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Anaerobic Reduction of a Sulfonated Azo Dye, Congo Red, by Sulfate-Reducing Bacteria

Patricia Esteves Diniz,[1,2] Ana Teresa Lopes,[2] Ana Rosa Lino,[1] and Maria Luisa Serralheiro*,[2]

[1]Instituto de Tecnologia Química e Biológica, 2780 Oeiras and [2]Centro de Ciências Moleculares e de Materiais, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1700 Lisboa, Portugal, E-mail: mls@fc.ul.pt

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Abstract

The capacity for anaerobic decolorization of a sulfonated azo dye, Congo Red, by a strain of a sulfate-reducing bacterium was evaluated. After optimizing the growth rate of the bacteria on a simple carbon source and terminal electron acceptor pair, lactate and sulfate, respectively, the effect of the dye concentration on their growth rate was analyzed. The decolorization rate was affected by the dye concentration in the growth medium. The azo-bond cleavage mechanism of reductive decolorization with the formation of benzidine was consistent with the results, as this metabolite was identified by high-performance liquid chromatography. Several fractions of the culture medium, including lysed cell extracts, were examined for the capacity to reduce the azo dye. This reduction capacity was found in the culture medium in which the cells had previously grown. The results showed that the mechanism of reductive decolorization of this sulfonated azo dye was extracellular and nonenzymatic, consistent with the production of sulfide anion by the microorganisms while growing on lactate and sulfate. The sulfide anions were the cause of the reduction leading to the disappearance of color in the medium. To increase the rate of decolorization, the presence of ferrous ion was also necessary together with the lactate and sulfate substrates.

Index Entries: Sulfonated azo dye; Congo red; reduction; sulfate-reducing bacteria; central composite design; experimental planning.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

The organic dyes used in the textile industry are one of the major pollutants found in the industry's wastewaters *(1)*. Among these dyes, the azo group containing compounds can be referred to as one of the most important *(1)*. These compounds are not easily removed under the aerobic conditions found in most of the biologic wastewater treatment units *(2)*. Several reports on the complete degradation of azo dyes by yeasts and fungi have been published *(3–12)*. Azo dyes can easily be decolorized by microbial mixed cultures growing in anaerobic conditions *(2)*. This process can be attributed to the reduction in the azo group with the concomitant formation of aromatic amines *(5)*. These compounds can be highly toxic and resistant to further anaerobic degradation. However, several aerobic bacteria have been referred to as being able to mineralize amine derivatives of aromatic compounds *(5,13)*. Sequences or the coexistence cycles of anaerobiose and aerobiose are thus a way of completely converting azo compounds into carbon dioxide and biomass. Examples have been described with facultative anaerobic microorganisms such as *Sphingomonas* sp. BN6 strain *(14–16)* and mixed cultures of anaerobes and aerobes *(5,14)*.

The mechanisms involved in the degradation of the azo compounds are not completely elucidated. In the anaerobic bacteria, two main mechanisms have been proposed. In the decolorization process, azo reductase enzymes take part. These enzymes have as cofactors the riboflavins $FMNH_2$ and $FADH_2$ *(17)*. Some of the anaerobic bacteria have been found to produce extracellular azo reductases that do not require the presence of dye to be produced *(17)*. Most of the producing azo reductase bacteria are of the anaerobic facultative type *(18,19)*. Recently, a gene from *Clostridium perfringens* coding for these enzymes was cloned in an *Escherichia coli* and the azo reductases were expressed. The investigators concluded that this gene is present in some of the anaerobic microorganisms *(17)*. The other hypothesis for the anaerobic degradation of the azo compounds proposes a nonenzymatic electron transfer between the cofactors $FADH_2$ and the azo group, as first stated by Gingell and Walker *(20)*. Some of the results show the azo dye decolorization to be an intracellular process *(21)*, although a mechanism for the dye transport has not been found. However, most of the reports seem to indicate that the mechanism of decolorization is extracellular with the involvement of the cells themselves and some factors excreted to the culture medium *(5,15,16)*. According to these results, the dye functions as an oxidant relatively to the bacteria or to the compounds they produce in the medium, and the decolorization rate is dependent on the rate of formation of electron donors. The redox potential between the electron donor and acceptor is very important, and the presence of oxygen will reduce the dye decolorization because it will be the electron acceptor of choice. An increase in the concentration of carbon source and the presence of an alternative carbon source and quinone derivatives have been shown to raise the rate of the dye decolorization *(22,23)*.

There are only a few studies on azo dye decolorization by sulfate-reducing bacteria (SRB), and they were carried out with mixed cultures immobilized in polyurethane foams *(18)*. The decolorization mechanism proposed involves the degradation of the carbon source lactate with the transferring of the reducing power to the sulfate anion with the concomitant formation of sulfide ions. These ions can then reduce the azo bond *(18)*.

In the present work, the reduction of an azo compound, Congo Red dye, in a strictly anaerobic environment was studied. The microorganism used was a strictly anaerobic strain of sulfate-reducing bacterium: *Desulfovibrio alaskensis*. The objective of this study was to contribute to the knowledge of the azo bond degradation process by these anaerobic microorganisms, so that this process could be used in a wastewater treatment plant, in cycles of anaerobiose/aerobiose.

