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Descoloração e degradação de azocorantes por bactérias

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“O segredo de progredir é começar.
O segredo de começar, é dividir as tarefas árduas e complicadas em tarefas pequenas e fáceis de executar, e depois, começar pela primeira”
(Mark Twain)

"Todas as verdades são fáceis de entender
uma vez que são descobertas; o ponto é,
descobri- lás"
(Galileo Galilei)

“ Feliz aquele que transfere o que
sabe e aprende o que ensina”
(Cora Coralina)

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Resumo

Azocorantes são compostos aromáticos com um ou mais grupos azo (-N=N-). São os maiores e a mais importante classe de corantes sintéticos usados em aplicações industriais. Eles são considerados compostos xenobióticos recalcitrantes aos processos de biodegradação, e a presença destes azocorantes nos ecossistemas aquáticos é a causa de sérios problemas ambientais e relacionados com a saúde.

Neste trabalho, a habilidade em degradar azocorantes de 62 linhagens bacterianas previamente isoladas de efluente industrial foi investigada. A seleção das linhagens foi realizada através de testes de descoloração visual em meio líquido com azocorantes suplementados com diferentes fontes de carbono. O processo de descoloração foi realizado sob condições microaerofílicas ou estáticas até que nenhuma cor fosse observada, seguido de agitação para promover a biodegradação dos metabólitos produzidos.

A descoloração e a biodegradação dos azocorantes bem como dos metabólitos produzidos foram monitoradas por análises de UV-vis, Carbono Orgânico Total (COT), Espectroscopia no Infravermelho com Transformadas de Fourier (FTIV) e Cromatografía Líquida de Alta Eficiência e Espectrometria de Massa (CLAE-EM).

A atividade de enzimas oxidativas (peroxidase, lacase and tirosinase) foram analisadas para verificar se estas estavam envolvidas no metabolismo de biodegradação dos azocorantes.

Análises de toxicidade foram realizadas antes e após a degradação dos azocorantes utilizando o organismo teste *Daphnia magna.*

As aminas aromáticas geradas da biodegradação dos azocorantes foram testadas com o propósito de obter a polimerização. A enzima lacase de *Myceliophthora Thermophila* foi usada para catalizar reações de acoplamento entre as aminas aromáticas produzidas.

As análises de UV-vis mostraram que os corantes foram descoloridos (80%) em condições microaerofílicas ou estáticas. Não houve nenhuma mudança significativa na cor, no estágio aeróbio seguinte.

O tempo de descoloração mostrou relação com o meio de cultura utilizado e com a estrutura química dos corantes. Os corantes monoazo foram descoloridos entre 8 a 120 hs. Os diazo e os triazo foram descoloridos após 120 e 168 hs.

As linhagens bacterianas descoloriram os corantes somente quando o meio foi suplementado com glicose e piruvato ou extrato de levedura. Na ausência destes compostos, as culturas foram incapazes de descolorir, indicando um requerimento obrigatório de uma fonte suplementar de carbono para alcançar a descoloração.

Resultados mostraram que, quando o meio foi incubado em condições microaerofílicas ou estáticas a redução no COT (Carbono Orgânico Total) foi menor do que em condições aeróbias, onde 70% de redução foi observado. A presença de altas concentrações de aminas aromáticas em condições microaerofílicas ou estáticas confirma que houve redução das ligações azo. Porém, houve a confirmação da oxidação destas aminas no estágio aeróbio, indicando que um proceso oxidativo foi responsável pela biodegradação dos metabólitos.

Foi observada atividade de tirosinase para na linhagem de *Brevibacterium sp*, sugerindo o papel desta enzima no processo de descoloração dos azocorantes, não sendo observada atividade de lacase e peroxidase.

Nas análises de Espectroscopia no Infravermelho com Transformadas de Fourier (FTIV) após condições microaerofílicas ou estáticas foram observadas bandas em regiões atribuidas a grupamentos amina. Estas bandas desapareceram no estágio aeróbio e foram observadas novas bandas nas regiões associadas com ácido carboxílicos e íons NH_3^+, confirmando mineralização parcial dos produtos de degradação dos azocorantes, bem como dos metabólitos do meio de cultura.

Os metabólitos produzidos pela biodegradação do azocorante RR 198 foram analisados por CLAE-EM, para tentar identificar alguns metabólitos desconhecidos. Entre os possíveis compostos produzidos da biodegradação do RR198, 4-cloro-N-o-toluil-1,3,5-triazina-2-amino; sódio 4-aminonaftaleno-2-sulfonado e 3,6 -dimetil-7-(o-toluildiazenil) naftaleno-1-amino, tiveram razoável semelhança com os metabólitos aromáticos encontrados na amostra. Após condições aeróbias, a intensidade dos íons presentes nestes metabólitos foram reduzidos. Estes resultados também foram confirmados pelas análises de FTIV e poderia ser explicado pela diminuição dos compostos aromáticos gerados nas condições microaerofílicas ou estáticas.

Foi observado que após um longo período de tempo, a lacase catalizou a polimerização das aminas aromáticas presentes nas soluções descoloridas. Os produtos gerados precipitaram e adquiriram cor, como confirmado pelas análises de UV-Vis. Os tamanhos das partículas foram significativamente maiores após o tratamento com lacase, como mostra as análises de Espectroscopia de Correlação de Fótons.

Todas as linhagens bacterianas usadas neste estudo foram capazes de descolorir e degradar os azocorantes em condições microaerofílicas ou estáticas. Em condições aeróbias, ocorreu parcial mineralização dos produtos de degradação dos azocorantes, bem como dos metabólitos do meio, como confirmado para o organismo teste *Daphnia magna* e Carbono Orgânico Total (COT). Após o estudo, estas bactérias foram identificadas através de análises de sequência de rDNA 16S como *Staphylococcus arlettae*, *Klebsiella sp*, *Microbacterium* sp, *Leucobacter albu*s e *Brevibacterium* sp.

Abstract

Azo dyes, which are aromatic compounds with one or more azo (-N=N-) groups, are the most important and largest class of synthetic dyes used in commercial applications. They are considered as xenobiotic compounds that are very recalcitrant to biodegradation processes. The presence of these dyes in the aqueous ecosystem are a cause of serious environmental and health concerns.

In this work, the ability of 62 bacterial strains previously isolated from an industrial activated sludge process treating effluent containing azo dyes was investigated. The selection was undertaken, through visual decolorization, in liquid media with azo dyes supplemented with diferent carbon sources.

Decolorization process was performed under microaerophilic or static conditions until no color was observed. The medium was then aerated to promote the biodegradation of the metabolites produced.

The azo dyes decolorization and biodegradation and the aromatic amines produced were monitored by UV-Vis, Total Organic Carbon (TOC), Fourier Transformed Infra Red (FTIR) and High Performence Liquid Chromatography (HPLC- MS).

Activity of the oxidoreductase enzymes (peroxidase, laccase and tyrosinase) was evaluated in cultures of the bacterial isolates.

Acute toxicity tests with D*aphnia magna* (Crustacea, Cladocera) were carried out after and before microaerobic or static and aerobic conditions.

The aromatic amines generated from the biodegradation of the azo dyes were tested for their ability to undergoing polymerization using a laccase from *M. thermophila* to catalyze the coupling reactions of the aromatic amines.

The decolorization time showed a relationship with the culture medium and chemical structure of the dyes. The monoazo dyes were decolourized within 8 to 120 h. The diazo and triazo were decolourized required 120 to168 h, approximately.

UV-Vis analysis showed complete decolorization (>80%) in the microaerophilic or static conditions. No significant color changes were detected in the following aerobic stage. However the bacterial strains could only decolourize the dyes effectively when the medium was supplemented with glucose and pyruvate or yeast extract. In the absence of these compounds, the cultures were unable to decolorize the dyes, thus indicating an obligate requirement for a supplementary carbon source for dye decolorization.

When the medium was incubated under microaerophilic conditions, the reduction in TOC was low even after 7 days of incubation. Conversely, a significant increase in TOC reduction (>70%) was observed in the aerobic stage.

The reduction of azo bonds is known to yield the production of high concentrations of amines in the microaerophilic or static stage therefore confirmed the azo bond was reduced. Therefore the oxidation of these aromatic amines was confirmed by the absence of amine in the aerobic stage indicating that an oxidative process was responsible by metabololite biodegradation.

Tyrosinase activity was observed for *Brevibacterium sp,* suggesting the role of this enzyme in the decolorization process, but no-activity was observed for laccase and peroxydase.

In the Fourier Transformed Infra Red (FTIR) analysis after microaerophilic or static decolorization, new bands were observed in region attributed to amine groups. These bands disappeared in the aerobic stage and a new broad region associated with carboxylic acid and NH_3^+ ions were observed. However, in the aerobic stage the partial mineralization of the dye degradation products and of the medium metabolites was confirmed.

The decolorization products of the RR198 dye were analyzed by High Performence Liquid Chromatography (HPLC- MS) for tentative identification of the unknown metabolites tentating identifield compounds included, 4-chloro-N-o-tolyl-1,3,5-triazin-2-amine; sodium 4-aminonaphthalene-2-sulfonate and 3,6-dimethyl-7-(o-tolyldiazenyl) naphthalen-1-amine. After aerobic conditions the intensity of these metabolites was reduced. These results were also confirmed by FTIR and could be explained by degradation of these aromatics coumpounds previously generated in microaerophylic or static stage.

After an extended period of time, laccase catalyzed polymerization of the aromatic amines in the destained solutions. The products generated precipitated spontaneously from the solution and acquired some color as confirmed by the UV-Vis analysis. The particle size was also significantly higher after laccase treatment as show by Photon Correlation Spectroscopy analysis (PCS).

The bacterial strains used in this study were able to totally destain the azo dyes under microaerophilic or static condition. In the aerobic stage, partial mineralization of the dye decolorization products as well as of the medium metabolites was also confirmed by toxicity testing and TOC measurements. The strains were identified by 16S rDNA gene sequence analysis as *Staphylococcus arlettae*, *Klebsiella sp*, *Microbacterium* sp, *Leucobacter albu*s e *Brevibacterium* sp.

1. Introdução

Desde o início da humanidade as cores sempre exerceram fascínio sobre as pessoas, que usavam corantes e pigmentos para colorir seu ambiente, pele, roupas e alimentos.

Uma das características principais do ser humano é utilizar as forças e os materiais do meio ambiente em benefício próprio, e o enorme e rápido crescimento das populações humanas em todo mundo levam a uma necessidade crescente de aumentar esta produção. Devido à moda dos descartáveis e a tendência de possuir sempre os modelos mais modernos dos diversos materiais oferecidos para uso, à chamada sociedade de consumo somados a necessidade de produção, faz com que as indústrias se proliferem, consumindo quantidades cada vez maiores de matérias primas, gerando poluição e outros inconvenientes para o meio ambiente.

O interesse no uso de corantes menos poluentes tem crescido nos últimos anos. Isto é o resultado das severas regras ambientais impostas por alguns países em resposta às reações tóxicas e alérgicas associadas aos corantes sintéticos.

Por outro lado, a escassez quantitativa e qualitativa de recursos hídricos, frente à elevadíssima demanda para atender a usos conflitantes nas regiões metropolitanas de São Paulo e de Campinas, e a iminência da cobrança pela captação de água e pelo lançamento de efluentes nos corpos d'água, tem acarretado a introdução de novas prioridades no planejamento estratégico de grandes indústrias, bem como das companhias de saneamento aí atuantes. Enfrentam-se graves problemas nas bacias hidrográficas dos rios Piracicaba, Capivari e Jundiaí (onde o município de Campinas está inserido) decorrente da restrição hídrica. No setor industrial, a falta de garantia na disponibilidade de água impossibilita a ampliação da produção industrial, seja em unidades já existentes, seja por meio da implantação de novos empreendimentos, impactando negativamente a economia da região.

Neste contexto está inserido o problema de poluição, devido a efluentes industriais contendo corantes. Dentre os vários setores indústriais responsáveis por este tipo de poluição estão as indústrias de tintas e pigmentos, alimentos, cosméticos, têxteis, couro etc.

Os azocorantes, caracterizados pela presença de uma ou mais ligação azo (-N=N-), são os corantes sintéticos mais utilizados pelas industrias e geram grandes problemas para o

gerenciamento de efluentes. Estes corantes, mesmo em pequenas concentrações (1mg/L), causam coloração na água inaceitável para a população e prejudicam a solubilidade de oxigênio, além de muitos corantes serem fabricados a partir de carcinógenos como aminas aromáticas e fenóis que acabam sendo liberados no meio ambiente.

Os azocorantes não são degradados pelos tratamentos convencionais de lodos ativados utilizados em 80 % das indústrias e, necessitam de tratamento físico-químico para remover a cor dos efluentes. Estes, além de serem custosos, apenas transferem o problema, devido a formação de grandes quantidades de lodo gerado no processo, criando um sério problemas de disposição, o que vem a inutilizar grandes áreas usadas como depósito de lodo.

Com o aumento da preocupação em preservar o meio ambiente e, com a metade da população mundial enfrentando problemas de abastecimento de água, é fundamental que todos os setores usuários o façam de uma forma sustentável adotando práticas como o uso racional e eficiente da água, implantando sistemas fechados para a sua utilização, que até então era considerada como resíduo descartável. Na indústria de corantes, novas tecnologias para remover a cor dos efluentes visando o reuso da água tem ganhado atenção. No ambiente natural, os corantes podem ser transformados ou degradados por uma variedade de microrganismos, incluindo bactérias aeróbias, anaeróbias, leveduras, fungos e consórcios microbianos destes organismos.

Recentemente, novos processos incluindo condições anaeróbias e aeróbias tem sido desenvolvidos. Além do mais, o potencial biotecnológico de bactérias eficientes na degradação de corantes e matéria orgânica, aliada a pouca produção de biomassa produzindo efluente final sem toxicidade, tem sido amplamente procurado e estudado. No entanto, ainda pouco se sabe sobre o metabolismo dos corantes e sobre o sistema enzimático produzido pelos microrganismos que atuam na suas estruturas. Condições aeróbias, microaeróbias ou anaeróbias e a produção de enzimas intra e extracelulares, bem como a participação de mediadores redox têm sido investigado.

A seleção de linhagens capazes de metabolizar os corantes, bem como o estudo da formação e caracterização de seus metabólitos e a redução de toxicidade serão importantes contribuições para o desenvolvimento de novas biotecnologias envolvendo a degradação de corantes.

2. Revisão Bibliográfica

2.1. A história dos corantes

A percepção do mundo ao nosso redor é dada pelos nossos sentidos. Destes sentidos, o impacto visual é o mais marcante, não sendo definido apenas pela forma dos objetos, mas também pela cor. Dentre as percepções sensoriais do homem, 87% são captados pela visão, 9% pela audição e 4% pelo olfato, paladar e tato, sendo assim, a cor tem papel dominante em nossa vida diária, embora passe despercebida na maior parte do tempo.

Não se sabe bem ao certo o século, mas, desde o início da humanidade, pessoas usam corantes para colorir e tingir seu ambiente, pele, roupas e alimentos. A primeira evidência de uso de corantes pelo homem foi nas paredes da caverna de Altamira na Espanha e remonta 15000-9000 a.C. Os desenhos eram feitos com pigmentos inorgânicos como fuligem, óxido de mangânes, hematita e ocre (Clark et al., 1993).

As civilizações antigas também já tinham o hábito de retirar substâncias da natureza para colorir e melhorar a aparência de seus alimentos. Egípcios adicionavam extratos naturais e vinhos para melhorar a aparência de seus produtos (Downham et al., 2000). Muitas substâncias de origem animal, vegetal, ou mineral utilizadas como especiarias e condimentos, já tinham o objetivo de colorir os alimentos (Queija et al., 2001; IFT., 1986).

Muitas das técnicas de tingimento usadas no século XIX foram desenvolvidas pelos antigos egípcios, usando extratos de plantas em associação com um mordente. Outras civilizações desenvolveram métodos de tingimento, não somente com planta como o Indigo extraído da *Tinctoria isatis* e o vermelho alizarina de *Rubia tinctorum* mas também a partir de insetos *Persian scarlet*, moluscos *Tyrian purple*, fungos e líquens. Devido ao fato destas plantas e materiais serem geralmente nativos de suas regiões onde eram usados, a difusão destes métodos não foi possível por longo tempo (Carr, 1995). Naturalmente, as propriedades de muitos destes corantes estavam longe do ideal e este fato, juntamente com a indisponibilidade comercial das fontes de suprimento, encorajaram a busca por corantes sintéticos com propriedades superiores.

O primeiro corante orgânico sintetizado com técnica controlada foi o Mauve, obtido em 1856, por William H. Perkin. O cientista trabalhava em seu laboratório caseiro, estudando a oxidação da fenilamina, também conhecida como anilina, com dicromato de potássio ($K_2Cr_2O_7$). Certa vez, ao fazer a reação entre estes compostos, obteve um resultado surpreendente. Após jogar fora o precipitado, resultante da reação, e lavar os resíduos do frasco com álcool, Perkin admirou-se com o aparecimento de uma bonita coloração avermelhada. Ele repetiu a reação, sob as mesmas circunstâncias, e obteve de novo o corante, ao qual chamou de Púrpura de Tiro e que, posteriormente, passou a ser denominado pelos franceses de Mauve. Imediatamente, Perkin patenteou sua descoberta e, com ajuda financeira do pai e do irmão, montou uma indústria de malva (Abiquim, 1997). Nos anos seguintes, outros corantes foram desenvolvidos e somente em 1865, com a descoberta da estrutura molecular do benzeno, as pesquisas tomaram sentido sistemático e não empírico (Welham, 2000). Com esta descoberta vieram também os abusos, onde vários alimentos eram coloridos com substâncias altamente tóxicas para melhorar a aparência na tentativa de mascarar produtos de má qualidade. Na Inglaterra, no início do século XIX, foram relatados casos do uso de sulfato de cobre para colorir de verde as conservas de picles, chumbo negro em folhas de chá, para parecerem novas e chumbo vermelho para realçar a coloração alaranjada de alguns queijos (Downham et al.,2000; IFT,1986).

No início de século XX, os corantes naturais foram quase que totalmente substituídos pelos sintéticos. Hoje, a indústria de corantes dos Estados Unidos é a maior fonte exportadora destes produtos, colocando no mercado aproximadamente 2.000 tipos diferentes de corantes sintéticos.

Durante grande parte do século XIX, o Brasil foi a maior fonte natural do indigo e atualmente supre 60% de sua demanda doméstica (Nilsson, 1993). Com pequenas exceções, estas indústrias localizam-se basicamente no eixo Rio - São Paulo e a maioria é dependente de produtos intermediários importados, tais como: derivados de benzeno, naftaleno, tolueno, etc. Nos últimos anos, a exportação de corantes no Brasil tem mostrado um aumento anual ao redor de 40% (Beckmann and Sewekow, 1991). Por causa do seu baixo custo de síntese, estabilidade e variedade de cores quando comparado aos corantes naturais, os corantes sintéticos tem sido extensivamente usados, na indústria têxtil, papel, borracha, plástica, cosméticos, farmacêutica e de alimentos (Chagas and Durrant, 2001).

