the other dyes [45]. Therefore oxidation of the aromatic amines, as confirmed by the absence of amine in the aerobic stage (Table 1), was necessary to diminish the toxicity of the medium. The 10% of mortality for the triazine containing RR198 in the aerated samples could be attributed to the triazine reactive group that persisted in the aerobic treated effluent due to its slower reaction rates [46]. In addition the effectiveness of the microaerophilic–aerobic process by a facultative *Klebsiella* sp. strain VN-31 was evaluated by the biodegradation of the Total Organic Carbon, as a complementary indicator of the treatment efficiencies.

As shown in Table 2, when the medium was incubated under microaerophilic conditions, the TOC reduction was only ~50% even after 7 days of incubation. Conversely, a significant increase in TOC reduction (~80%) was observed during the aerobic stage. It was concluded that even if the microorganisms were able to decolourize the dye under microaerophilic conditions, the aerobic microorganisms required aeration not only for amine removal but also for TOC stabilization [3].

In conclusion, the strain VN-31 isolated from the dye effluent was identified by 16S rRNA gene as *Klebsiella* sp. All the dyes tested were totally decolourized under microaerophilic conditions with some difference in the decolourization time depending on the dye structure, as confirmed by the UV–vis analysis. The formation of

amines during the microaerophilic stage and their disappearance during the aerobic stage was confirmed by direct measurement and by FT-IR analysis. In the aerobic stage, partial mineralization of the dye degradation products as well as of the medium metabolites was confirmed by the FT-IR, toxicity and TOC measurements. 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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