Materials and Methods

Chemicals

All chemicals used for culture media and buffer solution were from Merck AG (Mannheim, Germany) or Sigma (St. Louis, MO). The dye was from Sigma.

Sources of Bacteria

The bacterial strain used was the sulfate-reducing bacterium *D. alaskensis*, a gift from Dr. Iwone Beech of the Chemistry Department of Porthsmouth University, UK. Subcultures were routinely made and were not previously exposed to any of the dyes being tested.

Culture Conditions

The bacteria were cultivated under anoxic conditions in a mineral medium containing lactate as the carbon source and sulfate as the electron acceptor. Cultures were grown in liquid culture medium (100 mL) composed of lactate; sulfate (varied according to Table 1); casamino acids (2 g/L); tryptone (2 g/L); and a solution of inorganic salts known as Wolf Elixir, composed of nitriloacetic acid (7.8×10^{-6} *M*), magnesium sulfate (1.2×10^{-5} *M*), manganese sulfate (3.0×10^{-6} *M*), sodium chloride (1.7×10^{-5} *M*), iron (II) sulfate (3.6×10^{-7} *M*), cobalt sulfate (3.6×10^{-7} *M*), nickel chloride (4.2×10^{-7} *M*), copper (II) chloride (5.9×10^{-7} *M*), zinc sulfate (3.5×10^{-7} *M*), copper (II) sulfate (4.0×10^{-8} *M*), aluminum potassium sulfate (2.1×10^{-8} *M*), boric acid (1.6×10^{-7} *M*), sodium molybdate (4.1×10^{-8} *M*), and sodium selenite pentahydrate (3.8×10^{-9} *M*). A solution of vitamins composed of riboflavin (5.3×10^{-7} *M*), nicotinic acid (4.1×10^{-6} *M*), thiamine (1.8×10^{-6} *M*), d-pantothenic acid (2.5×10^{-6} *M*), pyridoxine (2.9×10^{-6} *M*), cyanocobalamin (3.7×10^{-8} *M*), ascorbic acid (1.1×10^{-5} *M*), and biotin (4.1×10^{-8} *M*) was also added. The values given in parentheses refer to the final concentrations in 100-mL bottles. The medium was dispensed to tubes or bottles and flushed

Table 1
CCD Experimental Plan to Study Influence
of Lactate and Sulfate on Growth Rate of *D. alaskensis*

Codified levels of factors		Uncodified levels of factors	
Sulfate	Lactate	Sulfate (m*M*)	Lactate (m*M*)
–1	–1	30	53
+1	–1	50	53
–1	+1	30	89
+1	+1	50	89
–2	0	20	71
+2	0	60	71
0	–2	40	35
0	+2	40	107
0	0	40	71

with N_2 to obtain the anoxic conditions. Then, the culture vessels were sealed with rubber stoppers that were fixed with open-top screw caps and sterilized by autoclave. The final pH of the medium was approx 7.5.

Before inoculation, 1 mL of the filter-sterilized vitamin mixture was added per liter of medium. The cultures of *D. alaskensis* were incubated at 37°C in the dark. The inoculum used in all studies was 10% (v/v).

Optimal growth conditions of *D. alaskensis* in the absence of dyes was determined according to the central composite design (CCD) method, as described next.

Optimal Growth Conditions

The influence of lactate and sulfate on the bacterial growth was studied by applying a factorial design known as CCD *(24)*. The culture media were prepared with sodium lactate (35–105 m*M*) and sodium sulfate (24–60 m*M*) according to Table 1. A second-order model relating the two factors and taking into account the interaction between the two factors was applied to the experimental results *(25)*. This mathematical treatment allowed the drawing of the response surface curves in which the influence of both substrates on the bacterial specific growth rate could be analyzed.

Influence of Concentration of Azo Dye on Specific Growth Rate

Cultures of *D. alaskensis* were grown in the presence of Congo Red with concentrations between 20 µ*M* and 1 m*M* dye. The dye was added to the culture medium before sterilization by autoclaving. Congo Red decolorization in the culture supernatant was monitored by measuring the absorbance of filtered samples at the wavelength of maximum absorptivity (478 nm). Abiotic controls were run in the same conditions but without bacterial inoculation.

Congo Red Degradation Assays by Different Fractions of Bacterial Culture

The Congo Red decolorization assays were carried out under anaerobic conditions in culture tubes with 9 mL of culture medium containing 50 μ*M* dye. After oxygen removal by N_2 flushing, the culture tubes were sealed with rubber stoppers and screw caps. The decolorization reactions were started by the addition of 1 mL of culture fractions, prepared as described in the next section. Dye decolorization was followed for 12 h. The assays were performed at least in duplicate and incubated at 37°C. The decolorization rate of Congo Red was expressed in micromoles of dye removed per hour per milliliter of reaction mixture. The calculations were based on the value 0.00149 μM^{-1} cm^{-1} for the extinction coefficient ε_{479nm} of a Congo Red solution in culture medium (pH 7.5).