Em anos recentes, tem-se manifestado uma consciência crescente em torno das características possivelmente tóxicas de muitos corantes, incluindo a saúde e sua liberação no meio ambiente. Essa tendência tem-se refletido em alguns países, mediante o ajuste gradativo de sua utilização em produtos alimentares, cosméticos, medicinais e têxteis. O uso de corantes naturais tem sido visto como uma alternativa ambientalmente correta em vários processos de tingimento. Já existem vários grupos de pesquisa estudando o uso de corantes naturais não só em alimentos, mas também em tecidos (Angelini et al., 2003; Bermejo et al 2003; Singh et al., 2005). Algumas das vantagens deste tipo de corante é a ausência de toxicidade para humanos, uso de fontes sustentáveis, e a adequação aos caminhos naturais de biodegradação quando liberados nos efluentes (Ramalho, 2005).

2.2. Classificação e estrutura dos corantes

A matéria prima básica dos corantes sintéticos são compostos que, como o benzeno, derivam da destilação seca ou destrutiva do carbono. Por isso os corantes são conhecidos popularmente como derivados do alcatrão da hulha. A partir da matéria prima se elabora produtos intermediários mediante diversos processos químicos que normalmente implicam na substituição de elementos específicos ou radicais químicos por um ou mais átomos de hidrogênio da substância básica.

Todas as moléculas absorvem radiação eletromagnética, mas diferem no comprimento de onda absorvido. Algumas moléculas têm a habilidade de absorver luz no espectro visível (400-800 nm) e como resultado elas possuem cor.

Os corantes são materiais normalmente aplicados em solução e se fixam de alguma maneira a um substrato, que pode ser um tecido, papel, cabelo, couro, alimentos ou outros materiais. Devido à quantidade usada, o uso industrial mais significante é o da indústria têxtil com processos de tingimento. Idealmente, os corantes devem ser estáveis à luz e aos processos de lavagem. Também deve apresentar fixação uniforme com as fibras em todo o substrato.

O maior responsável pela absorção de luz na molécula do corante é o grupo cromóforo, um sistema de elétrons delocalizado com duplas ligações conjugadas. Em muitos casos os corantes

apresentam grupos adicionais chamados auxocromos, que podem ser doadores ou receptores de elétrons. Estes deslocam o comprimento de onda de absorção característico do grupamento cromóforo, intensificando a cor e promovendo a solubilidade e fixação do corante ao substrato (Rocha Gomes, 2001). Os grupos mais importantes de auxocromos são: NH_2, NR_2, NHR, COOH, SO_3H, OH e OCH_3 (De Las Marías, 1976).

Tendo em vista que corantes são compostos muito complexos, muitas vezes é impossível traduzi-los por uma fórmula química - alguns são misturas de vários compostos e outros não possuem estrutura química definida. Por esse motivo, a nomenclatura química usual raramente é usada, preferindo-se utilizar os nomes comerciais.

Para identificar os mesmos corantes, comercializados com diferentes nomes, utiliza-se o Colour Index (CI), publicação da American Association of Textile Chemists and Colorists e da British Society of Dyers and Colorists, que contém uma lista organizada de nomes e números para designar os diversos tipos. Os números de Colour Index são atribuídos quando a estrutura química é definida e conhecida (Abiquim).

Exemplo:

Tipo de Corante: Disperso Antraquinona
Nome Sistemático: 1-(2-Hidroxietilamino)-4-metilaminoantraquinoma
Nome Comum: Fast Blue FFR
Nomes Comerciais: Altocyl Brilliant-Blue B; Artisil Direct Blue BSQ e Cibacete Brilliant Blue BG
CI Nome: Disperse Blue 3
CI Número: 61505

Existem duas maneiras de se classificar os corantes sintéticos artificiais: de acordo com a estrutura química do grupo cromóforo (Tabela 1) ou através do método pelo qual o corante é fixado ao substrato (Tabela 2). Baseados na estrutura química ou cromóforo, aproximadamente 20-30 tipos diferentes de corantes são identificados (Ciência Hoje, 2001).

Tabela 1. Classificação dos corantes de acordo com seus respectivos grupos cromóforos

Classificação segundo as classes químicas	
Classe	**Classificação por aplicação**
Acridina	Básicos, pigmentos orgânicos
Aminocetona	À tina, mordentes
Antraquinona	Ácidos, mordentes, à tina, dispersos, azóicos, básicos, diretos, reativos, pigmentos orgânicos
Ao enxofre	Enxofre, à cuba
Azina	Ácidos, básicos, solventes, pigmentos orgânicos
Azo	Ácidos, diretos, dispersos, básicos, mordentes, reativos
Azóico	Básicos, naftóis
Bases de oxidação	Corantes especiais para tingimento de pelo, pelegos, cabelos
Difenilmetano	Ácidos, básicos, mordentes
Estilbeno	Diretos, reativos, branqueadores ópticos
Ftalocianina	Pigmentos orgânicos, ácidos, diretos, azóicos, à cuba, reativos, solventes
Indamina e Indofenol	Básicos, solventes
Indigóide	À tina, pigmentos orgânicos
Metina e Polimetina	Básicos, dispersos
Nitro	Ácidos, dispersos, mordentes
Nitroso	Ácidos, dispersos, mordentes
Oxazina	Básicos, mordentes, pigmentos orgânicos
Quinolina	Ácidos, básicos
Tiazina	Básicos, mordentes
Tiazol	Branqueadores ópticos, básicos, diretos
Triarilmetano	Ácidos, básicos, mordentes
Xanteno	Ácidos, básicos, mordentes e solventes

Fonte: ABIQUIM Associação Brasileira da Indústria Química

Tabela 2. Classificação dos corantes segundo a afinidade pelo substrato

CLASSIFICAÇÃO SEGUNDO A UTILIZAÇÃO POR SUBSTRATO	
Classe	**Principais campos de aplicação**
Branqueadores ópticos	Detergentes, fibras naturais, fibras artificiais, fibras sintéticas, óleos, plásticos, sabões, tintas e papel
Corantes	
À Cuba Sulfurados	Fibras naturais e fibras artificiais
À Tina	Fibras naturais
Ácidos	Alimentos, couro, fibras naturais, fibras sintéticas, lã e papel
Ao Enxofre	Fibras naturais
Azóicos	Fibras naturais, fibras sintéticas, alimentos,cosméticos
Básicos	Couro, fibras sintéticas, lã, madeira e papel
Diretos	Couro, fibras naturais, fibras artificiais e papel
Dispersos	Fibras artificiais e fibras sintéticas
Mordentes	Alumínio anodizado, lã, fibras naturais e fibras sintéticas
Reativos	Couro, fibras naturais, fibras artificiais e papel
Solventes	Ceras, cosméticos, gasolina, madeira, plásticos, solventes orgânicos, tintas de escrever e vernizes
Pigmentos Orgânicos	Tintas gráficas, tintas e vernizes, estamparia têxtil, plásticos
Pigmentos Inorgânicos	Tintas gráficas, estamparia têxtil, plásticos

Fonte: ABIQUIM Associação Brasileira da Indústria Quimica

Os grupo azo (monoazo,diazo, triazo, poliazo), antraquinona, ftaleína e trifenilmetano são os cromóforos mais importantes (Tabela 3).

Os corantes antraquinônicos, são basicamente uma só molécula e são estáveis eletronicamente. São resistentes à degradação devido a suas estruturas aromáticas fundidas, que permanecem coloridas por longos períodos de tempo (Banat et al.,1996).

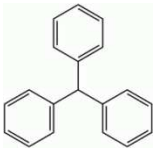
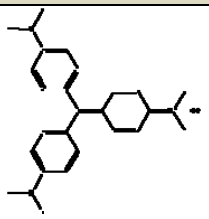
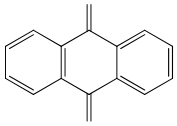
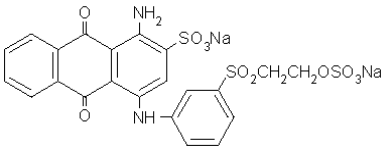
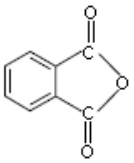
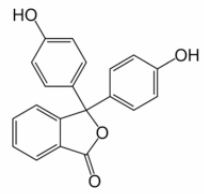
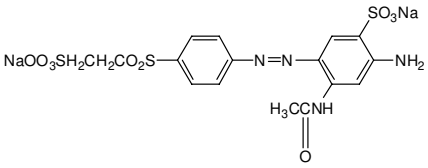
As ftaleínas são derivadas do aquecimento do anidrido ftálico com resorcinol (1,3-dihidroxibenzeno) em solução aquosa, produzindo um composto fluorescente chamado fluoresceína. A fluoresceína é um xanteno, uma classe de compostos também largamente empregados como corantes. Este composto é empregado nas placas de sinalização em rodovias

brasileiras. Vários derivados da fluoresceína são hoje utilizados como corantes, tal como a eosina (tetrabromofluoresceína), que é empregado como corante vermelho em cosméticos, tintas e papéis. Seu análogo eritrosina (tetraiodofluoresceína) é usado como corante vermelho em alimentos.

O trifenilmetano é um sólido incolor que apresenta três grupos fenil e um átomo de hidrogênio unidos a um átomo de carbono. Os primeiros corantes sintéticos eram derivados do trifenilmetano, que em geral era obtido a partir da anilina ou da toluidina. São compostos orgânicos solúveis em água e contêm um cátion responsável pela cor (cromóforo), devida à extensão da conjugação do seu sistema de ligações duplas (Ansel et al., 1998). O trifenilmetano se encontra em muitos corantes como a fenolftaleína, o verde de bromocresol e o verde malaquita.

Os azocorantes são considerados como a classe química mais importante para a indústria de corantes com participação de 50 a 65 % das formulações comerciais (Chung e Stevens,1993). Possuem a ligação azo como grupo cromóforo e são caracterizados pela presença de uma ou mais ligações azo — N=N — ligados a sistemas aromáticos e podem conter de um a três grupos sulfônicos (Marmion, 1991). Além de serem os mais aplicados na indústria têxtil, esta classe de corantes é também bastante utilizada pelas indústrias alimentícias, farmacêuticas, e de cosméticos (Rafii e Cerniglia, 1997). Os azocorantes mais importantes na industria alimentícia são: Amaranto, Ponceau 4 R, Vermelho 40 e Amarelo Crespúsculo.

Tabela 3. Classificação do corante segundo o grupo cromóforo.

Classe	Cromóforo	Exemplo
Trifenilmetano		 C.I.violeta básico 3
Antraquinona		 C.I.Azul Reativo 19
Ftaleina		 Fenolftaleína
Azo	—N=N—	 C.I. Amarelo Reativo 107

2.3. Azocorantes e sua liberação no ambiente

Em decorrência das tendências e demanda dos consumidorores, o setor industrial tem cada vez mais desenvolvido novos reagentes, processos, maquinaria e técnicas para a confecção de seus produtos, colocando o ambiente em contato com diversos poluentes e interrompemdo o equilíbrio natural, devido à toxicidade destes compostos. Entre as indústrias mais problemáticas em termos de

liberação de contaminantes no meio ambiente estão aquelas que produzem efluentes coloridos em seus processos. A liberação não controlada destes compostos no meio ambiente causam sérios problemas. Alguns corantes são compostos xenobióticos, pois não existem naturalmente no meio ambiente e contém grupos químicos que não podem ser sintetizados bioquimicamente (Rieger et al., 2002; Stolz, 2001). Dessa forma, os corantes utilizados no processamento de várias indústrias, chegam ao meio ambiente, diminuindo a absorção da luz e afetando a atividade fotossintética dos organismos autótrofos quando em meio aquático, e causando toxicidade pela presença de anéis aromáticos e metais pesados em suas estruturas (Banat et al. 1996, Carneiro et al. 2004). Em condições anaeróbias, encontradas nos sedimentos dos rios e em partes poluidas com alta Demanda Bioquímica de Oxigênio (DBO), os azocorantes são degradados por bactérias que liberam aminas aromáticas mutagênicas e potencialmente carcinogênicas no processo de biodegradação (Paszczynski et al., 1995).

Os 2-fenilbenzotriazóis (PBTA) são outra classe conhecida de compostos genotóxicos formados a partir de corantes azóicos, utilizados principalmente em unidades de tingimento de produtos têxteis, e podem ser produzidos durante o próprio processo industrial, ou em estações de tratamento de efluentes ou esgotos. As etapas do processo industrial de tingimento, bem como do tratamento de efluentes resultantes, incluem redução com hidrossulfito de sódio para descoloração dos corantes remanescentes, levando à formação dos PBTA não-clorados. Estes, no processo de cloração realizado para desinfecção dos efluentes, são convertidos nos PBTA. Os PBTA, não clorados e os seus corantes azóicos precursores apresentam atividade mutagênica para diferentes linhagens de *Salmonella typhimurium* do teste de Salmonella/microssoma Nukaya et al., 2001; Watanabe et al., 2006).

As análises dos efluentes utilizando testes de toxicidade têm se tornado uma prática comum e incluída na legislação brasileira. Estes testes ajudam a compreender os mecanismos dos efeitos de impactos sobre os compartimentos bióticos, utilizando-se organismos vivos que atuam como biossensores que podem prever impactos de determinados poluentes sobre a biota.

Os organismos mais utilizados na avaliação da toxicidade de substâncias são as algas e o zooplâncton, porém uma grande variedade de outros organismos aquáticos são comumente usados em testes de toxicidade, incluindo invertebrados (espécies *de Daphnia*) e peixes *(Danio rerio,*

Cyprinus carpi) Masutti et al., 2006. Vários estudos têm demonstrado que efluentes contendo azocorantes apresentam toxicidade aguda e crônica frente ao microcrustaceo *Daphnia magna,* e que alguns tipos de tratamento como microaeróbio são conhecidos por remover a cor dos efluentes, porém não são suficientes para remover a toxicidade das amostras (Immich et al.,2009; Franciscon et al.,2009 a,b). Há também a possibilidade de bioacumalação em peixes onde fatalmente ocorreria a entrada destes compostos na cadeia alimentar. Estudos recentes demostraram que efluentes têxteis contendo azocorantes foram citotóxicos e mutagênicos para eritrócitos de peixes, reduzindo sua quantidade e causando anomalias progressivas com a dose e tempo de exposição (Sharma et al., 2009). Amostras ambientais coletadas na área do Ribeirão dos Cristais, região metropolitana de São Paulo, apresentaram atividade mutagênica sistemática durante os últimos anos. De acordo com estudos já publicados em literatura, essa contaminação estava relacionada ao lançamento de efluentes líquidos de uma indústria de tingimento neste corpo d'agua (Oliveira, 2005). O mais preocupante é que estes corantes apresentam grupos quimicamente ativos, capazes de reagir covalentemente com celulose e com grupos de proteínas biologicamente importantes (Abeta et al., 1984). Deste modo, resíduos destes corantes poderiam ser altamente nocivos quando presentes em qualquer organismo vivo.

Alguns autores têm demonstrado que estes compostos na forma não-hidrolisada apresentam alta estabilidade hidrolítica em meio neutro, permitindo um tempo de vida de 50 anos em ambientes aquáticos, causando expressiva preocupação quanto aos aspectos ecológicos (Weber et al., 1993). Por isso, é fundamental fazer novos testes de toxicidade ecológica e confrontá-los com estudos já existentes na literatura sobre efluentes coloridos pois, ainda é extremamente necessário entender mais sobre os mecanismos de transporte e transformação destes compostos em organismos vivos.

2.4. Aspectos toxicológicos e saúde.

Os riscos toxicológicos de corantes sintéticos à saúde humana estão intrinsicamente relacionados ao modo (ingestão oral, sensibilização da pele, sensibilização das vias respiratórias) e tempo de exposição, (Clarke and Steinle, 1995). A análise do grau de toxicidade oral de corantes,

medido através de 50% da dose letal (LD_{50}) tem demonstrado que apenas um número reduzido de corantes pode apresentar toxicidade aguda (LD_{50} < 5g/Kg) e são encontrados principalmente nos azocorantes (Clarke e Anliker,1980). Quando há exposição oral, os azocorantes são metabolizados por mecanismos de biotransformação baseados principalmente em processos de oxidação, hidrólise, conjugação e redução, cuja velocidade de degradação é acelerada através de processos catalíticos enzimáticos (Hunger, 1994). Desse modo, a formação de aminas aromáticas podem ser geradas rapidamente, uma vez que estas enzimas contribuem na degradação, sendo incapazes de diferenciar se os produtos gerados são nocivos ou não ao organismo (Figura 1).

Em 1906, surgiram as primeiras suspeitas da ação cancerígena dos corantes. Ao injetar um corante azóico sob a pele da orelha de um coelho, observou-se crescimento celular atípico sob a pele. Em 1924, foi observado que a ingestão deste azocorante por camundongos podia provocar a formação de adenomas hepáticos (Lederer, 1990).

Os corantes que apresentam o grupo azo (-N=N-), são também usados nas indústrias alimentícias para intensificar a coloração dos alimentos, tornando-os mais atrativos visualmente. Esses corantes podem ser metabolizados pela microbiota intestinal e muitos desses compostos se mostraram mutagênicos no teste de Ames (Chung and Cerniglia, 1992). O Ministério da Saúde, por meio da Agência Nacional de Vigilância Sanitária – ANVISA, permite a utilização de corantes artificiais em alimentos em quantidades controladas, devido à possibilidade de toxicidade destes produtos (Kapor et al., 2001). Apesar dos corantes sintéticos terem menores custos de produção, maior estabilidade e capacidade tintorial, o que se constata é que, a despeito dessas vantagens, o número de aditivos sintéticos permitidos nos países desenvolvidos vem diminuindo a cada ano. Recentemente, a Austrália proibiu a circulação de todos eles, e os Estados Unidos e o Japão prevêem sua retirada total do mercado até o início da próxima década. No Brasil, em julho/agosto de 1987, a ANVISA, pela Portaria número 2, proibiu a utilização dos seguintes corantes sintéticos: Amarelo Ácido, Azul de Indatreno, Vermelho Sólido E, Escarlate GN e Laranja GCN. Apenas os corantes Amarelo Crepúsculo, Azul Brilhante FCF, Bordeaux S ou Amaranto, Eritrosina, Indigotina, Ponceau 4R, Tartrazina e Vermelho 40 tiveram a autorização mantida.