Preparation of Culture Fractions

D. alaskensis cultures grown up to the stationary phase were used. The cells of 10 mL of culture were removed by centrifugation (15 min, 9800*g*). The culture supernatants were collected and filter sterilized. To prepare resting cells, cell pellets were resuspended in an anaerobic buffer of Tris-HCl (10 m*M*, pH 7.2). Cell extracts were prepared using the alkaline lysis method. These cell-extract culture fractions were sterilized by filtration on cellulose acetate filters (pore size of 0.22 μm; Millipore).

Studies of Congo Red Decolorization by Cell-Free Supernatant

To determine Congo Red decolorization rates by the cell-free supernatants previously exposed to a protein denaturation condition, these supernatants were subjected to a process of autoclaving at 121°C for 20 min on two consecutive days. Between the two consecutive processes, the culture supernatants were stored at 4°C. These culture supernatants were used in the dye decolorization assays.

The effect of using a cell-free supernatant, previously flushed with nitrogen, on the dye decolorization was studied. The flushing process lasted for 10 min, and the supernatant was filter sterilized before being used in the dye decolorization assay.

Both these studies were carried out by adding 1 mL of the referred cell-free supernatant to 9 mL of culture medium containing 50 μ*M* Congo Red dye.

Influence of Components of Medium on Decolorization Rate

To evaluate the influence of components of the medium on the rate of dye decolorization, different media were prepared with all but one of the normal components of the medium. To 9 mL of these media containing also 50 μ*M* Congo Red dye, 1 mL of the cell-free supernatant was added, and the rate of decolorization was determined spectrophotometrically.

Table 2
CCD Experimental Plan to Study Effect
of Lactate, Sulfate, and Iron on Rate of Congo Red Decolorization

Codified levels of factors			Uncodified levels of factors		
Sulfate	Lactate	Iron	Sulfate (m*M*)	Lactate (m*M*)	Iron (μ*M*)
–1	–1	–1	30	26	13
+1	–1	–1	90	26	13
–1	+1	–1	30	80	13
+1	+1	–1	90	80	13
–1	–1	+1	30	26	39
+1	–1	+1	90	26	39
-1	+1	+1	30	80	39
+1	+1	+1	90	80	39
–2	0	0	0	53	26
+2	0	0	120	53	26
0	–2	0	60	0	26
0	+2	0	60	107	26
0	0	–2	60	53	0
0	0	+2	60	53	52
0	0	0	60	53	26

Development of Medium to Increase Rate of Decolorization

Several media with different concentrations of lactate, sulfate, and ferrous ion were prepared according to Table 2. These values were established according to an experimental CCD plan *(24,25)*. To 9 mL of this medium, containing 50 μ*M* Congo Red, 1 mL of the supernatant was added and the rate of decolorization was evaluated.

Analytical Methods

The decolorization of dyes was followed spectrophotometrically (UV-160 A Shimadzu) in the visible range, against a blank of medium without the dye. The formation of one of the corresponding reduction products (benzidine) was analyzed by high-performance liquid chromatography (HPLC) (Merck Hitachi Pump L-7110 equipped with a Merck Hitachi UV L-7400 Detector). A reversed-phase column (250 × 4 mm id) filled with 5-μm-diameter particles of Lichrosphere RP 18 (Merck AG) was used as the stationary phase. Peaks were detected spectrophotometrically at 220 nm. The mobile phase was 50% (v/v) methanol, 49.7% (v/v) H_2O, and 0.3% (v/v) H_3PO_4 at a flow rate of 1 mL/min at pH 3.6 *(15)*.

The product of Congo Red degradation, benzidine, was extracted from the aliquots (1 mL), of the culture supernatant with 1 mL of methylene chloride, and 20-μL aliquots of the methylene chloride extracts were used, after dilution (1:10), for HPLC analysis.

Cell growth was quantified by two methods: microscopic cell counts and protein concentration. Cell counting was performed by phase-contrast

microscopy with a counting chamber (Weber Scientific). Protein concentration was measured by the Bradford *(26)* method with bovine serum albumin as standard.

The sulfide ion was measured by the colorimetric method with methylene blue adapted for this study *(27)*.

Results and Discussion

Effect of Congo Red Dye on Growth of D. alaskensis

The effect of the dye Congo Red, used in the textile industry, on the growth of the anaerobic bacterium *D. alaskensis* was studied. This dye is a diazo compound whose structure is shown in Fig. 1. Before analyzing the effect of Congo Red on the growth of this anaerobic bacteria, the culture medium was optimized on lactate and sulfate, the carbon source and the terminal acceptor, respectively.

An experimental plan involving the carbon source, lactate, and the electron terminal acceptor, sulfate, was set up. The influence of these two substrates on the specific growth rate was measured, and the response surface methodology allowed drawing of the curves shown in Fig. 2, with a relative error of 6%. These results indicate that increasing the lactate and sulfate concentration increases the specific growth rate up to limit values above which both factors inhibit growth, probably owing to an excess of sulfide that is toxic to the cells. In fact, fermentation of lactate by these bacteria leads to the reduction of sulfate to sulfide, according to Scheme 1.

From Fig. 2 it can be seen that when the lower concentration of lactate is used, the sulfate does not show an inhibitory effect on the growth of bacteria, although a constant level of growth rate is reached. The best conditions for the bacterial growth, under the conditions tested, are thus 60 m*M* sulfate and 54 m*M* lactate. All the other subsequent growth studies were done with these values of sulfate and lactate in the culture medium. Carrying out the growth studies in the presence of the dye under the best conditions of lactate and sulfate ensured that the results can be attributed to the dye's presence and not to any deficiency in the supply of nutrients.

After we checked that *D. alaskensis* was able to grow in the presence of 5–50 µ*M* Congo Red and also to decolorize this compound within 24 h, we tested higher concentrations of the dye. The specific growth rates for six different concentrations were measured and the results are shown in Fig. 3. An increase from 20 µ*M* to 0.5 m*M* in the dye concentration decreased the specific growth rate by 70%, and a concentration of 1 m*M* completely inhibited the growth of *D. alaskensis*. The presence of the dye in the culture medium also increased the growth lag phase. However, the dye was completely decolorized in all the conditions, except when there was no growth at all. Above 0.5 m*M* the dye was toxic to the cells, inhibiting growth. The inhibition kinetic was linear up to a dye concentration of 50 µ*M*. An inhibition rate of the specific growth rate of 0.42% per micromolar Congo Red was found. Most of the strains used to decolorize these dyes had

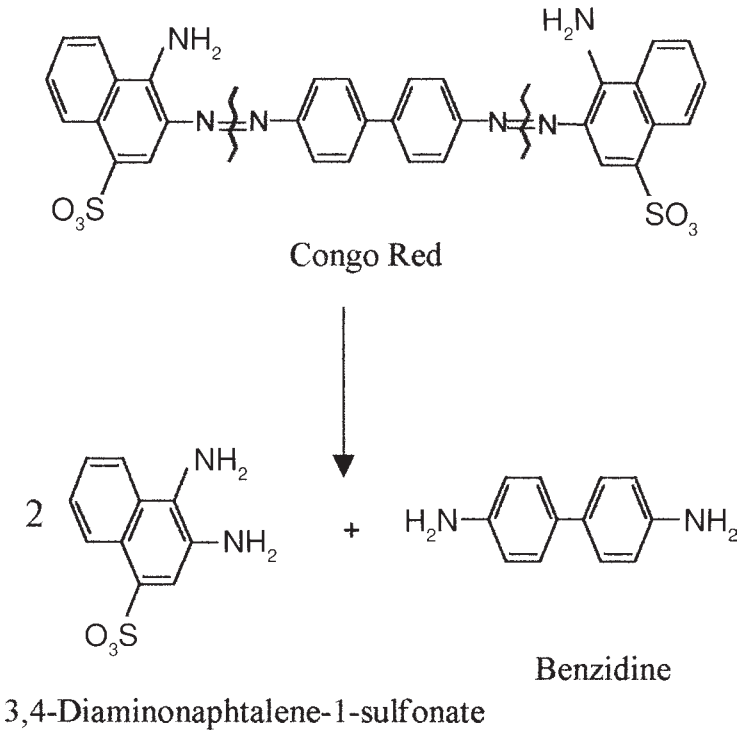

Fig. 1. Chemical structure of Congo Red dye and its degradation products obtained in medium in which sulfate-reducing bacteria were growing.

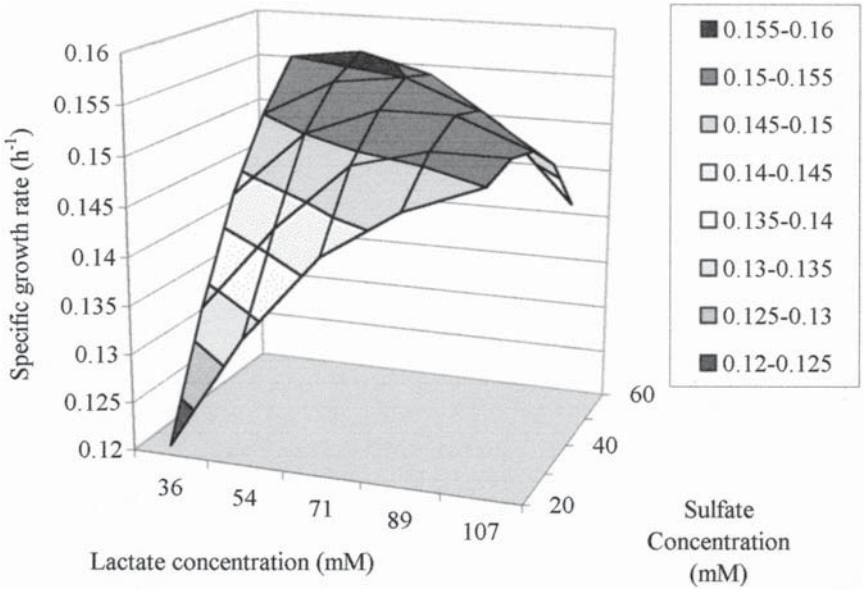

Fig. 2. Effect of lactate and sulfate concentrations on specific growth rate of *D. alaskensis* in absence of dye.

$$2\ CH_3\text{-}CHOH\text{-}COOH + 2\ H_2O \rightarrow 2\ CH_3\text{-}COOH + 2\ CO_2 + 8H^+ + 8e^-$$

$$SO_4^{2-} + 8H^+ + 8e^- \rightarrow S^{2-} + 4\ H_2O$$

Scheme 1. Final reactions resulting from fermentation of lactate and sulfate by sulfate-reducing bacteria *(28)*.