A Diretiva 2004/21/CE, em vigor desde 1 de janeiro de 2005, limita a colocação no mercado europeu de artigos acabados como têxteis ou couro, tingidos com corantes azóicos, que por

clivagem redutora de um ou mais grupos azo (-N=N-), possam liberar determinadas aminas aromáticas, detectáveis em concentrações superiores a 30mg/L, conforme métodos de ensaio estabelecidos. Esta Diretiva tem como principal objetivo proteger a saúde da população européia que, numa exposição prolongada a estas substâncias, se tornaria suscetível a contrair doenças cancerígenas (Directiva 2004/21/CE.,2004).

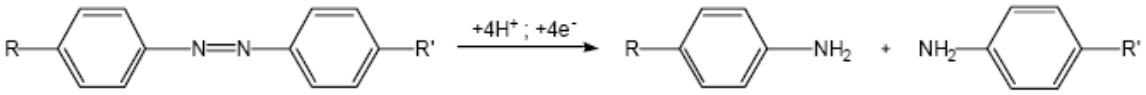


Figura 1. Redução de azocorantes e formação de aminas aromáticas

2.5. Tecnologias usuais de tratamento para efluentes com corantes

A cor de uma amostra de água está associada ao grau de redução de intensidade que a luz sofre ao atravessá-la e esta redução dá-se por absorção de parte da radiação eletromagnética, devido à presença de sólidos dissolvidos, principalmente material em estado coloidal orgânico e inorgânico.

A resolução do CONAMA (Conselho Nacional de Meio Ambiente) nº 357/05, que dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, e estabelece as condições e padrões de lançamento de efluentes, não fixa valores máximos para o parâmetro de cor. Entretanto, estabelece que o lançamento não poderá modificar a característica original do corpo receptor, ou seja, visualmente não é permitida a presença de corantes provenientes de fontes antrópicas que não sejam removíveis por processos de coagulação, sedimentação e filtração convencionais. Os valores máximos permitidos para o padrão de cor em cada classe de corpo hídrico, medido em miligramas de platina-cobalto por litro não deverão ser ultrapassados após o lançamento.

A Lei Federal n° 9.433/97, que instituiu a Política Nacional de Recursos Hídricos, estabelece que serão cobrados o uso da água e o lançamento de efluentes em corpos hídricos em todo país. É crescente, portanto, a preocupação dos consumidores industriais em reduzir o volume de água utilizada. Neste contexto, o reuso de água no processo produtivo torna-se uma meta a ser alcançada. Os efluentes dos processos que utilizam corantes apresentam uma limitação evidente para a reutilização no próprio processo, pois a qualidade requerida da água para o processo é alta em termos de ausência de cor. O processo de tratamento empregado deverá ter alta eficiência em remoção de cor para tornar possível a reutilização da água. Deste modo, métodos para remoção da cor das águas de rejeito têm recebido enorme atenção nos últimos anos. O desenvolvimento de tecnologias adequadas para o tratamento de efluentes tem sido objeto de grande interesse nos últimos tempos, devido ao aumento da conscientização e rigidez das leis ambientais. As principais técnicas disponíveis na literatura para descoloração dos efluentes envolvem principalmente processos químicos, físicos e biológicos.

2.5.1. Químicos

As técnicas de tratamento utilizando-se degradação química baseiam-se principalmente na reação oxidativa pelo cloro, peróxido de hidrogênio ou ozônio.

As técnicas de destruição baseadas no uso de ozônio têm se demostrado mais efetivas do que aquelas com cloro, que são insatisfatórias para alguns tipos de corantes (corantes dispersos e diretos), além de apresentarem a vantagem adicional de não produzir íons inorgânicos, como no tratamento com cloro. Considerado um bom agente oxidante devido a sua grande instabilidade, possui potencial de oxidação igual a 2,08. Degrada um grande número de poluentes presentes em efluentes como fenóis, pesticidas, hidrocarbonetos aromáticos e corantes (Robinson et al., 2001; Pera-Titus et al., 2004). Para efluentes coloridos, o método é baseado na remoção da cor através da clivagem das moléculas do corante em processo catalítico ou radiação ultravioleta.

Durante o processo de ozonização ocorre a descoloração do efluente devido à clivagem oxidativa dos grupos cromóforos, seja por via direta ou indireta . A clivagem destas ligações duplas conjugadas e de outros grupos funcionais faz com que as moléculas percam a habilidade de absorver luz na região visível do espectro eletromagnético.

As desvantagens do processo estão relacionandas com a limitada remoção de matéria orgânica expressa como COT e DBO, explicado pela capacidade do ozônio de converter os compostos orgânicos de cadeia longa para compostos menores e biodegradáveis. O tempo curto de vida útil do ozônio, aproximadamente 20 minutos, também é um aspecto negativo, pois requer ozonização contínua, inviabilizando economicamente a aplicação. A demanda de ozônio é muito influenciada pela presença de matéria orgânica no efluente. As dosagens aplicadas ao efluente dependem da demanda Química de Oxigênio (DQO) residual a ser removida, da cor ou de outro parâmetro a ser oxidado (Robinson et al., 2001). Diversas pesquisas têm relatado que a ozonização pode produzir compostos mutagênicos tóxicos ou cancerígenos na água ozonizada (White,1999).

Os efluentes coloridos também podem ser químicamente oxidados por compostos contendo cloro. Os principais compostos de cloro usados em sistemas de tratamento de efluentes são o cloro elementar (Cl_2), o hipoclorito de sódio (NaOCl), o hipoclorito de cálcio [$Ca(OCl)_2$], e o dióxido de cloro (ClO_2). Dentre estes compostos o dióxido de cloro é um dos mais usados como oxidante, pois é altamente solúvel em água e não tem sabor e odor ofensivo aos sentidos (Junli et al.,1997). No processo de descoloração, o Cloro Cl^+, inicia um ataque eletrofílico no grupo amino acelerando a quebra da dupla azo (Robinson et al., 2001). Este método não é eficiente para descolorir corantes sintéticos e requer um longo tempo de contato para corantes reativos, além da preocupação da formação de compostos organoclorados (Slokar and Le Marechal, 1998).

Os impactos ambientais associados ao uso do ClO_2 em efluentes não são muito bem conhecidos. O ClO_2 não se dissocia ou reage com água como faz o cloro. No entanto, devido sua produção envolver reação entre cloreto de sódio e cloro, o cloro livre pode permanecer como resultado da solução de ClO_2 e o ambiente aquático receber este impacto, como faz o cloro residual (Metcalf e Eddy, 2003).

2.5.2. Físicos

Os processos físicos são caracterizados pela separação de fases (sedimentação, decantação, filtração, centrifugação e flotação), transição de fases (extração por solventes e adsorção), além de separação molecular, que utiliza membranas seletivas (hiperfiltração, ultrafiltração, osmose reversa e diálise) Barreto , 2001.

Em geral, o tratamento físico permite a depuração dos efluentes. Para a redução das cargas de difícil decomposição, como é o caso dos corantes, freqüentemente se utilizam processos de coagulação-floculação, onde através da adição de determinados produtos químicos ao efluente se forma um floco que contém os produtos indesejáveis, os quais são retirados por meio de sedimentação, flotação e filtração. Normalmente utiliza-se um excesso de polieletrólito como $Al_2(SO_4)_3$ e amônia, por exemplo, que por sua vez irá acrescentar um resíduo a mais no efluente.

O carvão ativado também pode atuar no tratamento de efluentes, extraindo assim a cor, odor, matéria orgânica, compostos tóxicos e servindo de suporte para a biomassa. A economia na aplicação do carvão ativado depende de um eficiente meio de regeneração e reativação do carvão para remoção do material adsorvido depois que sua capacidade de adsorção tenha se esgostado (Eckenfelder, 1989). Normalmente, uma parte da capacidade de adsorção do carvão é perdida durante o processo de regeneração (Crittenden, 2000).

O processo de separação por membranas (SPM) se baseia também em mecanismos físicos, isto é, não envolve processos químicos, biológicos ou trocas térmicas. Refere-se apenas a separação dos componentes de uma mistura pela rejeição daqueles que não possuem tamanho para atravessar os poros da membrana. A eficiência deste tipo de filtração depende inteiramente da diferença de tamanho entre o poro e a partícula a ser removida (Eckenfelder, 1989). Os processos com membranas incluem várias técnicas, nas quais a pressão é utilizada para passar o efluente sobre uma membrana semi-permeável. Essa membrana permite que a água atravesse, e impede a passagem das substâncias solúveis que estão contidas no efluente. Variando as membranas, pode-se eliminar da água substâncias de distintos pesos moleculares (Sanin, 1997).

No entanto, as substâncias contaminantes não são degradadas ou eliminadas, mas apenas transferidas para uma nova fase. E nesta nova fase, embora o volume seja reduzido, continua persistindo o problema. A disposição de concentrados produzidos por processo de separação em membranas representa o maior problema que deve ser considerado no uso destas aplicações. Na maioria dos casos os processos físicos para remoção de cor apenas transferem o problema, pois o lodo colorido gerado ainda será disposto em aterros, inviabilizando grandes áreas.

2.5.3. Biológicos

Dentre as opções mais econômicas e viáveis para o tratamento de efluentes coloridos, os sistemas biológicos se apresentam como os mais práticos e de baixo custo, entretanto, podem ser bastante complexos (Méndez-Paz et al., 2005).

O papel de plantas em tratamento de xenobióticos chamado fitorremediação tem aumentado o interesse de pesquisadores . As plantas possuem um sistema enzimático mais complexo que podem agir juntamente com enzimas de microrganismos existentes nas raízes destas plantas em diferentes condições e levar a parcial ou a total degradação por enzimas redutivas, oxidativas e hidrolíticas (Schwitzguébel and Vanek, 2003;). Pesquisa com *Phragmites australis* e *Blumea malcolmii* demostraram serem eficientes na descoloração e degradação de vários azocorantes, porém em concentrações não muito altas (20 mg L^{-1}). Estes sistemas têm a desvantagem de necessitar de grandes áreas para implementação do tratamento. Entretanto a grande maioria dos processos biológicos são baseados na nutrição de microrganismos pelo substrato a ser tratado. Devido a sua versatilidade, podem ser usados em uma ampla classe de compostos orgânicos poluentes (Freire et al.,2000).

Conforme Crueger e Crueger (1993), os sistemas de tratamento de efluentes baseados nos princípios da biodegradação devem atender a aspectos importantes, como remoção da matéria orgânica, redução da DBO, do resíduo a ser tratado bem como a degradação de compostos químicos orgânicos de difícil remoção, resultando no fornecimento de um efluente em condições que não afete o equilíbrio dos sistemas receptores finais como rios e lagos (Crueger and Crueger, 1993).

O uso de processos aeróbios denominados de lodos ativados são muito comuns em indústrias para tratar efluentes com corantes. Estes processos são eficientes na remoção da matéria orgânica, porém não são capazes de degradar os corantes, que ficam adsorvidos na massa bacteriana, resultando em grande quantidade de lodo colorido a ser disposto em aterros (Franciscon, 2005).

2.6. Metabolismo de degradação de corantes por microrganismos

Dentre as múltiplas estratégias aplicadas para a eliminação de corantes, a biodegradação ocupa um lugar de destaque por seu apelo ecológico. Esta consiste na modificação estrutural ou adsorção de substratos poluentes por diferentes organismos (bactérias, leveduras e fungos) levando à introdução destes compostos no fluxo de nutrientes na natureza. A aplicação de processos biológicos na remoção de compostos químicos recalcitrantes na natureza é designada biorremediação (Banat et al.,1996; Gianfreda e Rao, 2004; Ramalho et al., 2005). Muitas enzimas microbianas são utilizadas no tratamento de efluentes e resíduos industriais, para resolver problemas específicos. Assim, pode-se citar a remoção por precipitação ou transformação de compostos tóxicos ou recalcitrantes, e a alteração das características de um determinado efluente, como o aumento da degradabilidade ou diminuição da toxicidade, permitindo o tratamento posterior por processos biológicos convencionais ou a formação de materiais em produtos de valor agregado (Karan and Nicel,1997).

2.6.1. Degradação de corantes por Leveduras

Na literatura há poucos relatos sobre degradação de azocorantes por leveduras. Os primeiros trabalhos envolveram a levedura *Candida zeylanoides* isoladas de solo contaminado (Martins *et al*. 1999, Ramalho *et al.,* 2002). A caracterização de uma atividade enzimática para degradação de azocorantes foi descrita para *Issatchenkia occidentalis* (Ramalho *et al.,* 2004) e *Saccharomyces cerevisiae* (Ramalho *et al.,* 2005).

2.6.2. Degradação de corantes por Fungos

Muitos trabalhos tiveram como objetivo isolar e selecionar fungos que apresentaram capacidade de descolorir diferentes corantes comerciais (Okino *et al.,* 2000; Levin *et al.,* 2004; Dhouib *et al.,* 2005). Os fungos, por exemplo, possuem grande capacidade em degradar parcialmente, e em alguns casos completamente, uma variedade de poluentes resistentes a

degradação, através da ação de enzimas específicas produzidas por estes microrganismos como lignina peroxidase (LiP) e mangânes peroxidases (MnP), ou ainda fenol-oxidases como lacases (Lac) e tirosinases.

As lacases, que possuem 4 átomos de cobre em sua estrutura molecular, oxidam muitos fenóis utilizando o O_2 como receptor final de elétrons (Bon et al., 2008). Estas enzimas apresentam grande capacidade em despolimerizar a lignina e uma grande variedade de outros compostos, como os corantes presentes nos efluentes devido a sua falta de especificidade ao substrato (Forgacs et al.,2004; Novotný et al., 2004). A degradação de estruturas aromáticas pelos fungos acontece no metabolismo secundário e começa quando nutrientes como carbono, nitrogênio e enxofre se tornam limitantes (Kirk e Farrel 1987)**.** As lacases são particularmente interessantes no tratamento de águas residuárias, onde os poluentes fenólicos oxidados pela lacase originam polímeros insolúveis em água, os quais são de remoção mais fácil. As lacases são fenol-oxidases que catalisam a oxidação de várias substâncias aromáticas e inorgânicas com concomitante redução de oxigênio para água. Têm sido extensivamente estudada pela degradação de azocorantes, pois descolorem estes compostos através de um mecanismo de radical livre não específico. Após um período de tempo pode haver um acoplamento entre os produtos da reação e mesmo uma polimerização (Figura 2) Zille et al, 2005.

A estrutura química dos corantes e grupos laterais podem influenciar a degradação pelos fungos, sendo que esta dificuldade pode ser ainda maior quando consideramos a variabilidade na composição dos efluentes contendo corantes de cada indústria (Fu e Viraraghavan 2001).

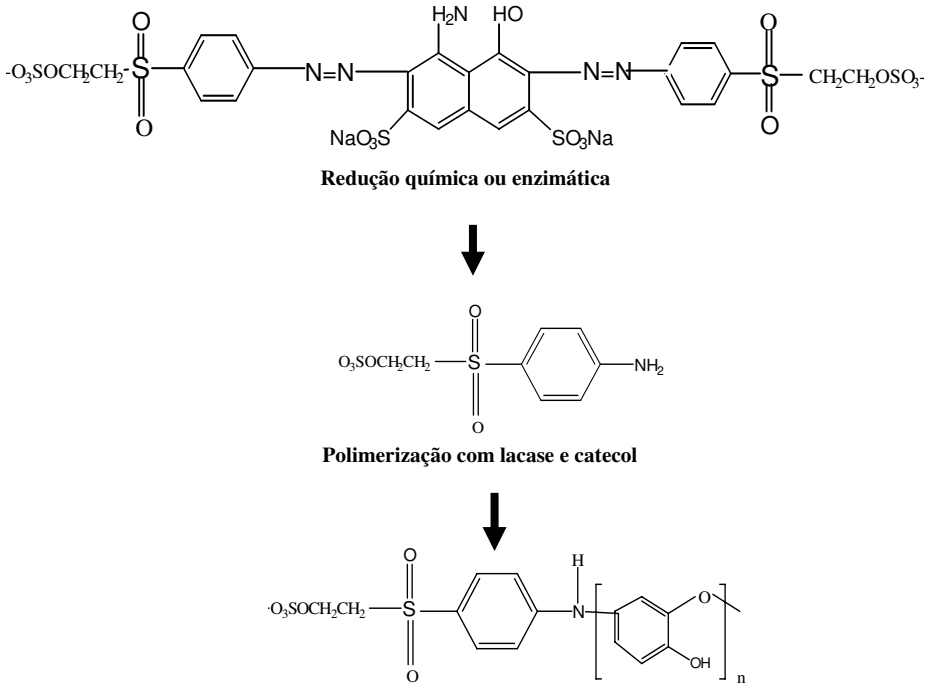


Figura 2. Polimerização enzimática do corante Preto Reactivo 5

2.6.3. Degradação de corantes por Bactérias

A redução bacteriana das ligações azo não é específica e usualmente é mais rápida quando comparada a degradação por fungos. Este tipo de descoloração é ambientalmente correto e mais barato quando comparado com a degradação por processos químicos (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).

Os processos que utilizam bactérias para degradação de azocorantes são classificados como anaeróbios e aeróbios. Há alguns relatos sobre bactérias que descolorem azocorantes aerobicamente (*Xenphylus azovorans* KF46F, *Bacillus* strain, *Kerstersia* sp strain VKY1 e *Staphylococcus* sp), porém é em condições anaeróbias que a descoloração é mais efetiva (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).

Para bactérias aeróbias é necessária uma adaptação para ocorrer processos redutivos significantes. Esta adaptação envolve um longo tempo de crescimento aeróbio em culturas contínuas na presença de um composto azo simples. A bactéria sintetiza uma azoredutase específica para estes compostos, que sob condições controladas, podem clivar o grupo azo na presença de oxigênio. Porém, a presença de oxigênio normalmente inibe a atividade de azoredução, uma vez que a respiração aeróbia pode dominar o uso de carreadores de elétrons (NADH, FADH) impedindo estes de serem entregues a ligação azo, ocasionando uma ineficiente descoloração (Stolz, 2001).

Por outro lado, em condições anaeróbias, muitas bactérias podem reduzir os azocorantes através da atividade de redutases inespecíficas. Este tipo de degradação anaeróbia faz com que os azocorantes sejam convertidos em aminas aromáticas tóxicas, mutagênicas e possivelmente carcinogênicas para mamíferos (Pinheiro et al., 2004). Alguns estudos têm sugerido outras formas de redução de azocorantes em condições reduzidas de oxigênio, o qual não requer transporte do azocorante ou de carreadores de elétrons como as flavinas, pois estes não teriam facilidade de ultrapassar a membrana celular (Russ et al., 2000).

Estudos mais recentes fornecem evidência de que a azoredução anaeróbia por bactérias esteja ligada a cadeia de trasporte de elétrons e sugere uma azoredução dissimulatória como uma forma de respiração microbiana anaeróbia (Hong et al, 2007).