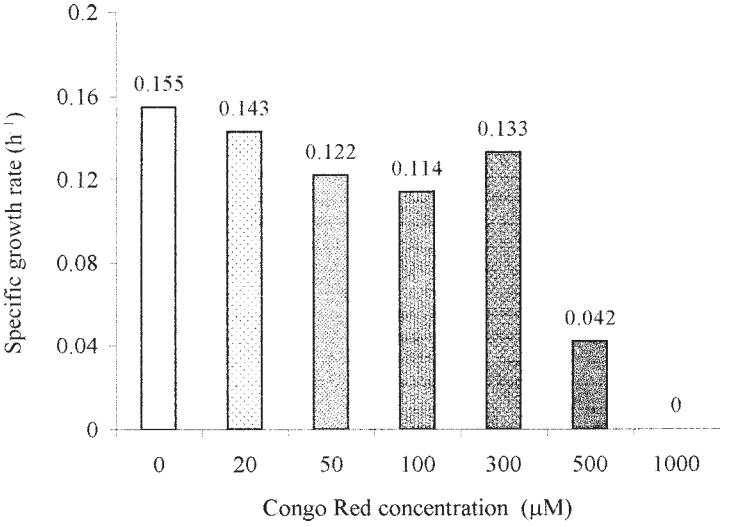

Fig. 3. Effect of Congo Red concentration on specific growth rate of *D. alaskensis* in medium containing lactate (71 m*M*) and sulfate (60 m*M*).

to be acclimated to the toxic compound in order to withstand higher doses than those used in the present work *(5,15)*. When using nonadapted strains *(18)*, the maximum value under which the strains could grow was similar to the one used in the present study.

The influence of the dye concentration on its degradation rate was analyzed (Fig. 4). The results show that most of the color disappeared from the culture medium within the first 24 h of growth, irrespective of the initial concentration of dye, a faster process than the one found in the literature *(18)*. The decolorization of the culture medium was not owing to adsorption of the dye to the bacteria because they remained with the same color as in the beginning of the experiments. A change in the culture pH could be another explanation for the dye decolorization, but the observed shift in the pH during bacterial growth (from 6.9 to 7.3) is not enough to account for the drastic change in the dye structure needed for decolorization. This was investigated by making spectra in the UV/VIS range of the dye dissolved in the culture medium at pH values between 6.5 and 7.5. The results showed that the maximum wavelength was approximately the same for the values under analysis, 478 and 479 nm, respectively. At these pH values, the dye maintains its original color. The decolorization of the dye must be produced by the culture itself and not by a simple change in the pH of the medium.

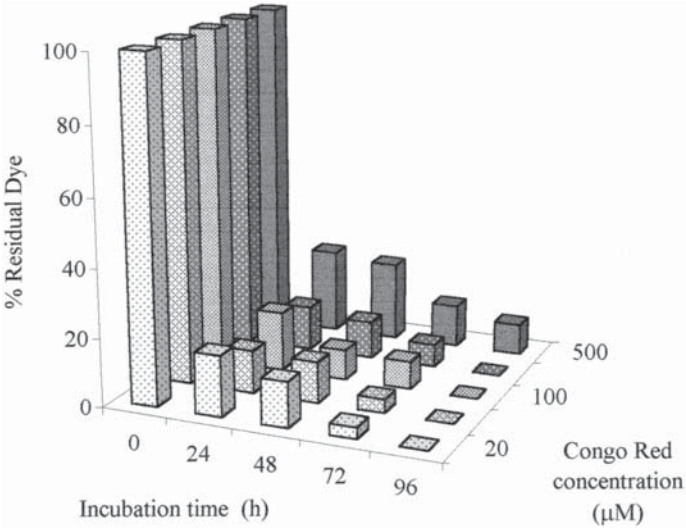

Fig. 4. Effect of incubation time and dye concentration on decolorization capacity of *D. alaskensis* while growing in lactate (71 m*M*) and sulfate (60 m*M*).

Identification of Culture Fraction Responsible for Dye Decolorization

To identify the fraction of the bacterial culture responsible for the dye decolorization process, different fractions of the culture (cell extracts, resting cells, and cell-free supernatant) were separately assayed for their capacity to decolorize the Congo Red dye. For this study, a series of test tubes containing the culture medium with the dye were prepared as described in Materials and Methods. To these test tubes, an aliquot of the culture fractions was added and the dye decolorization was followed for up to 12 h of incubation. As can be seen from Fig. 5, the cell-free culture medium was the only fraction able to decolorize the dye present in a fresh culture medium. The agent responsible for the reductive degradation of the azo group is thus extracellular and is only formed by growing the bacteria in that medium, even in the absence of the dye. After growth, there is apparently no need for the bacteria to be present in the culture medium for the decolorization to occur. The clarified medium was able to decolorize the dye at a rate of 0.49 μmol/(h·mL). The results obtained with the resting cells indicate that the dye cannot penetrate the cell wall to be reduced inside the cell or that, although it may penetrate the cell, it is not an intracellular enzyme that catalyzes the dye reduction. The negative results found with the cellular extract reveal that the reducing power is not found inside the cells but must be located in the culture medium.