Para alcançar a completa degradação dos azocorantes, alguns estudos tem incluído um estágio aeróbio depois do anaeróbio, para biodegradar as aminas aromáticas produzidas (Libra *et al.* 2004; Sponza e Isik, 2005, Franciscon et al., 2009 a,b).

Além destes trabalhos, pode-se encontrar estudos sobre a oxidação enzimática de muitos corantes sintéticos, das classes azo, heterocíclicos, poliméricos e trifenilmetanos. A utilização de peroxidases e fenol-oxidases como lacases e tirosinases tem sido umas das principais aplicações destas enzimas no tratamento de efluentes industriais (Bon et al, 2008).

O envolvimento destas enzimas na oxidação de azocorantes sulfonados foi relatado anteriormente (Kandelbauer et al, 2004). Peroxidases bacterianas extracelulares que oxidam azocorantes foram caracterizadas em *Streptomyces chromofuscus* (Pasti-Grigsby et al,1996). Recentemente, um estudo realizado por Kalme et al (2006), sugeriu que enzimas como lignina

peroxidase, laccase e tirosinase estiveram envolvidas diretamente na descoloração do corante Direct Blue 6 em 72 hs e também de seus intermediários.

O envolvimento de tirosinase na degradação de compostos aromáticos é menos estudado do que o papel das lacases. Entretanto, a degradação de compostos pela tirosinase tornou-se recentemente o foco de várias investigações. Tirosinase tem a vantagem de não requerer mediador para degradação de compostos aromáticos e envolve ambas reacões monofenolase e difenolase, enquanto a lacase envolve apenas reações de difenolase (Dec J and Bollag, 2000).

Tirosinase (o-difenol, oxigênio oxidoredutase) EC 1.10.3.1; é uma enzima que contém cobre no seu sítio ativo largamente distribuída na natureza, conhecida também como fenol monoxigenase, catecol oxidase ou polifenol oxidase. É muito utilizada em várias reações com derivados de catecóis e fenóis, pela sua capacidade em oxidar estes compostos a quinonas em presença de oxigênio (Brown *et al.*, 1994).

Estas pesquisas sugerem que azoredução é um mecanismo específico para cada linhagem bacteriana, e que pode ser realizada por azoredutases, oxidoredutases ou por um caminho metabólico mais complexo.

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3. OBJETIVOS

3.1. Objetivos Gerais

O principal objetivo deste estudo foi observar as diferentes formas de degradação de quatro azocorantes realizadas por diferentes linhagens bacterianas isoladas de efluentes industriais contendo corantes, utilizando processos microaeróbios e aeróbios sucessivamente, procurando desta forma, fornecer mais detalhes as pesquisas realizadas nesta área.

3.2. Objetivos Específicos

- Avaliar a dinâmica do processo de descoloração dos azocorantes com a utilização de diferentes substratos como fonte de carbono e concentrações;
- Determinar a descoloração dos azocorantes presentes no meio de cultura por análises de Espectrofotômetro UV-Vis e medir se a atividade enzimática de lacase, peroxidase e tirosinase estavam presentes durante a degradação;
- Avaliar a degradação dos corantes e a produção de metabólitos por Espectrofotômetro de Infravermelho com Transformadas de Fourier (FTIV), e por Cromatografia Líquida de Alta Eficiência e Espectometria de Massa (CLAE-EM);
- Monitorar a toxicidade dos azocorantes, bem como seus produtos de degradação após o tratamento redutivo e oxidativo, através da utilização do microcrustáceo *Daphnia magna*;
- Investigar a possibilidade de polimerização das aminas aromáticas formadas após biodegradação dos azocorantes, através do uso da enzima lacase, bem como a redução da DQO após tratamento;
- Identificar os microrganismos selecionados através de análises de sequência de rDNA 16S.

4. Capítulo 1

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

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

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.

Keywords: azo dyes, *Staphylococcus arlettae*, biodegradation, textile effluents, aromatic amines

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, 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 decolorization. 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., 2005; 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 decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using (Abadulla et al., 2000). In contrast, manganese-peroxidase was reported as the main enzyme involved in dye decolorization 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 decolorize dyes have also been reported (Kim and Shoda, 1999; Cha et al.,

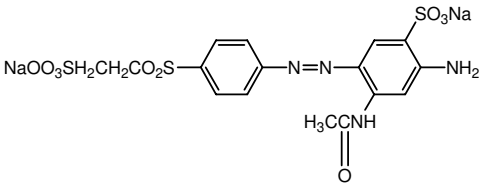
2001; Ambrósio and Campos-Takaki, 2004; Bumpus, 2004; Tetsch et al., 2005). However, the long growth cycle and the complexity of the textile effluents, which are extremely variable in composition, limit the performance of these fungi. Although stable operation of continuous fungal bioreactors for the 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 decolorization 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, 2001; 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 (Işık and Sponza, 2003; Işık 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; Bras 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.

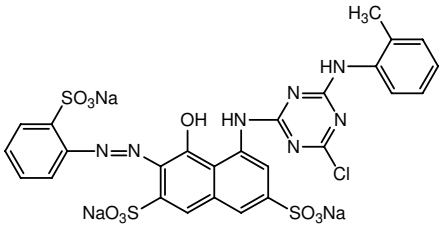
2. Material and methods

2.1. Chemicals and media

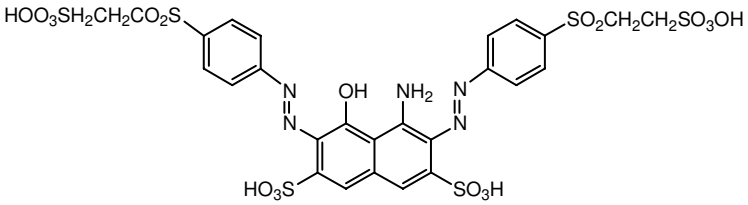
The azo dyes (100 mg L^{-1}) C.I. Reactive Yellow 107 (RY107, 0.18 mM), C.I. Reactive Black 5 (RB5, 0.11 mM), C.I. Reactive Red 198 (RR198, 0.13 mM) and C.I. Direct Blue 71 (DB71, 0.1 mM) were kindly provided by the Vicunha textile company, Itatiba, Brazil. The structures of the dyes are shown in Figure 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}), $MgSO_4x7H_2O$ (0.2 g L^{-1}), $FeSO_4$ $7H_2O$ (0.01 g L^{-1}), NaCl (0.1 g L^{-1}), $CaCl_2$ x 2 H_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.



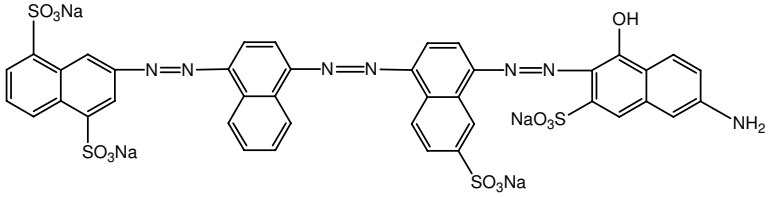
C.I. Reactive Yellow 107 (RY107)



C.I. Reactive Red 198 (RR198)



C.I. Reactive Black 5 (RB5)



C.I. Direct Blue 71 (DB71)

Figure 1. Chemical dye structures.

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} to 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 (0.1g L^{-1}) and mixed with azo dyes (100 mg L^{-1} each dyes) and incubated under microaerophilic conditions at 30 °C for seven 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 94ºC, followed by 10 cycles of 1 min each at 94ºC, 30 s at 69ºC, decreasing 0.5ºC each cycle, and 3 min at 72ºC, followed by another 10 cycles of 1 min each at 94ºC, 30 s at 63ºC and 3 min at 72ºC, 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 incubation under the microaerophilic and aerobic conditions, frozen and freeze-dried (FTS System model Dura-Dry MP). The samples (5mg) were dissolved in 5 mL of a 0.4% solution of chloranil in dimethylformamide (DMF) and heated to 100ºC 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 calculated in mM. 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 for 168 hs. Dye decolourization was measured in a UV-visible 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 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 60ºC for 24 hours and pressed to obtain IR-transparent pellets. The absorbance spectra of the samples were recorded using a Fourier transform infrared spectroscopy (FTIR) 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 FTIR 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 to 2000μL) (Shimadzu Corporation,1997). Samples were centrifuged (20.000 x g for 15 min) and filtered through a 0.45µm 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 to 4000 $mg.L^{-1}$. 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 x g for 20 min and filtered through a 0.45µm pore size filter. The acute toxicity tests with D*aphnia 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 (0.1g 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 numbers for GenBank access are represented in parenthesis. *Sulfobacillus acidophilus* DSM 10332^T was used as the outgroup. The phylogenetic tree (Figure 2) showed the grouping of VN-11 within *Staphylococcus arlettae,* confirming its identification due to the high bootstrap value.

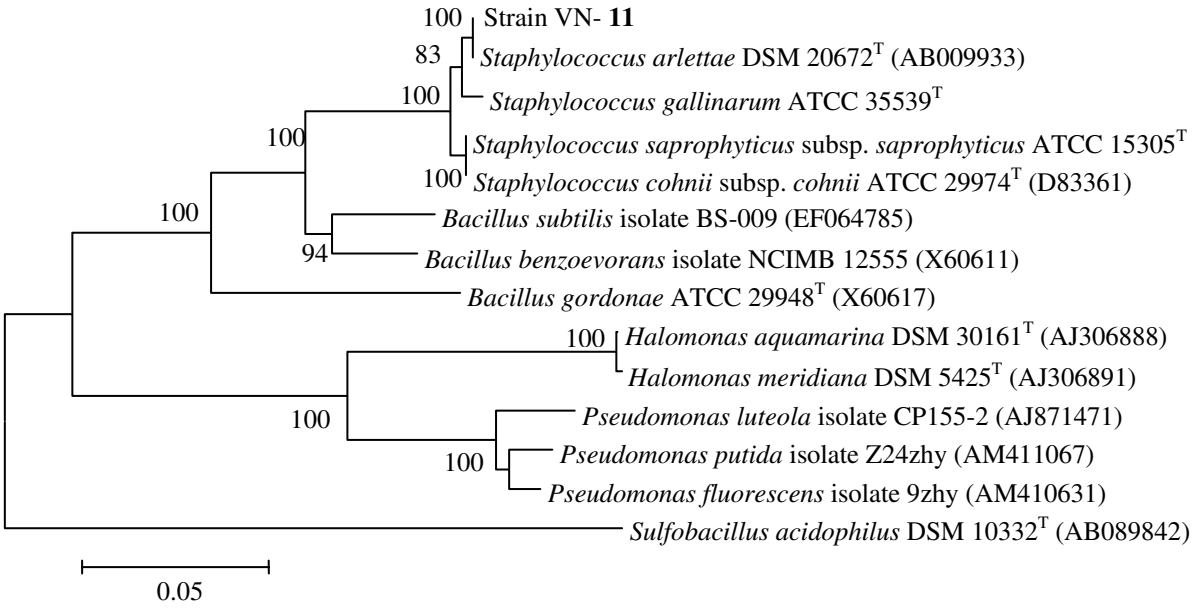


Figure 2. Phylogenetic tree of the *Staphylococcus arlettae*. strain VN-11 for the partial sequences based on 16S rDNA.

3.2. Decolourization

The strain *Staphylococcus arlettae* was tested to separately decolourize four azo dyes (C. I. Reactive Yellow 107, C. I. Reactive Red 198, C. I. Reactive Black 5 and C. I. 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).

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

	Amine concentration (mM)		Decolourization time (h)	Decolourization (%)	
Dyes	**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

n.d. – not detected

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 of, respectively 0.21 and 0.13 mM. 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 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 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. Untreated dyes: As shown in Figure 3(a), RY107 presented two absorbance peaks at 301 and 550 nm. As shown in Figure 3(b) RR198 presented absorbance peaks at 513, 380, 290 and 220 nm and a shoulder at 325 nm. Figure 3(c) C. I. 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 Figure 3(d), DB71 presented one large peak at 412 and a smaller peak at 300 nm. Wide band absorption near 220 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 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 (Figure 3).

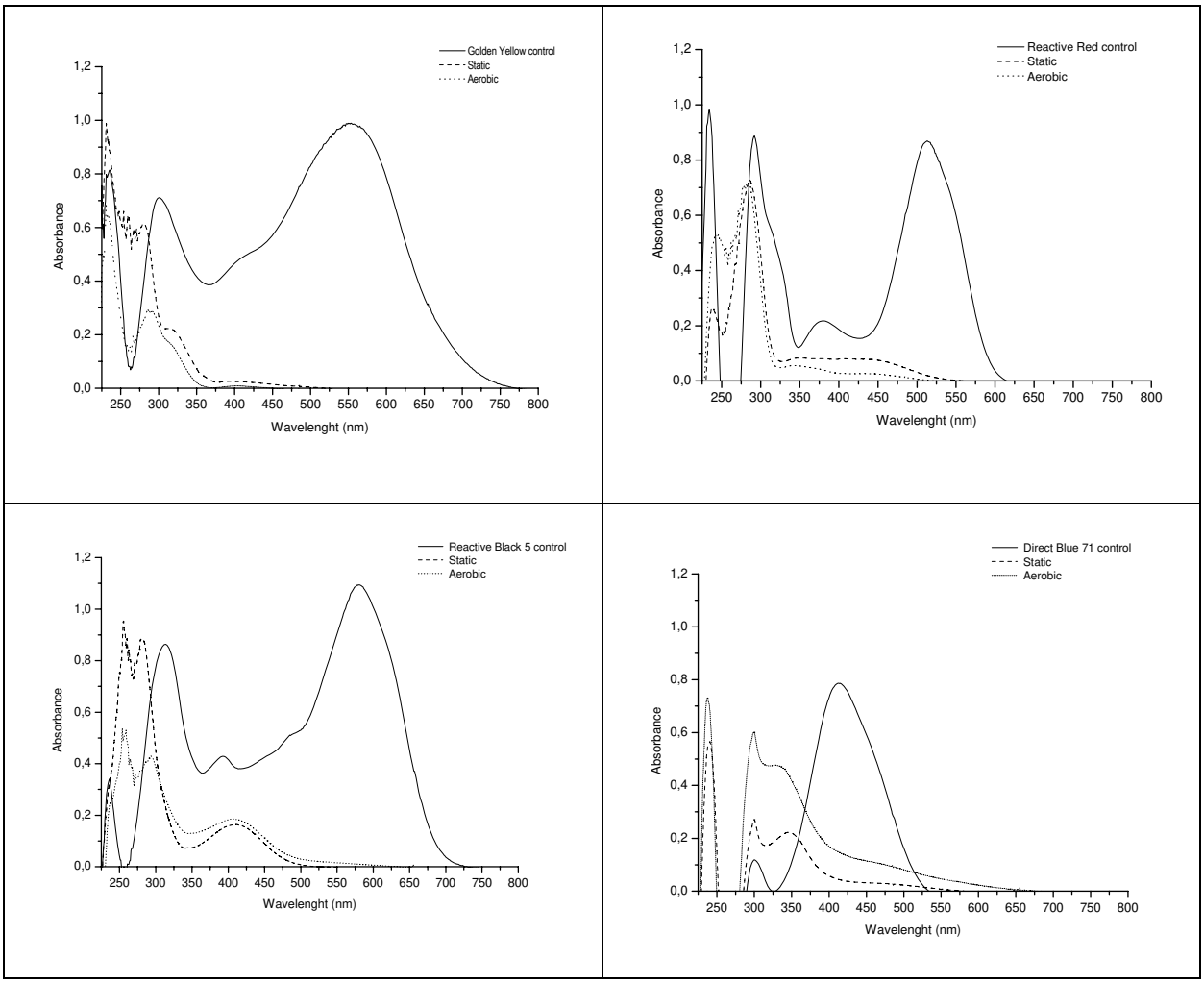


Figure 3. UV–vis spectra of the azo dyes before (straight line) and after microaerophilic (dashed line) and aerobic (dotted line) treatments: (A) RY107, (B) RR198, (C) RB5, (D) DB71.

3.5. FT-IR characterization

The FTIR 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 1,402 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 (Figure 4).

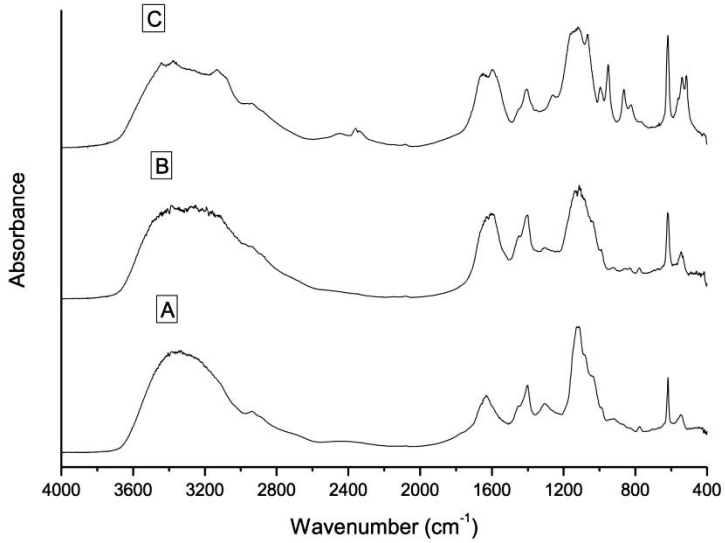


Figure 4. FT-IR spectra of the azo dye RR198 before (A) and after microaerophilic (B) and aerobic (C) treatments.

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 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).

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 (%) *			TOC reduction (%)**	
	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

*S.D. ± 11% for all the data

**S.D. ± 2% for all the data

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.

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 hours 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 pnuemoniae* 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, sulpho 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 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 (Figures 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 FTIR 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 figure 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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5. Capítulo 2

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. 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.

Keywords: azo dyes, *Klebsiella* sp., biodegradation, textile effluents, aromatic amine, toxicity

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 (Anjali and Leela, 2007). 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 to 80 % of the organic dyes used in industries such as the textile, leather, plastic, cosmetic and food industries (Vandevivere et al, 1998). Recent studies have shown that azo dyes contribute to the mutagenic activity of ground and surface waters polluted by textile effluents (Sponza and Isik, 2005). 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 (Pinheiro et al., 2004). 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 (Gogate and Pandit, 2004). In recent years, new processes for dye degradation and wastewater reutilization have been developed (Santos et al., 2007). 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 (Pandey et al., 2007; Khalid et al., 2008; Dhanve et al., 2008; Whiteley, 2007). 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 (Hsueh and Chen, 2008; Kudlich et al., 1997; Scheline et al., 1970; Wong and Yuen, 1996)

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 (Pinheiro et al., 2004). 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 (Lodato et al., 2007; Field et al., 1995; Van der Zee and Villaverde, 2005). 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 (Sandhya et al., 2004; Xu et al., 2007).