The active compound released to the extracellular medium could be a protein with enzymatic activity. To analyze this possibility, the cell-free culture supernatant was inactivated by autoclaving twice and tested again for its decolorization capacity. Both the autoclaved and the nonautoclaved

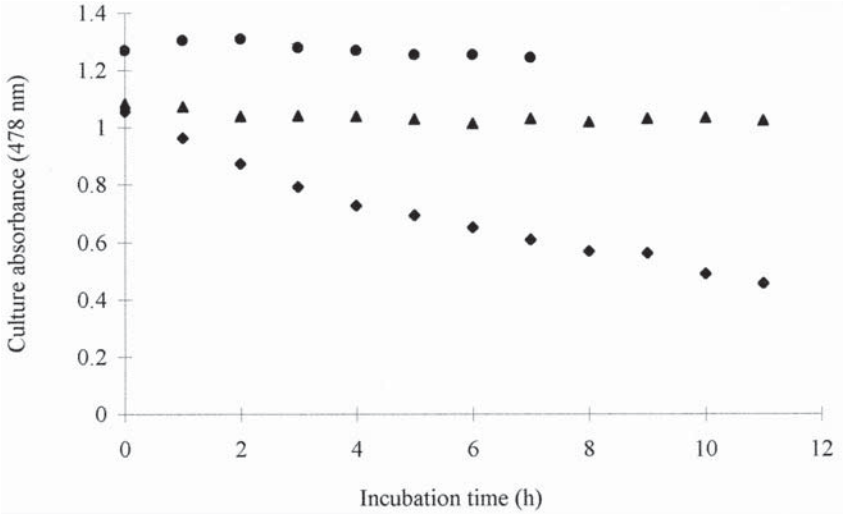

Fig. 5. Decolorization of Congo Red by different fractions of a *D. alaskensis* cell culture. (◆), Cell-free supernatant; (▲), alkaline lysed extract; (●), resting cells.

media had approximately the same decolorization rate, 0.49 and 0.50 µmol/(h·mL), respectively. These results seem to indicate that neither the cells nor an extracellular enzyme is directly involved in the decolorization process.

To determine the presence of volatile substances in the clarified culture medium after cell growth, the medium was flushed with nitrogen gas for 10 min, and the rate of decolorization of dye was subsequently measured. A 93% decrease in the decolorization rate was found. The degradation process thus involves a volatile substance that is apparently stripped out by the nitrogen flux.

The volatile product that may be formed during the microbial growth is the hydrogen sulfide, coming from the metabolism of the bacteria growing on lactate and sulfate anion. The sulfide anion, once in water at pH 7.5, is in equilibrium with hydrogen sulfide *(18)*. The sulfide ion was measured before and after flushing the medium with nitrogen (Fig. 6, column a and column b, respectively). The sulfide ion concentration was drastically reduced with flushing of the medium (Fig. 6, column b). The sulfide anion present in the medium in which the dye decolorization took place was reduced relatively to a medium without the presence of dye (Fig. 6, column c). Flushing this medium with nitrogen decreased the sulfide anion even more (Fig. 6, column d). It may be concluded that the hydrogen sulfide was one of the compounds producing the azo bond reduction, leading to the formation of aromatic amines (Scheme 2).

To confirm decolorization by a volatile substance, an experiment in which the decolorization by this substance was set up. In this experiment, the bacteria were grown in a medium without the dye, and the volatile compound produced was bubbled through a sterilized silicon tube into the bottle containing the dye dissolved in water. Thus, the hydrogen sul-

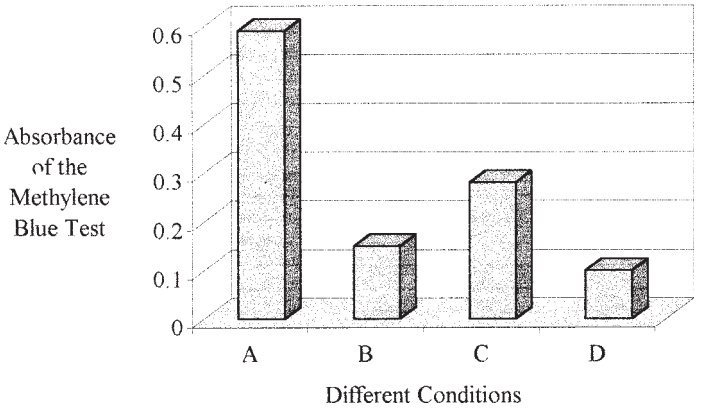

Fig. 6. Effect of nitrogen flushing on sulfide anion disappearance measured by methylene blue method. A, Cell-free supernatant; B, same medium as in A after flushing with nitrogen; C, medium in which *D. alaskensis* had been growing while decolorizing the Congo Red dye (cell-free supernatant); D, same medium as in C after flushing with nitrogen.

$$4\ H_2S + R\text{-}N{=}N\text{-}C_{12}H_8\text{-}N{=}N\text{-}R \rightarrow 4\ S + 2\ R\text{-}NH_2 + NH_2\text{-}C_{12}H_8\text{-}NH_2$$

Scheme 2. Reduction equation of diazo compound by hydrogene sulfide.

fide could be transferred directly into the bottle containing the dye while it was being produced. A complete decolorization of the dye solution could be seen.

The sulfide anions produced by the bacteria while growing on lactate and sulfate can be found in the culture medium by the methylene blue method, showing that this anion can permeate the cell wall and be found in the culture medium. Once outside the cells, the sulfide anion is converted into HS^- and H_2S *(18)*, and it seems to be the hydrogen sulfide responsible for the dye decolorization, because the reducing power is found in the supernatant.

To determine whether the decolorization process could be accelerated by using cultures of *D. alaskensis* previously grown in a culture medium with Congo Red (50 μ*M*), these cells were resuspended in a fresh medium containing the dye, but the decolorization rate found was similar, 0.49 and 0.51 μmol/(h·mL), respectively. The decolorization process seems to be independent of an inductive mechanism that may be initiated by the presence of the dye.

Studies done with sulfate-reducing bacteria for azo-dye degradation are scarce, but there are some reports of studies done with facultative anaerobic bacteria in which both the culture medium and the cellular extracts were necessary to produce decolorization *(15,29)*. The present work seems to corroborate the results found by Libra et al. *(18)* in which the reduction of an azo compound was also accomplished by the hydrogen sulfide produced from the metabolism of these bacteria.

Table 3
Effect of Absence of Different Medium Components
on Decolorization Rate of Congo Red by Cell-Free Supernatant[a]

Substance absent from medium	Effect on rate of dye decolorization (%)
KH_2PO_4	–3
Na_2SO_4	–30
NH_2Cl	+11
$MgSO_4$	–11
$CaCl_2$	–17
Lactate	–37
Citrate	+25
$FeSO_4$	–60
Wolf Elixir	+36
Casamino acids	+13
NaCl	–12
Tryptone	+2

[a]Values are calculated comparatively with the value obtained with the complete medium (0.49 µmole of dye/[h·mL of medium]).

Identification of Components of Medium Involved in Reduction Process

The formation of sulfide was also the main cause of the azo dye decolorization with a strain of SRB *(18)*. If the sulfide anion was the unique cause for the azo bond reduction, it should decolorize this dye also when the supernatant was added to a solution of the dye in Tris-HCl, for instance. When this is done, there is only a decrease in 50% in the dye decolorization rate. Other substances must be present in the medium that facilitate the electron transfer to the azo bond. To study which of the medium's components should be present in order to increase the decolorization rate, several media were prepared containing all but one of the compounds under evaluation. To these media one aliquot of the cell-free supernatant (medium in which the SRB had been growing and centrifuged afterward) was added. All these media were tested for their capacity to decolorize the Congo Red solution without introducing the medium in which the SRB had been grown, so each test had its one blank. The rate of decolorization was compared with the value obtained using the complete medium; table 3 presents the results. It can be seen that sodium sulfate, sodium lactate, and iron sulfate were needed for the azo dye reduction. The absence of citrate as well as the Wolf's Elixir was beneficial to the dye decolorization. A blank done with these salts in the culture medium without the presence of the supernatant was not able to cause decolorization of the dye.

To establish a medium in which the decolorization could take place at a faster rate, a study of the influence of lactate, sulfate, and ferrous ion on this rate was undertaken. The CCD methodology described in Materials and Methods was followed, allowing the drawing of the response surface