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 bond, into non-toxic metabolites. The degradation products were characterized by Fourier Transforms Infrared Spectroscopy (FT-IR) and UV-Vis techniques as well as their toxicity and 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. All other analytical grade reagents were purchased from Sigma and used without further purification. The mineral salts medium (MM) at pH 7 as previously described in previous chapter was used in all the batch experiments The medium was supplemented with 100 mg L^{-1} of dye, 3 mg L^{-1} of glucose and 1 mg L^{-1} 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 ºC for seven 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 (1989). Cultures were harvested at the end of the exponential growth phase by centrifugation at 18.600 x 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 x 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 x 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 94ºC, followed by 10 cycles of 1 min at 94ºC, 30 s at 69ºC, decreasing 0.5ºC each cycle, and 3 min at 72ºC, followed by another 10 cycles of 1 min at 94ºC, 30 s at 63ºC and 3 min at 72ºC, 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 (Godon et al., 1997).

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 (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 neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software (Saitou and Nei, 1987). The 16S rRNA partial sequence determined in this study was 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 (2003). Samples were taken after incubation under microaerophilic and aerobic conditions, frozen and freeze dried (FTS System model Dura-Dry MP). The samples (5mg) were dissolved in 5 mL of a 0.4% solution of chloranil in dimethylformamide (DMF) and heated at 100ºC 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 (Marik et al., 2003).

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^{-1} (dyes TOC ~ 60 mg L^{-1}). 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-visible 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 60ºC for 24 hours and compression molded in a uniaxial hydraulic press under a load of 0.9 MPa to obtain IR-transparent pellets. The absorbance FTIR spectra of the samples were recorded using a FTIR Spectrum 2000 Perkin-Elmer 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 FTIR 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 Total Organic Carbon (TOC) under microaerophilic conditions and after agitation using a TOC analyzer (Shimadzu 5000A) with direct injection of the samples after centrifugation (20.000 x g for 15 min) and filtration through a 0.45µm pore size.

2.8. Toxicity test

The samples taken after treatment with *Klebsiella* sp strain VN-31 were centrifuged at 20,000 x g for 20 min and filtered through a 0.45µm pore size filter. Acute toxicity tests with D*aphnia magna* (Crustacea, Cladocera) were carried out according to the ABNT norms (ABNT/NBR 1271327, 1993). 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 ºC 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 neighbour joining method on the program Mega 2.0. The bootstrap and values higher than 70 % were indicated on the tree (Figure 1). The evolutive distance was based on the Kimura 2p model (Kimura, 1980). 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.

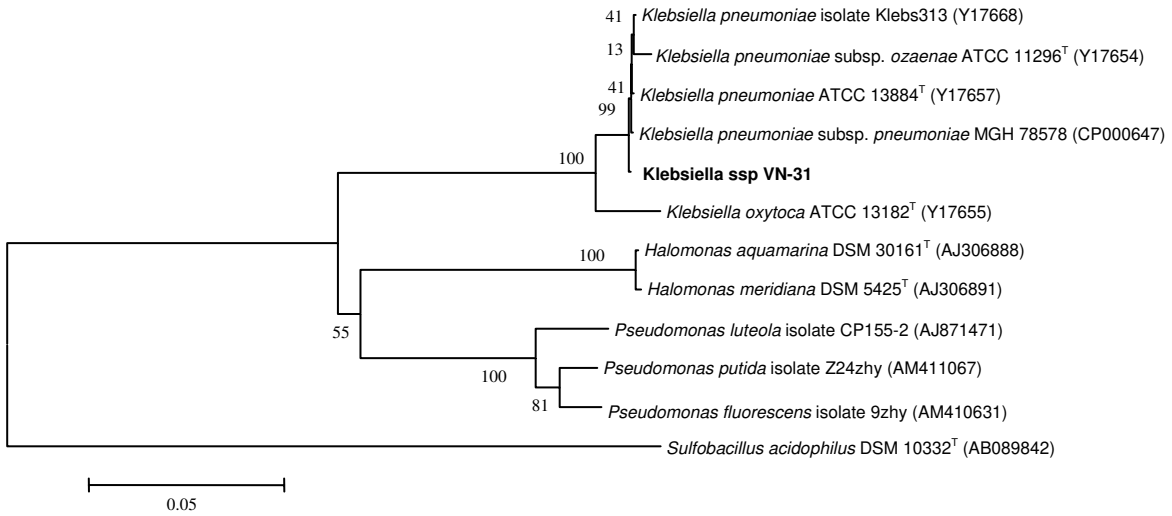

Figure 1. Phylogenetic tree of the *Klebsiella sp.* strain VN-31 for the partial sequences based on 16S rDNA.

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 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.

3.3. Aromatic amine determination

All the decolorized 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. 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.

	Amine concentration (mM)		Decolourization time (h)	Decolourization (%)	
Dyes	**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

3.4 UV-Vis characterization

The biodegradation of the four azo dyes was monitored by UV-Vis analysis. Untreated dyes: Figure 2(a) shows that RY107 presented two absorbance peaks at 285 and 410 nm. Figure 2(b) shows that RR198 presented absorbance peaks at 510, 380 and 285 nm and a shoulder at 320 nm. Figure 2(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. Figure 2(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 (Figure 2).

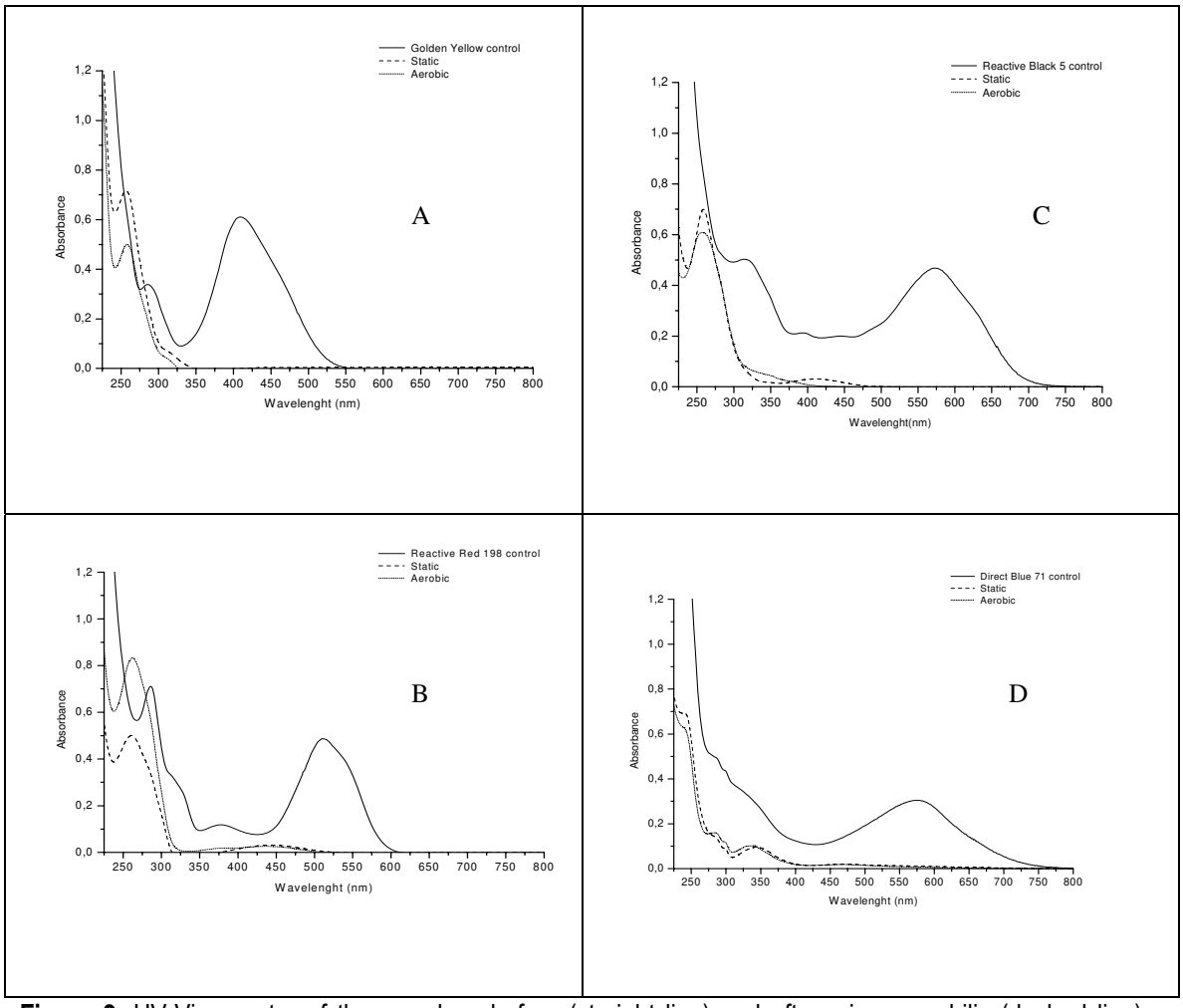

Figure 2. UV-Vis spectra of the azo dyes before (straight line) and after microaerophilic (dashed line) and aerobic (dotted line) treatments - A: RY107; B: RR198; C: RB5; D: DB71.

3.5. FT-IR characterization

The FTIR 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}) Figure 3. 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}.

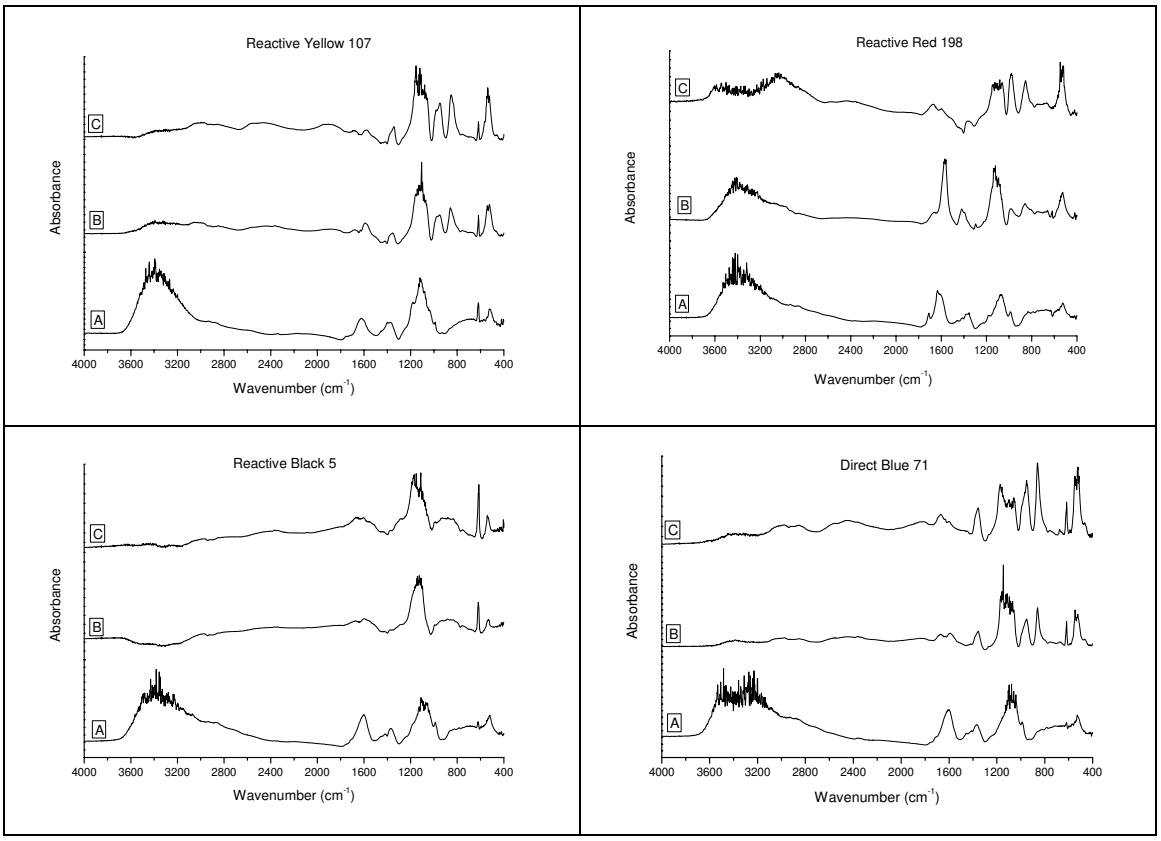

Figure 3. FT-IR spectra of the azo dyes before (A) and after microaerophilic (B) and aerobic (C) treatments

3.6. Toxicity test and TOC reduction

The results for *Daphnia 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. 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 ~50%. However, after shaking (aerobic condition), a significant increase in TOC reduction (~80%) was observed.

Table 2. Mortality for *Daphnia magna* exposed to a 1:4 dilution of the supernatant containing azo dyes and incubated with *Klebsiella sp* strain VN-31 and the % TOC removal, under microaerophilic and aerobic conditions.

Dyes	Mortality (%) *			TOC reduction (%)**	
	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

*SD ± 11% for all the data; **SD ± 2% for all the data

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 microbiota intestinal , there are numerous reports about the presence this strain in contaminated soil and wastewaters indicating its ability to metabolize toxic compounds (Wong and Yuen, 1996; Kao et al.,2003).

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 (Kao et al., 2003). Wong et al 1996, isolated five bacteria

from dye-contaminated sludge and found that two bacteria, identified as *Klebsiella* ssp. and *Klebsiella pneumonae*, showed decolourization ability with respect to the Methyl Red dye.

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 (Moutaouakkil et al., 2003). 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 (Stolz, 2001). 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 (Kudlich et al., 1997). 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 (Hong et al., 2007). In addition, different models for the nonspecific 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 (Jurgen 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 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 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 (Pasti-Grigsby et al., 1992). 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 (Pearce et al., 2003). For this reason, the highly substituted diazo RB5 and the triazo DB71 showed longer decolourization times (120 and 168 h respectively). Moreover, the azo compounds with hydroxyl or amino groups are more likely to be degraded than those with methyl, methoxy, sulpho 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 their decolourization rates (Xu et al., 1996). In this context, the present decolourization times are 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 the 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 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 (Figure 2) and FT-IR (Figure 3) 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 (Feng et al.,2000). 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. In the FTIR 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 (Coates, 2000). 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 (Coates, 2000). The fact that no new peaks appeared between 3300-3500 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 (Gavril and Hodson, 2007). 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 (Gavril and Hodson, 2007). 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, contrary to expectations that biorecalcitrant aromatic amines would tend to autoxidise, 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 (Coughlin et al.,2003), 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 (Van der Zee and Villaverde, 2005).

The toxicity results 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 in aquatic organisms as compared to basic, acid and disperse dyes. 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 (Abadulla et al., 2000). 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 (Mahmoodi et al., 2006). 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 [Sponza and Isik, 2005)

5. Conclusion

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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6. Capítulo 3

Decolourization/Biodegradation of textile azo dyes by a isolated of *Brevibacterium sp* strain VN-15

Decolourization/Biodegradation of textile azo dyes by a newly isolated of *Brevibacterium sp* strain VN-15

Abstract

Different azo dyes were decolourized and biodegraded in a sequential static–agitated treatment by a *Brevibacterium* sp, isolated from activated sludge process containing dyes. Dye decolourization was performed under static conditions until decolourization (reached percentage >95%). The medium was then aerated to promote the oxidation of the residual carbon source and metabolites produced. The enzymatic activities as well as the Total Organic Compound (TOC) were monitored during the biodegradation process. Tyrosinase activity was observed suggesting the role of this enzyme in the decolorization process, but no-activity was observed for laccase and peroxydase. The TOC reduction for the growth medium plus dyes was ~70% in the static stage and ~80% in the aerobic stage. The degradation products were also characterized by UV-Vis and also HPLC- MS techniques, and their toxicity measured using *Daphnia magna*. The results demonstrate that the successive static/aerobic process using a *Brevibacterium* sp was successful in achieving a complete decolourization and degradation of azo dyes. The toxicity test revealed metabolites nontoxic indicating the high potential of the bacterium for the use in azo dyes removal.

Keywords: azo dyes, *Brevibacterium* sp, biodegradation, enzymes, aromatic amine, toxicity

1. Introduction

Azo dyes account for the majority of all dye produced and have been the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries (Chang and Lin, 2001).

Colored wastewaters are frequently complex and highly variable due to mixture of many dyes and polluting substances (Hai et al., 2007). The amount of dye lost in industrial applications depends on the class of dye applied and varies from 2% loss for basic dyes to about 50% loss in certain reactive sulfonated dyes due to the relatively low levels of dye fiber fixation (Mcmullan et al., 2001; Pearce et al., 2003).

Because color is highly visible and affect esthetics, water transparency and gas solubility in water bodies, and especially because many dyes are made from known carcinogens, such as aromatic compounds, dye wastewaters have to be treated (Banat et al, 1996). Government legislation, especially in the developed countries is very stringent for the allowable concentration of dye in industrial effluents. Environmental policy in the United Kingdown (UK), since September 1997 has stated that zero synthetic chemicals should be released into the marine environment. European community (EC) regulations are also becoming more stringent (O' Neill et al 1999). The conventional treatment of wastewater in aerobic conditions from dyestuff factories cannot remove most azo dyes effectively and low removal is achieved primarily through adsorption to the sludge (Brik et al., 2006). Additional processes for color removal such as physical and chemical methods have the disadvantages of being highly expensive, coupled with the formation of large amount of sludge and the emission of toxic substances. In addition, the accumulation of concentrated sludge creates a disposal problem (Franciscon E, 2005.). In the natural environment, the dyes can be transformed or degraded by a variety of microorganisms, including aerobic/anaerobic bacteria, fungi and mixed microbial consortia. Moreover in recent years, new biological processes, including aerobic and anaerobic bacteria and fungi, for dye degradation and wastewater reutilization have been developed (Chung and Stevens, 1993).

The wood rotting fungi have interesting properties in the sense that they are capable to degrade lignin which is a polymeric structure consisting of cross-linked aromatic rings. Lignin is regarded as stable. The fungi have been shown to excrete certain enzymes that catalyze the

formation of activated oxygen initiating the process. The lack of selectivity among these enzymes with regard to the aromatic compounds that are attacked is the basis for the interest in this funghi for treating textile dyes. Problems associated with the biodegradation dyes by funghi include rather slow degradation requiring long treatment time, bacterial contamination into de process destabilizing fungal decoloration and loss of the extracellular enzymes (Libra et al., 2003).

Bacterial decolourization is normally faster and one of the promising microorganisms able to degrade various dyes have been identified. Research on bacterial strains that are able to decolourize azo dyes under aerobic (*Xenphylus azovorans* KF46F, *Bacillus* strain, and *Staphylococcus* sp) (Suzuki et al., 2001; Olukanni et al, 2006; Vijaykumar et al 2007) and anaerobic conditions (Eubacterium sp, *Clostridium* sp, *Butyrvibrio* sp or *Bacteroides* sp) have been extensively reported (Rafii et al., 1990; Dos Santos et al., 2007; Lin and Leu, 2008). Under microaerobic or anaerobic conditions the azo bond are broken completely decolourizing the azo dyes with the formation of aromatic amines. Some aromatic amines have been related to bladder cancer in humans, to splenic sarcomas, and nuclear anomalies in experimental animals, and to chromosomal aberrations in mammalian cells (Medvedev et al., 1988; Percy et al., 1989).

Various biological systems have also been known to possess the capability to cleave the azo bond reductively under anaerobic conditions. In mammalian tissues, azo reductase activity is primarily found in the liver. Consequently intestinal microbiota have also been found to reduce azo bonds (Chung et al., 1978). The toxicity of these aromatic amine makes it essencial generally requires aerobic condition (Singh et al., 2007; Van der Zee and Villaverde, 2005). Therefore, in addressing the problem of decolourization of azo dyes, a primary issue is the formation of toxic amines during the biodegradation process and toxicity tests are essential for confirming dye detoxification (Bafana et al., 2008). *Daphnia magna* has been extensively used to determine the toxicity of azo dyes and metabolites from degradation in microaerophilic-aerobic treatment (Franciscon et al., 2009 a,b). Azoreductases appear to be the key enzymes responsible for reductive azo dye degradation in bacterial species, but there are numerous studies on bacterial strains that produced oxidative enzymes such as peroxidase, laccase and tyrosinase that completely decolorize azo dyes and their metabolites in static and aerobic conditions (Moutaouakkil et al., 2003; Kalme et al., 2007a). The enzyme tyrosinase is also capable of oxidizing aromatic amines and *o*-aminophenols

(Toussaint and Lerch, 1987). Therefore a combination of static and aerobic stages seem to be the best option for effective decolorization of dyes in biological treatment and is also a good choice to simultaneously remove Total Organic Carbon (TOC) in textile wastewater.

The present study was focused on the degradation of of four azo dyes in a successive static/aerobic process using, exclusively, an aerobic *Brevibacterium* sp isolated from textile dye effluents. Dye degradation was carried out under static conditions until no colour was observed. The medium was then aerated by stirring to promote oxidation of the metabolites formed by reductive break-down of the azo bond, into non-toxic metabolites. The enzymatic activities as well as the TOC were monitored during the biodegradation process. The degradation products were characterized by UV-Vis and HPLC-MS techniques and their toxicity was measured.

2. Material and methods

2.1. Chemicals and culture 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 a plastic and textile company in Brazil. All other analytical grade reagents were purchased from Sigma and used without further purification. The mineral salts medium (MM) at pH 7 was prepared as previously described by Franciscon et al, (2009a) was used in all the batch experiments. The medium (MM) was supplemented with 100 mg/L of dye glucose (1 and 3 g/L), sodium pyruvate (1 g/L) and yeast extract (1 g/L) were tested as alternative carbon sources. The medium that showed the highest degree of decolorization in the shortest time (3 g/L of glucose and 1 g/L of sodium pyruvate) was used in all batch experiments and is referred to as mineral medium rich (MMR).

2.2. Strain isolation and characterization

The bacterium was isolated as described in a previous publication (Franciscon et al., 2009 a,b). Isolated strains were inoculated onto Nutrient Agar plus azo dyes (100 mg L^{-1} each dye) plates

and incubated under static conditions at 30 ºC for seven days. The strains that achieved the best decolourization were selected for this study. The strains were routinely maintained on slants of a medium Nutrient Agar Medium.

Identification of the isolated strain was performed using biochemical tests in accordance to Bergey´s Manual and confirmed by 16S rDNA sequence analysis. Genomic DNA was obtained according to Ausubel et al, (1989).

The 16S rDNA gene was amplified by PCR using primers specific, for the universal Bacteria Domain. Identification was achieved by comparing the contiguous 16S rDNA sequences obtained with the 16S rRNA sequences available in the public databases GenBank and RDP (Ribosomal Database Project, Wisconsin, USA). Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA software using Kimura's DNA substitution model as previously described in Franciscon et al, (2009 a,b).

2.3. Dye decolourization

Decolourization experiments by growing cultures were performed in 500 mL Erlenmeyer flasks with 350 mL of sterile medium (MM) supplemented with 100 mg L^{-1} of dyes plus the carbon source. Samples were incubated under static conditions to provide conditions of oxygen limitation, at 30° C for 168 h or until total decolorization. The culture was then aerated by stirring without any further supplementation of the medium. Dye decolourization was measured in a UV-visible spectrophotometer (Shimadzu 2101) for the static and aerobic stages and the percentage of effluent decolourization was calculated.

2.4. Preparation of enzyme extract

During growth of *Brevibacterium* sp strain VN-15 in MMR containing dyes, samples were harvested (1 - 7 days) and the cells were sonicated with eight impulses of 5 seconds every 2 minutes (Sharp Silent Sonic Model UT-204). The sonifer was maintained at an amplitude output at 40 a

temperature of 4°C (Parshetti et al., 2006). The sonicated cells were then centrifuged (20,000 x g for 15 min) and the supernatant was used as enzyme source.

2.4.1. Determination of enzyme activity

Tyrosinase enzyme assay for enzymatic activity were assay modified from those described by Radliffe et al. (1994). Tyrosinase activity was determined using 0.1 mL of 1mM tyrosine solution (1 M phosphate buffer at pH 7) as substrate, 0.6 mL of enzymatic extract and 0.3 mL of distilled water in a final volume of 1 mL. The oxidation of tyrosine to dihydroxyphenylalanine was monitored spectrophotometrically by increased in absorbance at 280 nm. Laccase activity was determined using 0.1 mL of 0.5 mM Syringaldazine solution in ethanol (due to its limited solubility in aqueous solutions) as substrate, 0.2 mL of 0.05 M citrate phosphate at pH 5, 0.6 mL of enzymatic extract and 0.1 mL of distilled water in a final volume of 1 mL (Szklarz et al., 1983). The oxidation of substrate was monitored spectrophotometrically at 525 nm. Peroxydase activity was monitored by using the same substrate used for laccase with 0.1 mL of 2mM hydrogen peroxide solution instead of distilled water. All enzyme assays were run in triplicate.

2.5. High Performance Liquid Chromatography Mass Spectrometry analysis (HPLC-MS)

The biodegradation products of the RR 198 dye were analysed by HPLC- MS. Culture samples were centrifuged (20,000 x g for 15 min) and filtered through a 0.25 μm pore size filter. Then an aliquot of 25 μL was injected in a LC-MS system (LC-MS LC-MS-MS QToF micro). It consisted of an LC system (Waters, USA) coupled to a mass spectrometer comprising hybrid quadrupole (Q) and time-of-flight (ToF) mass analyzers from Micromass (Waters, USA), with an electrospray source interface (LC-ESI-MS-MS). Instrument control and data processing were carried out by Masslynx 4.0 software.The mobile phase components used were degassed in an ultrasonic bath (Model USC 700, Unique Thorton, Brazil) before use in the LC system.

A C_{18} HPLC column (150 x 2.1 mm, 5 mm marca) was used to identify biodegradation products. The column temperature was set at 25 ºC. The mobile phase was composed of water

(aqueous phase, AP) and methanol (organic phase, OP), using gradient elution. The gradient elution profile is shown in Table 1. A flow rate of 0.2 ml min^{-1} was employed. The injection volume was 20 ml.

Table 1. Gradient elution of mobile phase in the LC-MS system

Time (min)	Water (%)	Methanol (%)
0.0	100	0
20.0	0	100
25.0	0	100
30.0	50	50
31.0	50	50
33.0	100	0
35.0	100	0

In order to improve the signal-to-noise ratio, the quadrupole analyser was used as a filter before íon collection. In this way, the quadrupole analyser was programmed to select ions with m/z in the range from 50 to 1200 u.

The ionization conditions selected were: cone gas flow (150 L h^{-1}), dessolvatation gas flow (350 L h^{-1}), polarity (ESI+), capillary energy (2900 V), sample cone energy (30 V), extraction cone energy (2.0 V), dessolvatation temperature (350 ºC), source temperature (120 ºC), ionization energy (2.0 V), collision energy (4 V), and multi-channel plate detector energy (2700 V).

In the tentative identification of the metabolites from azo dye biodegradation the acquired mass spectra were compared to spectra in the MS Database using CambridgeSoftChemOffice 2008 program.

2.7. TOC measurement

The presence of organic carbon was monitored by measuring the Total Organic Carbon (TOC) under static conditions and after agitation, using a TOC analyzer (Shimadzu 5000A) as previously described in Franciscon E et al, (2009 a,b).

2.8. Toxicity test

Daphnia magna is a commonly bioindicators used as a test organism in acute and chronic toxicity studies of chemical compounds present in aquatic ecosystems (USEPA, 1985). The acute toxicity tests using *D. magna* were carried as previously described in Franciscon et al, (2009 a,b).

3. Results

3.1. Strain isolation and characterization

The bacterium was selected on the basis of the extent and time required for decolourization for the azo dyes. This bacterium showed good growth on a complete in the MMR medium at near neutral pH. The optimum growth temperature was 30 ºC.

The identification of the isolate was done by morphological biochemical and 16S rDNA analysis. Biochemical and morphological characteristics are presented in Table 2. The 16S rDNA gene sequence of the VN-15 strain was determined and compared with available 16S rDNA gene sequences from organisms in the Genbank and RDP databases. The VN-15 strain was phylogenetically positioned in the genus *Brevibacterium* sp (Figure 1). The bootstrap values higher than 97% were indicated on the tree. The evolutive distance was based on the Kimura 2p model (Kimura, 1980). The GenBank accession numbers are in parenthesis. *Rhodococcus percolattus* DSM 44240^T was used as the outgroup. The nucleotide alignment of strain VN-15 supported values of the boot strap value of 97% similarity to *Brevibacterium linens, B. epidermidis* e *B. iodinum* and other *Brevibacterium* sp. The partial sequence determined in this study was deposited in the Genbank database under the accession numbers FJ598007 (B*revibacterium* sp. strain VN-15).

Table 2. General morphological and physiological characteristics found of strain *Brevibacterium sp.*

Characteristics	*Brevibacterium* sp
Morphology	Smooth gray colony
Gram's staining test	Rod Gram positive
H_2S production	-
Spore staining test	-
Motility	-
Aerobic growth	+
Anaerobic growth	-
Nitrate reduction	-
Catalase	+
Oxidase	+
Citrate utilization	-
Urease	-
Indole production	-
Hydrolysis of gelatin	+
Starch	-
Oxidation/fermentation	O
Methyl red test	+
Voges Proskauer test	-
Acid/Gas Production from:	
Glucose	+
Mannose	- +
Mannitol	-
Rhamnose	-
Arabinose	-
Sucrose	
Xylose	-
Galactose	-

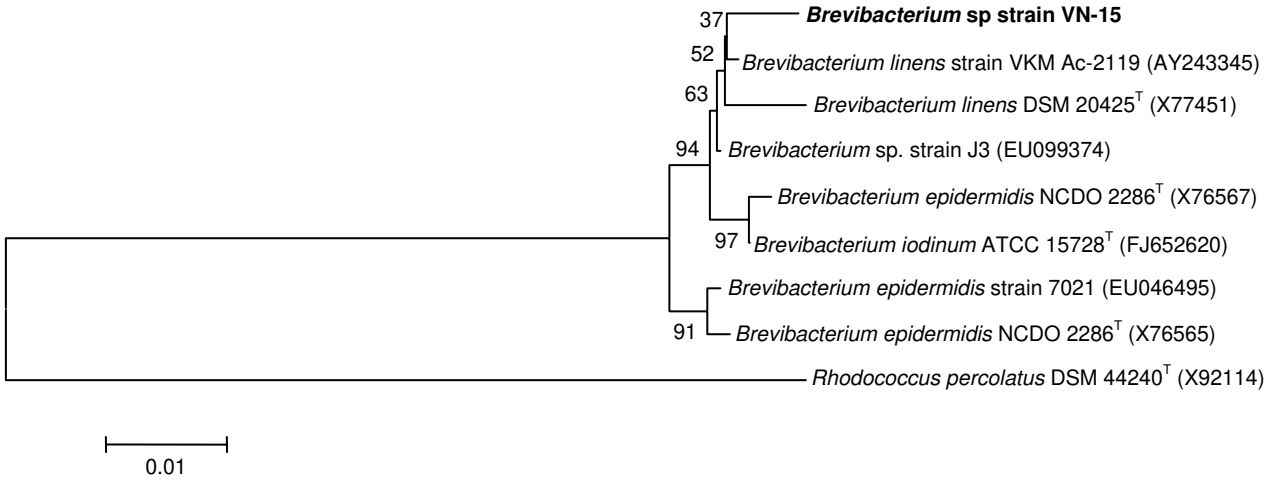


Figure 1. Phylogenetic tree of the *Brevibacterium sp* strain VN-15 for the partial sequences based on 16S rDNA gene.

3.2. Decolourization assays

The ability of *Brevibacterium sp* (strain VN-15) to separately decolourize four azo dyes (Reactive Yellow 107, Reactive Red 198, Reactive Black 5 and Direct Blue 71) was evaluated in a static/agitated sequential process. Complete decolourization (>95%) of all azo dyes was achieved in the static stage as shown in Table 3. *Brevibacterium* sp VN-15 could only decolourize the dyes efficiently when the medium was supplemented with carbon sources. When both glucose (1g L^{-1}) and sodium pyruvate (1g L^{-1}) were added to the medium the decolourization rate improved. When sodium pyruvate was substituted by yeast extract (1g L^{-1}) the bacterium showed a similar time and rate of decolorization. With only glucose present (1 and 3 g L^{-1}), the bacterium was able to decolourize the azo dyes, but presented lower decolourization efficiency (~ 30 and 50%) respectively in a longer time 168 hs. The monoazo dyes (RY107 and RR198) were decolourized in 96 and 120 hs and showed higher degradation rates. The more complex diazo RB5 and triazo DB71 dyes required after 144 to 168 hs, respectively, for complete decoloration.

Table 3. Decolourization times (h) ± S.D. and decolourization (%) ± S.D. in solutions incubated with Brevibavterium sp strain VN- 15 under static and aerobic conditions in the presence of azo dyes.

	Decolourization time (h)	Decolourization (%)	
Dyes	Static	Static	Aerobic
RY107	96 ± 1	98 ± 0.5	89 ± 0.3
RB5	120 ± 2	97 ± 0.2	100 ± 0.4
RR198	144 ± 2	95 ± 0.3	87 ± 0.2
DB71	168 ± 3	94 ± 0.1	97 ± 0.5

3.3. Determination of enzymatic activities

The activity of the oxidoreductases enzymes (peroxidase, laccase and tyrosinase) were during the decolourization process. In the static and stirring conditions laccase and peroxydase activities were very low and probably did not play much of a role in dyes decolourization/degradation (Figure 2). However, tyrosinase activity was observed in both conditions. Under static conditions the activity increased until decolourization and then decreased over of time for all dyes.

Induction of tyrosinase activity up to 96 -120 h suggests a role of this enzyme was envolved in the decolorization process. In addition under stirring conditions the activity remaned high for 168 hs suggest that the tyrosinase enzyme is involved in the biodegradation of dyes metabolites.

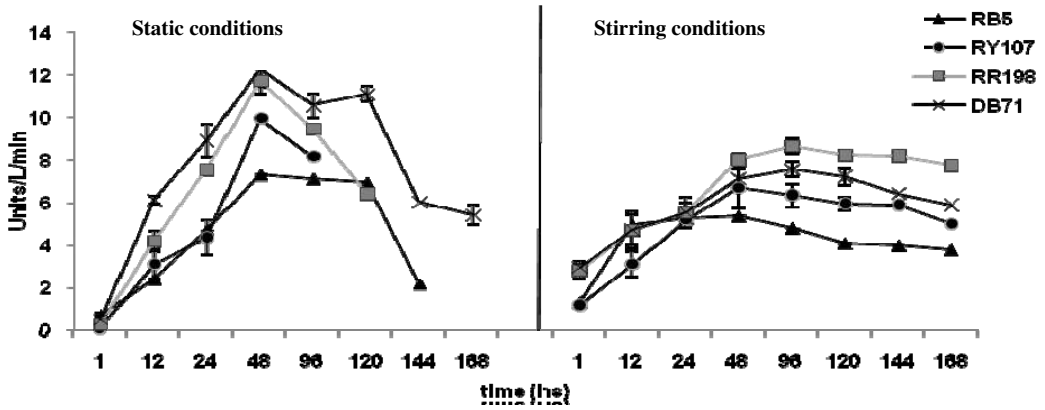


Figure 2. Time course of tyrosinase activity in Brevibacterium sp strain VN-15 during azodyes biodegradation.

3.4. UV-Vis characterization

The biodegradation of the four azo dyes was monitored by UV-Vis analysis. As shown in Figure 3(a), reactive yellow dye has two absorbance peaks at 280 and 410 nm. As shown in Figure 3(b) RR198 presented two large peak at 280 and 510 nm and a smaller peak at 378 and a shoulder at 320 nm. In Fig. 3(c), reactive black 5 has absorbance peaks at 320 nm and a large peak at 570 nm. As shown in Figure 3(d), DB71 presented one large peak at 560, a smaller peak at 280 and a shoulder at 320 nm.

After decolorization and degradation of the four azo dyes in the static/stirring reactions solutions, the absorbance peaks in the visible region disappeared for all dyes, indicating complete decolourization. The UV spectra of simple take in static conditions, the peaks at 280 observed in untreated RY 107, RR198 and DB71 diminished and were replaced by new peaks at 260 nm. A peak at 260 nm also emerged in the UV spectra in RB5 dye. The decrease in the absorbance of the peak at 280 and the formation of a new peak at 260 nm suggest that there were changes in the aromatic group. The peaks at 280 nm corresponding to benzene rings in compounds such as 4 amino-benzoic acid, and peaks near 260 nm is normally associated with the presence of phenolic and naphthoquinone compounds (Chung-Chuan and Hsueh, 2008, Mielgo et al.,2001). The peak at 320

nm (naphthalene rings) observed in the RR198, RB5 and DB 71 dyes were also removed. A new peak at 350 nm was observed in the DB 71 spectrum after static conditions. After stirring conditions, an increase in the UV-vis spectra absorption of RY107 and RR 198 was also observed.