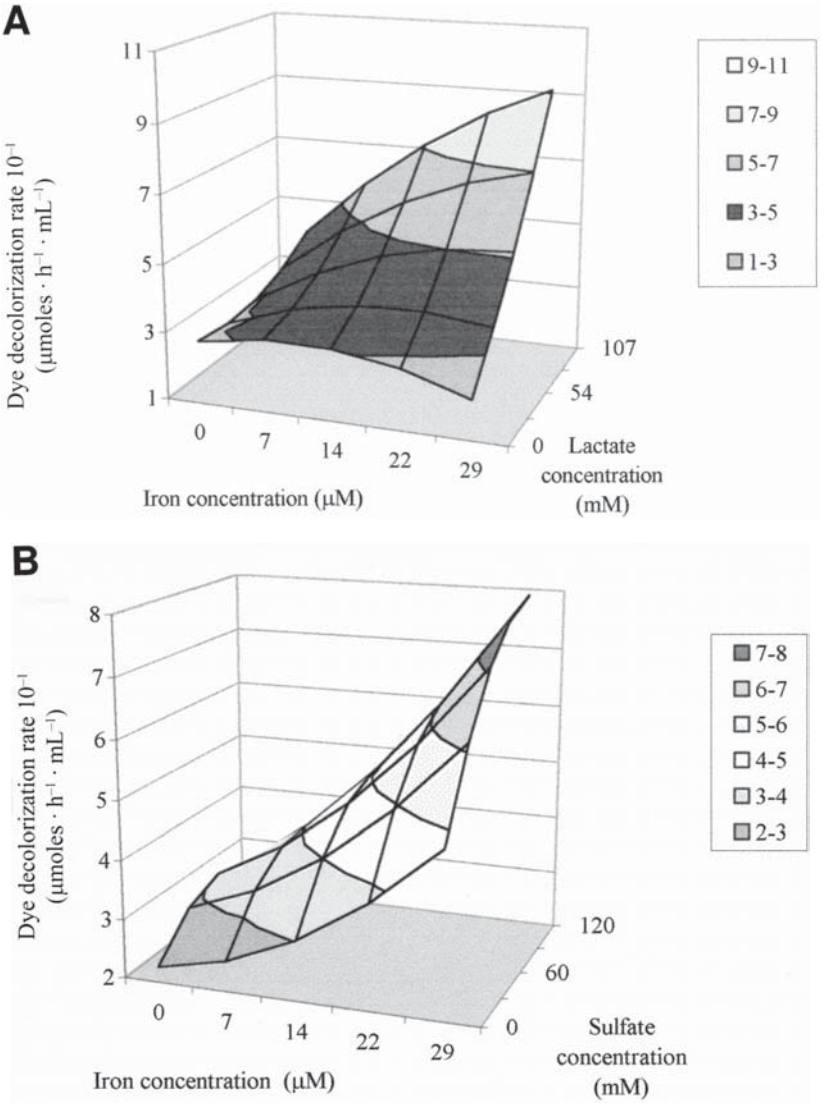

Fig. 7. Influence of lactate, sulfate, and iron (in ferrous form) on reduction rate, of Congo Red, by cell-free supernatant. **(A)** Influence of lactate and ferrous ion at fixed value of 60 m*M* sulfate; **(B)** influence of sulfate and ferrous ion at fixed value of 53 m*M* lactate; **(C)** influence of lactate and sulfate ion at fixed value of 23 μ*M* ferrous ion.

curves shown in Fig. 7. The influence of ferrous ion was analyzed at the central value of sulfate and lactate (Fig. 7A and B, respectively). It can be seen that the increase in this ion concentration brought about an increase in the rate of decolorization. The increase in lactate and iron concentration produced a higher rate of decolorization (Fig. 7C). Although the specific growth rate of the bacteria could be reduced by 8% when these values were

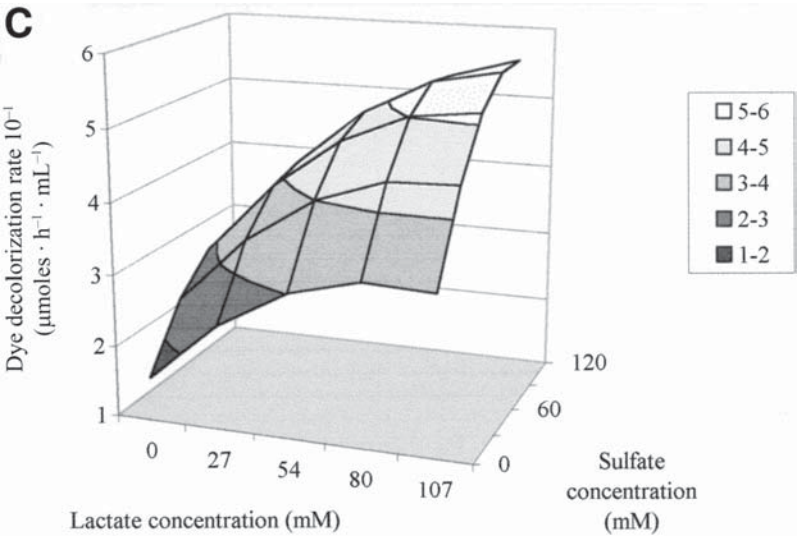

Fig. 7. *(continued)* **(C)** influence of lactate and sulfate ion at fixed value of 23 μ*M* ferrous ion.

used in the culture medium, a higher increase of 80% in the degradation rate of the dye could be obtained. The effect found for the presence of ferrous ion in solution can be attributed to its capacity for being oxidized to the ferric state while reducing the azo bond *(18)*.

These results indicate that to increase the decolorization rate, lactate, sulfate, and iron, in the ferrous state, should be present in solution together with the hydrogen sulfide in order to obtain a faster reduction rate.

Degradation Products

The first step in the degradation of azo compounds is usually the formation of amine derivatives. According to the Congo Red structure (Fig. 1), the amine derivative benzidine together with 3,4-diamino-naphtalene-sulfonate should be formed, during the azo group reduction. Using HPLC analysis, the possibility of the formation of benzidine in the culture medium was verified. Benzidine was soluble in the organic solvent dichloromethane and most of the medium's components were not. When benzidine was added to the medium as an internal standard, it was completely extracted to the organic phase. An aliquot of this phase was injected in the HPLC system, and a clear chromatogram in which the peak corresponding to this compound could be detected. The comparison is done against an external standard of benzidine. After extracting the supernatant in which the Congo Red had been reduced, with the organic solvent and injected in the HPLC, a peak with a retention time similar to the benzidine external standard could be detected. This peak was not visible in the beginning of the decolorization process.

Conclusion

The sulfate-reducing strain used in the present work, *D. alaskensis*, was able to reduce sulfonated azo dyes completely. The process was extracellular and noninducible, and it depended mainly on the presence of a volatile substance, hydrogen sulfide, formed during the bacterial growth. The existence of lactate, sulfate, and iron, in the form of ferrous anion, together with the medium in which the bacteria had previously grown, increased the rate of decolorization. This reaction led to the formation of aromatic amines, detected by HPLC.

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