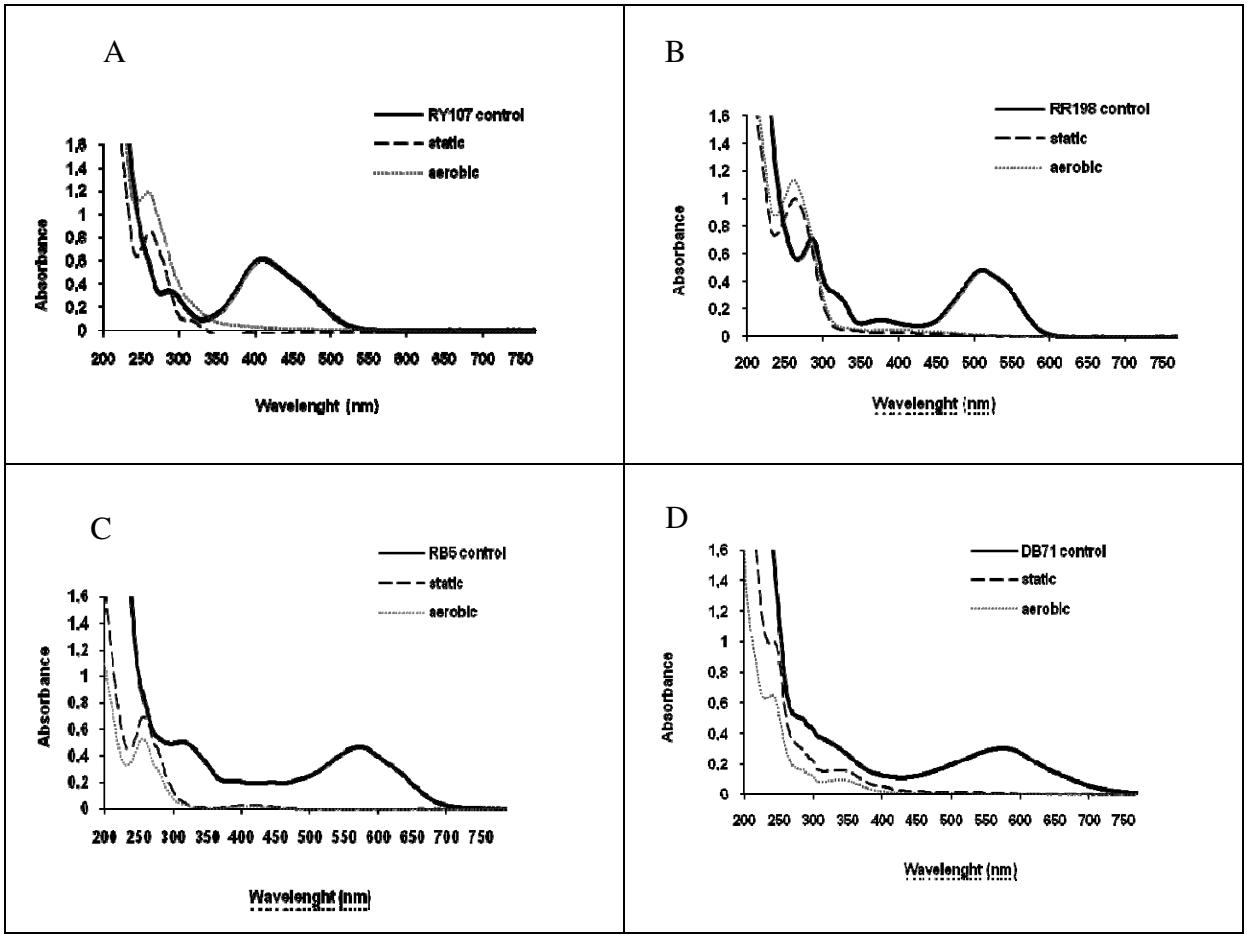


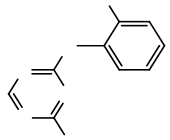
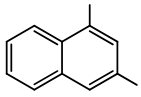
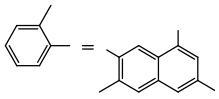
Figure 3. UV-Vis spectra of the azo dyes before (straight line) and after microaerophilic (dashed line) and aerobic (dotted line) treatments - A: RY107; B: RR198; C: RB5; D: DB71.

3.5. High Performance Liquid Chromatography Mass Spectrometry analysis (HPLC-MS)

The biodegradation products of the RR198 dye were analyzed by HPLC- MS for tentative identification of the unknown coupounds. Table 4 show aromatics compounds of the interest constructed by Cambridge SoftChemOffice, program based on RR198 dye structure and matches to the mass spectra from the samples after treatment under static conditions. Out of forty possible

compounds identified by the program and analysed, three had reasonable matchs in relation to metabolites presents in the sample and are show in Figures 4, 5 and 6.

Table 4. Aromatics compounds of interest constructed by Cambridge SoftChemOffice program based on RR 198 dye structure.

Chemical name	Chemical formula	Molecular weigh	Structure
4-chloro-N-o-tolyl-1,3,5-triazin-2-amine	$C_{10}H_9ClN_4$	220	
sodium 4-aminonaphthalene-2-sulfonate	$C_{10}H_8NNaO_3S$	245	
3,6-dimethyl-7-(o-tolyldiazenyl)naphthalen-1-amine	$C_{19}H_{18}N_3$	289	

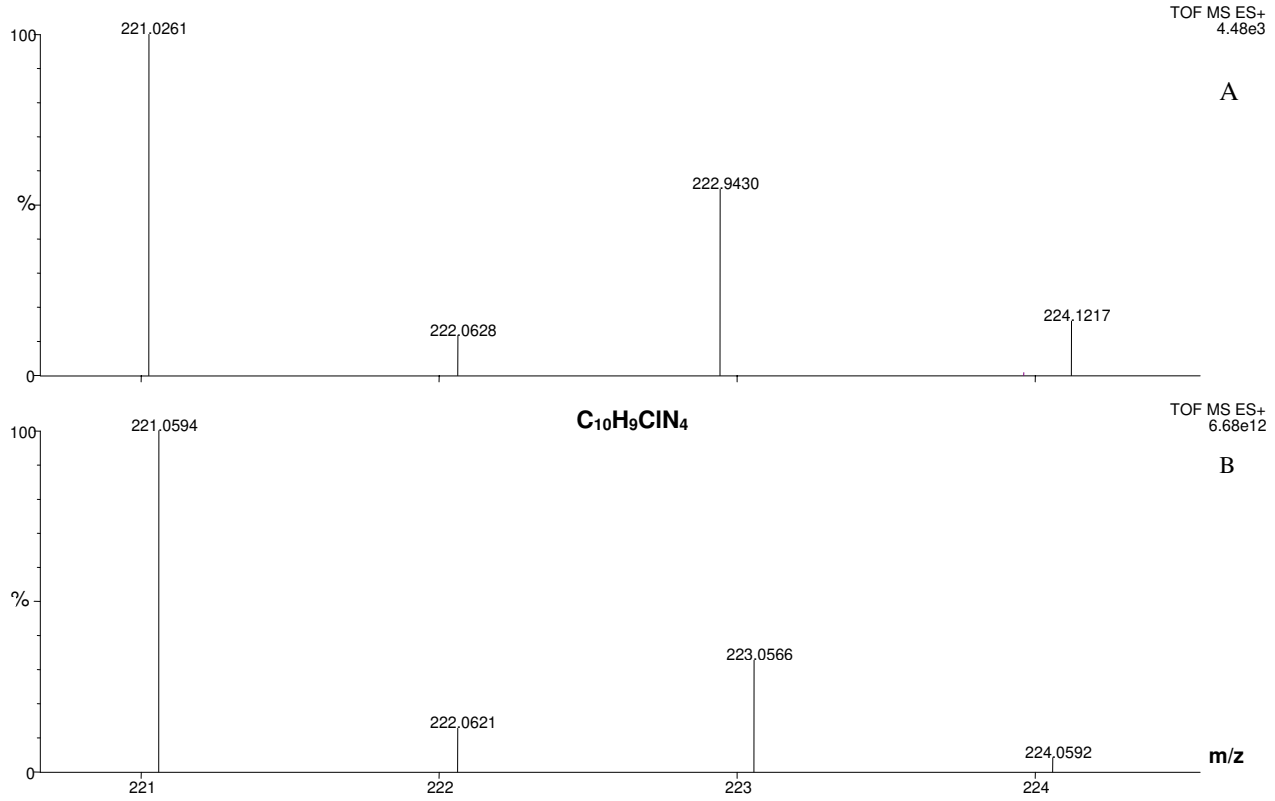


Figure 4. Mass spectra of the RR 198 azo dye metabolite (A) in comparison to standart 4-chloro-N-o-tolyl-1,3,5-triazin-2-amine (B).

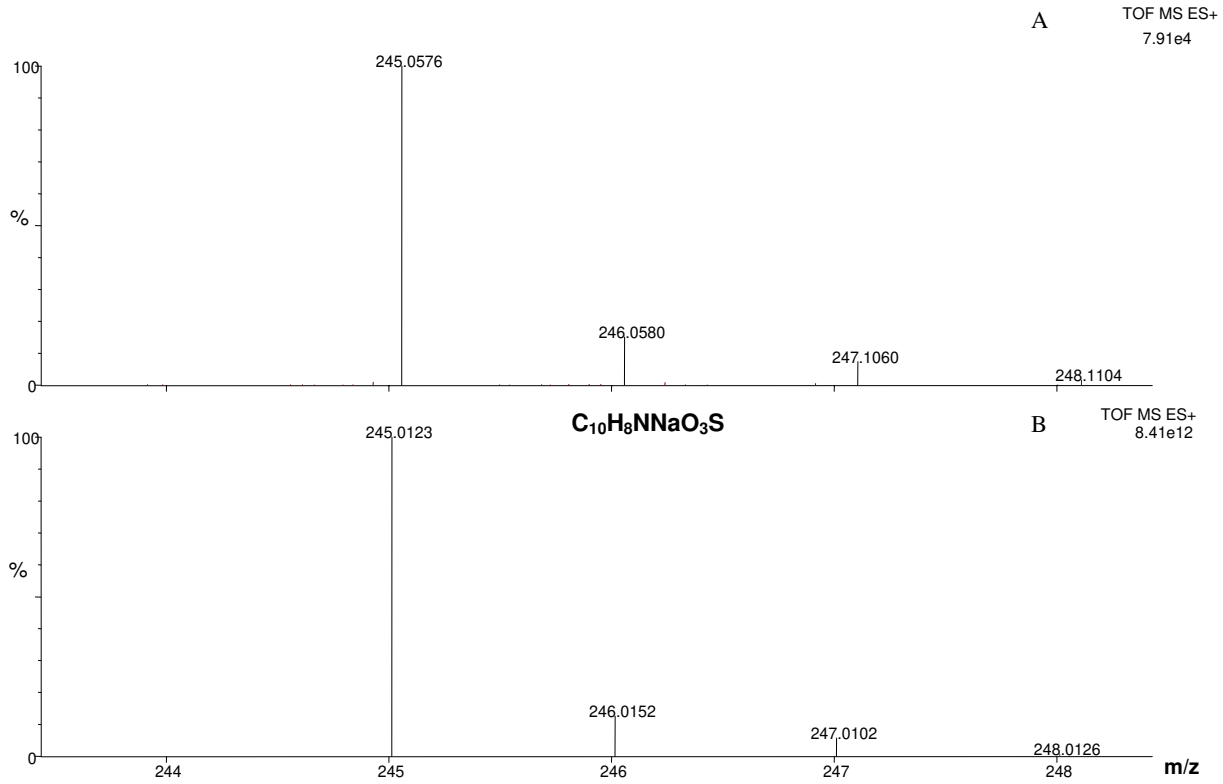


Figure 5. Mass spectra of the RR 198 azo dye metabolite (A) in comparison to standard sodium 4-aminonaphthalene-2-sulfonate (B).

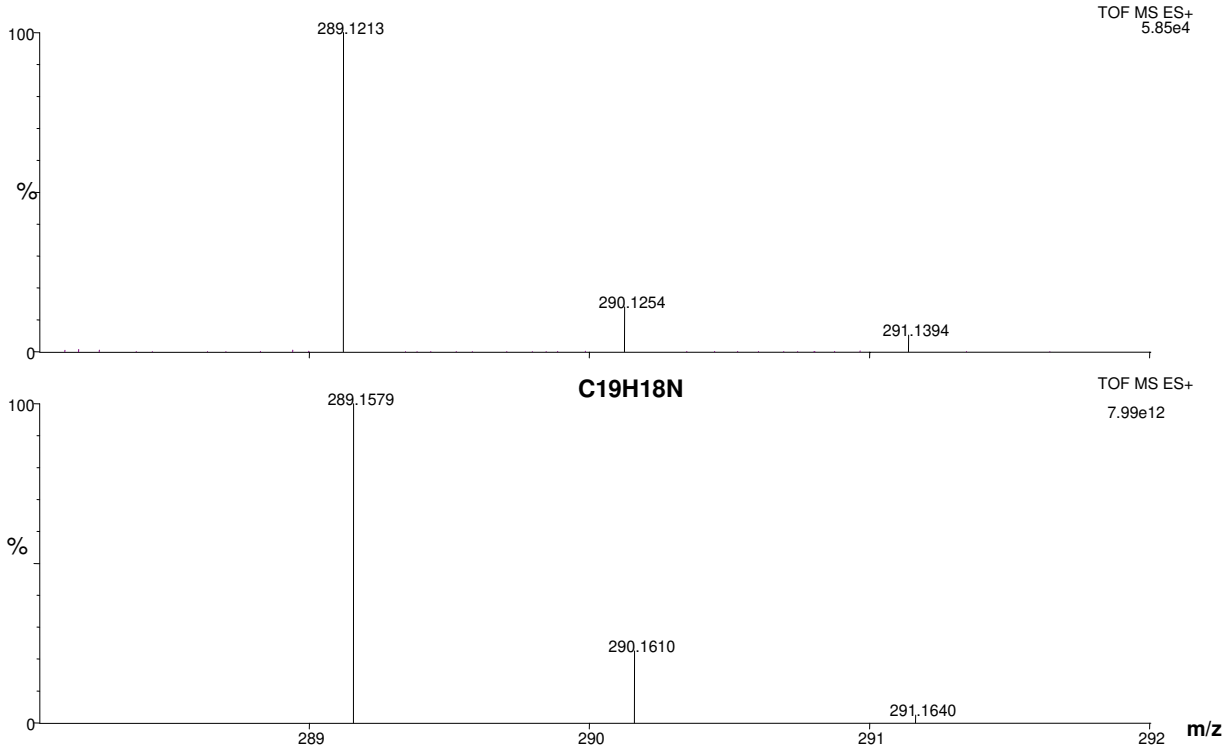


Figure 6. Mass spectra of the RR 198 azo dye metabolite (A) in comparison to standard 3,6-dimethyl-7-(o-tolyldiazenyl) naphthalen-1-amine (B).

3.5. Toxicity test and TOC reduction

The results for *Daphnia magna* toxicity are presented as the percentage of death in the presence of samples taken from the static and stirring treatments compared to controls composed of the dye culture medium without the bacteria (Table.5). The tests were carried out in a 1:4 dilution of the original supernatant concentration, because 100% mortality occurred in the undiluted and 1:2 diluted dye media. All the controls showed equal mortality for all the dyes (47%) at a dilution of 1:4.Samples take from static cultures had mortality values much lower for all the dyes (≤ 13%), and samples from stirred cultures had no detectable toxicity for any dyes.

The TOC reduction is show in Table 5 and based on Total Organic Carbon in the medium (MMR) including glucose, pyruvate and dyes. The TOC of the medium was around 2000 mg L^{-1}

(dye TOC≈ 60 mg L^{-1}). After 7 days the reduction in TOC under microaerophilic conditions was ~70%. After stirring a slight increase in TOC reduction (~80%) was observed.

Table 5. Mortality for Daphnia magna exposed to a 1:4 dilution of the supernatant containing azo dyes and incubated with Brevibacterium sp strain VN-15 and the % TOC removal under static and aerobic conditions.

Dyes	Mortality (%) *			TOC reduction (%)**	
	Control	Static	Aerobic	Static	Aerobic
RY107	40	9	0	75	87
RB5	40	13	0	70	83
RR198	47	10	0	82	85
DB71	47	13	0	65	76

*S.D. ± 11% for all the data
**S.D. ± 2% for all the data

4. Discussion

The genus *Brevibacterium* sp was first proposed by Breed in 1953, with *B. linens* as type species (Jones and Keddie, 1986). The *Brevibacterium* sp strain VN-15 belong to genus of the family of the Brevibacteriaceae and is a aerobic Gram positive, non motile rod (Onraedt et al., 2005).

Based on the biochemical tests performed in this study, strain VN-15 was identified as *B. iodinum* due to the differentiating characteristic in the oxidase test (Table 1). However, nowadays several reclassifications have been made and the genus has been restricted to those bacteria with a close resemblance to the type species *B. linens* based on 16S rDNA sequence analysis and DNA-DNA hybridization experiments and twelve species are currently classified in this genus (Euzeby, 2004).

Although the Brevibacterium isolate from this study showed considerable dye degradation ability as compared to other bacteria, there is no literature regarding dye decolourization using *Brevibacterium sp*. Some members of genus move to begining of this section *Brevibacterium* sp

tolerate are stimulated by the addition of NaCl to the medium (Jones and Keddie, 1986). Textile effluents have elevated amount of salts that may explain the presence of this bacterium.

Previous studies have shown that strains of *Brevibacterium* sp isolated from environmental samples such as different soils were able to degrade xenobiotics compounds such as PHAs, Dibenzofurans and nitroaromatic compounds (Strubel et al., 1991; Rakesh et al., 2005; Ningthoujam, 2005). Azo dyes, PAHs, Dibenzofuran and Nitroaromatic, are characterized by the presence of aromatic rings such as benzene, toluene and naphthalene rings. Thus, the presence of PAH, Dibenzofuran and nitroaromatic-degrading microorganisms may also enhance dyes degradation. Lester et al., 1979, isolated four strains from activated sludge and found that one bacteria, identified as *Brevibacterium* sp was the most resistant organism to heavy metal inhibition.

No pathogenicity towards mammals including humans is known for *B. linens, B. casei, B. iodinum and B. Epidermis* which can facilitate their use in water and wastewater treatment.

Brevibacterium sp strain VN-15 completely decolourized four azo dyes (Reactive Yellow 107, Reactive Red 198, Reactive Black 5 and Direct Blue 71) in a static/agitated sequential process. It is generally accepted that there are different ways by which bacteria decolorize azo dyes, in aerobic, anaerobic and limited oxygen conditions (e.g., intracellular azoreductases or extracellular processes, participation of redox mediators, oxidoreductives enzymes etc.). Bacteria capable of aerobic decolourization and mineralization of dyes, specially sulfonated azo dyes, have proven difficult to isolate and the bacteria need to be specially adapted (McMullan et al., 2001; Pearce et al., 2003). Azoreductase is the main enzyme responsible for azo dye degradation in bacterial species. Recent work described an *Enterococcus gallinarum* isolated from effluent treatment plant of a textile industry strain capable of decolourizing azo dye DB38 by azoreductase enzyme action under aerobic conditions (Amit et al., 2008). Generally aromatic amines generated by degradation of azo dyes are more cytotoxic with respiration-inhibition tests showns increased toxicity after anaerobic treatment (O'Neill et al., 2000).

An azoreductase gene from *Bacillus latrosporus* RRK1, able to decolorize several azo dyes in aerobic conditions, was isolated and cloned in vector and expressed in *E. coli,* which was able to decolorize Remazol Red, and a level of 0.8 mg L^{-1} of dissolved oxygen, was required (Sandhya et al., 2008). The role of intracellular azoreductases is to facilitate the transfer of electrons via soluble

flavins to the azo dye, which is then reduced. However, the role of these cytoplasmic azoreductases enzymes is uncertain (McMullan et al., 2001; Pearce et al., 2003). The mass transfer resistance of azo dyes across bacterial cell membranes might be the rate-determining step for color removal (Chang et al., 2001). The production of extracellular azoreductasesby bacteria is likely since it is highly improbable that highly charged sulfonated azo dyes or polymeric azo dyes pass through the bacterial cell wall (Keck et al., 1997). In contrast, anaerobic azoreductases have been described by various researches as class of enzyme involved in the reductive cleavage of the azo bond and helps the bacteria to decolorize high concentration of azo dye (Rafii and Coleman, 1999). Moreover, bacterial azoreductases that catalyze azo dye decolourization are inhibited in oxygen presence because of competition between the azo group and oxygen as electron acceptors.

Earlies studies showed that the addition of redox mediators to anaerobically incubated cultures of various bacterial species could result in significantly increased the reduction rate of azo dyes (Rau and Stolz, 2002). Theoretically, feasible redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary electron donor. Oxygen is a more effective electron acceptor than azo dyes, which may explain the low decolourization rates under aerobic conditions (Van der Zee., et al 2001).

Also oxidative enzymes such as laccase, tyrosinase, peroxidase and lignin peroxidase have been widely described in azo dye degradation using static conditions. Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyse the oxidation of phenolic and other aromatic compounds without use of cofactors. Dyes can be cleaved symmetrically and asymmetrically depending on the structure of the active site of the enzyme (Duran et al., 2002; Dawkar et al., 2008; Dhanve et al., 2008).

There are several studies that shown the induction of oxidative enzymes such as lignin peroxidase, laccase and tyrosinase throughout the decolorization process by strains fungi. In a recent study using *Agaricus bisporus,* tyrosinase activity play an important role, in the extent of decolourization of a textile effluent containing azo dyes (Shanmugam et al., 2005). In contrast, there are few studies about bacterial tyrosinase activity involved with the biodegradation azo dyes. In recent study, the Direct Blue decolourization by *P. desmolyticum* in 72hs, suggested that the

induction of tyrosinase was responsible for biodegradation and was better under static anoxic conditions (Kalme et al., 2007).

In the present work *Brevibacterium sp*. strain VN-15 efficiently decolorized four azo dyes in a sequential static/agitated process. These results show that supplements such as pyruvate and yeast extract play an important role in dye decolourization. Yeast extract, consisting of protein, free amino acids, B vitamins, minerals, nucleotides and other yeast cell components, has been the most commonly used as a nitrogen source for dye decolourization processes (Robert et al., 1998; Mohana et al., 2008).

Although pyruvate has been to shown considerable increase the dye degradation ability as compared to other carbon sources, there are few report on dye decolourization using pyruvate. Previous studies have shown that pyruvate was able to enhance the degradation of aromatic compounds (Chung and King, 2001). In contrast a recent study showed that in the Amaranth process of azoreduction, lactate and formate were the most effective electron donors while pyruvate also increased the reduction of amaranth but to a lesser extent (Hong et al., 2008).

The chemical structures of the dyes also greatly influence the decolourization rates (Pasti-Grigsby et al., 1992). 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 (Pearce et al., 2003). For this reason, the highly substituted diazo RB5 and the triazo DB71 showed longer decolourization times (144 and 168 h respectively).

These results suggest that the azo dye reduction can be a strain specific mechanism that can be performe by an azoreductase, oxidative enzymes or by a more complex metabolic pathway. Other factors such as dye structure, nutrients and potential redox of the compounds also play an important role on dyes decolourization.

In this study, induction of tyrosinase activity throughout both the static and aerobic conditions suggests this enzyme is envolved in decolorization process and in the subsequent biodegradation of the decolorized aromatic products.

Tyrosinases are copper-containing enzymes which are ubiquitously distributed in nature (Lerch, 1995). Tyrosinases are known in *Streptomyces glaucescens, Thermomicrobium roseum* and *B. thuringiensis* (Baumann et al., 1976; Kong et al., 2000; Liu et al., 2004). The majority of

Actinomycetes, gram-positive bacteria, produce tyrosinase activity. Members of the genus Streptomyces are involved in the formation and/or degradation of complex biopolymers like lignin, melanins, and humic substances, which posses many aromatic rings structures similar to azo dyes (Claus and Kutzner, 1985).

Several bacterial oxidative enzymes that can be used in biorremediation, such as mono and di-oxygenases have been reported. There are many reports on the presence of oxidative enzymes for decolourization dyes in fungi but little for bacterial strain (Shanmugam et al., 2005; Patil et al., 2008).

In the recents studies, the induction in the oxidative enzymes e.g. lignin peroxidase, laccase and tyrosinase were observed during the decolorization of sulfonated azo dyes by the bacteria *P. desmolyticum* and *Exiguobacterium* sp (Kalme et al., 2007; Dhanve et al., 2008). In others reports on dye decolororization the induction of laccase and LiP was observed but tyrosinase activity was not (Umesh et al., 2008).

Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyse the oxidation of phenolic and other aromatic compounds without the use of cofactors (Duran et al., 2002). However in this study cofactors such as sodium pyruvate and yeast extract were essentials for achieve an efficient decolourization. Redox mediators are well known for their effect on decolorization. A recent report showed that vaniline and riboflavin were good mediators for oxidative enzymes production (Ghodake et al., 2009). Tyrosinases use molecular oxygen to catalyse two different enzymatic reactions: (I) the ortho-hydroxylation of monophenols to o-diphenols (monophenolase, cresolase acticity) and (II) the oxidation of o-diphenols to o-quinones (diphenolase, catecholase activity). However, aromatic amines and o-aminophenols have also been recognized as tyrosinase substrates which undergo similar ortho-hydroxylation and oxidation reactions (Claus and Filip, 1990; Gasowska et al., 2004).

After biodegradation of the four azo dyes in the static/stirring treated solutions, the absorbance peaks in the visible region disappeared for all dyes, indicating complete decolourization. The decrease in the absorbance of the peak at 280 and the formation of a new peak at 260 nm suggest that there were changes in the aromatic group. The peaks at 280 nm corresponding, to the benzene rings in coumponds such as 4 amino-benzoic acid and the peaks near 260 nm are normally

associated with the presence of phenolic and naphthoquinone compounds (Chung-Chuan and Hsueh, 2008, Mielgo et al., 2001). The peak at 320 nm (naphthalene rings) observed in the RR198, RB5 and DB 71 dyes were removed. A new peak at 350 nm was observed in the DB 71 spectrum after static conditions.

In a recent study using diazo derivatives of phenol and catechol as substrates for tyrosinase activity theses substrates showed their λ max absorption near 350 nm (Kamahldin, and Tan, 2003). After stirring conditions an increase in the UV-vis spectra absorption of the RY107 and RR 198 was also observed. This fact can be explained by the action of the oxidoreductive enzymes in the aerobic stage.

Tyrosinase (also known as polyphenoloxidase) converts phenols using oxygen into *o*-quinones. These compounds are further converted into heavier oligomeric species (Duran and Esposito, 2000). The amines formed after the decolorization process are ideal substrates for tyrosinase oxidization. Moreover, the products obtained during the degradation reaction can undergo further reaction and couple between themselves, producing coupled products leading to a coloring of the solution (Zille et al., 2005).

The biodegradation products of the RR198 dye were analyzed by HPLC- MS for tentative identification of the unknown metabolites. Between possible compounds of the biodegradation RR198 azo dyes, three had reasonable match in relation to metabolites presents in the sample: 4-chloro-N-o-tolyl-1,3,5-triazin-2-amine; sodium 4-aminonaphthalene-2-sulfonate and 3,6-dimethyl-7-(o-tolyldiazenyl) naphthalen-1-amine. After aerobic conditions the intensity of íons metabolites present in the sample was reduced. These results could be a suggestion of diminution of these aromatics metabolites generated in the static condition.

In thi study under static conditions a significant toxicity reduction indicated that the dye biodegradation by *Brevibacterium* sp strain VN-15 could be due to the tyrosinase activity. In a similar study, after horseradish peroxidase treatment for decolorization of textile dyes, there was a significant toxicity reduction toward *D. magna* (Souza et al., 2007). Franciscon et al, (2009 a,b) showed that after aerobic treatment of four azo dyes, two bacterial strains were able to reduce the toxicity completely.

The *Brevibacterium* sp strain VN-15 was able to decolorized dyes and considerably diminished the toxicity as well as TOC in static conditions. The stirring conditions slightly increase this percentage.

5. Conclusion

The present study indicated that *Brevibacterium sp* strain VN -15 had potential to degrade azo dyes, it can be deduced that there was direct involvement of tyrosinase enzymes in the biodegradation of the azo dyes and reduction of intermediates of biodegradation. There exist limited informations about the exact reaction mechanisms for tyrosinase of azo dyes degradation. Some reasons for these deficits are difficulties in purification of sufficiently high amounts of tyrosinases and the identification of the metabolites produced from azo dyes. The addition of carbon substrates increased the percentage and reduced the decolorization time drastically. After aerobic conditions the continued presence of tyrosinase indicated its involvement in the degradation process of azo dye metabolites and also in the reduction of toxicity and TOC.

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7. Capítulo 4

Polymerization study of the aromatic amines generated by the biodegradation of azo dyes using the laccase enzyme

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Polymerization study of the aromatic amines generated by the biodegradation of azo dyes using the laccase enzyme

Abstract

Four different azo dyes were decolorized (color reduction >90%) by bacteria isolated from a textile effluent. The dye degradation was carried out under microaerobic conditions until complete decolorizing, and the aromatic amine concentration was monitored after destaining. A laccase from *Myceliophthora thermophila* was used to catalyze the coupling reactions of the aromatic amines produced from the biodegradation of the dyes. The reaction was carried out with stirring conditions in a weak acidic buffer solution at 45° C for three days. The presence of aromatic amines in the samples after bacterial decolorizing confirmed the azo bond was reduced in the process. The UV-vis spectrum showed dramatic changes after the sequential bacterial-laccase treatment further indicating a chemical transformation of the dyes. After laccase treatment the solutions formed colored and precipitated products. Photon Correlation Spectroscopy (PCS) indicated that the size of the particles formed after laccase treatment was higher than the initial size and varied between 105 nm – 483 nm. The laccase treatment was also capable of COD reduction (reduction percentage ~ 20 %) in the solutions. The results provided evidence that the successive bacterial-laccase treatment decolorized azo dyes by reduction of the azo bonds, and mediated coupling reactions between the aromatic amines formed. Coupling reactions between the aromatic amines using enzymes open new opportunities for the physical removal of these amines or new ways for reuse, depending on the characteristics of the particles formed.

Keywords: azo dyes, biodegradation, bacteria, aromatic amines, polymerization, laccase.

1. Introduction

Azo dyes constitute the largest and most versatile class of synthetic dyes used in the textile, pharmaceutical, food and cosmetics industries. These dyes are characterized by the presence of one or more azo bonds (-N=N-). These are prepared by azo coupling between a diazonium compound and aniline, phenol or other aromatic compound. During the dyeing process in the textile industry, approximately 10-15% of the dyes used are released into the wastewater.

Aromatic amines, with known carcinogenic potential, such as aniline and sulphanilic acid appear in liquid effluents as a consequence biological reduction of the azo bond in azo dyes (Bra´s et al., 2005). The presence of these dyes and their by-products in aqueous ecosystems leads to aesthetic and health problems due to the coloring of waters, the obstruction of light penetration and inhibition of oxygen transfer (Fang et al., 2004. Asad et al., 2007).

Dye wastewaters are usually treated by flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation (Lodha et al., 2007). These methods are often expensive and can generate large amounts of sludge, which increases process costs (Kumar et al., 2005). The use of biological methods in the treatment of textile wastewater may be a cost effective alternative to the physico-chemical and photochemical methods currently used. Aerobic biological methods are largely ineffective in the treatment of textile wastewater, resulting in little or no color removal in the case of most of the dyes, especially azo dyes. Anaerobic and microaerobic treatments are effective in removing the color, but the products from azo dye degradation are frequently carcinogenic aromatic amines and these amines can inhibit further aerobic degradation (Pinheiro et al., 2004; Chung and Stevens, 1993).

Conventional processes for the removal of aromatic amines from industrial wastewater include extraction, adsorption onto activated carbon, bacterial and chemical oxidation, electrochemical techniques and irradiation. All of these methods suffer from drawbacks including, high costs, formation of hazardous by-products and low efficiency (Samuelson et al., 2004; Gianfreda et al., 2003).

Laccases are multi-copper phenol oxidases, which reduce oxygen to water and simultaneously catalyze the oxidation of aromatic pollutants such as anilines and phenols (Bourbonnais et al., 1997; Robles et al., 2000). However, laccases can catalyze the coupling and polymerization of products resulting from the dye decolorizing process, and also of various halogen, alkyl and alkoxy substituted

anilines (Zille et al., 2005; Hoff et al., 1985). The enzymatic polymerization of aniline has been investigated for the removal of aromatic amines and aromatic compounds from effluents (Tomaz et al., 1984). The polymeric structures formed can precipitate spontaneously from solution due to their low solubility, and can be removed from the effluent in a further treatment process, or industrially reused, depending on the characteristics of the polymeric product formed (Thiele et al., 2002).
Recently a one-step, simple and environmentally friendly enzymatic synthesis of polymers derived from aromatic amines such as aniline, has been developed (Anand et al., 1998; Huang et al., 2003; Vasileva et al., 2007).

In the present paper the degradation of four azo dyes was performed under microarobic conditions, using a set of bacteria isolated from a textile activated sludge process. This was followed by a secondary treatment with laccase to determine if the biodegradation products from the azo dyes, such as aromatic amines, could be polymerized in coupling reactions. The enzymatic polymerization experiments were carried out for three days. The spectra and particle sizes of the products were analysed by UV-vis and Photon Correlation Spectroscopy (PCS) respectively, and the changes in Chemical Oxygen Demand (COD) were also determined to evaluate whether laccase could reduce the COD content.

2. Material and methods

2.1. Chemicals and media

The azo textile dyes investigated: C.I. Reactive Yellow 107 (RY107), C.I. Reactive Black 5 (RB5), C.I. Reactive Red 198 (RR198) and C.I. Direct Blue 71 (DB71), were kindly provided by a textile company in Brazil. The structures of the dyes are shown in Figure 1. Laccase (EC 1.10.3.2) from *Myceliophthora thermophila* (final activity 1.0 U mL^{-1}) was kindly provided by Novozymes, Denmark. 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}), $MgSO_4$ $7H_2O$ (0.2 g L^{-1}), $FeSO_4$ $7H_2O$ (0.01 g L^{-1}), NaCl (0.1 g L^{-1}) and $CaCl_2$ 2 H_2O (0.02 g L^{-1}). The medium was supplemented with 100

mg L^{-1} of dye, 3 g L^{-1} of glucose and 1 g L^{-1} of sodium pyruvate, and was autoclaved at 121°C for 15 min and designated rich mineral medium (MMR). Aniline-2-sulphonic acid (95% pure) was used as a model product of azo dye reduction, was purchased from Sigma.

2.2. Strain isolation and characterization

The bacteria were isolated from the activated sludge process of a textile company. Serial dilutions (10^{-1} to 10^{-6}) of the samples collected were inoculated into Nutrient Agar Medium using the spread plate technique. Isolated strains were inoculated into MMR with a mixture of azo dyes (100 mg L^{-1} / dyes) and incubated under microarobic conditions (15-5% oxygen) at 30°C for seven days. The strains that achieved the best decolorizing were selected for this study.

Identification of the isolated strains was performed using 16S rRNA gene sequence analysis. Genomic DNA was obtained according to Ausubel et al, (1989).

The cultures were harvested at the end of the exponential growth phase by centrifugation at 18,600 x g for 3 min. The cells were then re-suspended in 100 µL of fresh lysozyme (50 mg/ml) in TE buffer (10 mmol Tris-HC1; 1 mmol/l EDTA, pH 8) and incubated at 37°C for 30 min. The cells were lysed using 0.5 ml of guanidium thyocianate (5 mol/l guanidium thiocyanate (Sigma), 100 mmol/l EDTA and 0.5% v/v sarkosyl) and briefly vortexed. The lysates were cooled on ice, followed by the addition of 0.25 ml of cold 7.5 mol/l ammonium acetate with mixing, then held on ice for a further 10 min, followed by the addition of 0.5 ml of a chloroform and isoamilic alcohol (24: 1) mixture. The phases were mixed thoroughly, transferred to 1.5 ml Eppendorf tubes and centrifuged (18,600 x g) for 10 min. The supernatant fluids were transferred to Eppendorf tubes and 0.54 ml of cold 2-propanol added. The tubes were inverted for 1 min to mix the contents and the fibrous DNA precipitate was centrifuged at 10.000 x g for 20 s. The DNA pellets were washed in 70% ethanol, dried under vacuum and heated at 65°C with mixing until dissolved. The DNA samples were re-dissolved overnight at 4°C in 50 µL of sterile, deionized water.

The 16S rRNA gene was amplified by PCR using primers, 27f and 1401r specific for the Universal Bacteria Domain. Fifty microliter reaction mixtures were used containing 100 ng of total genomic 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 94ºC, followed by 10 cycles of 1 min at 94ºC, 30 s at 69ºC, decreasing 0.5ºC each cycle, and 3 min at 72ºC, followed by another 10 cycles of 1 min at 94ºC, 30 s at 63ºC and 3 min at 72ºC, in an Eppendorf thermal cycler (Eppendorf Mastercycler Gradient). The PCR product was purified using a 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 (Godon et al., 1997).

Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data obtained from the 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 version 4.0 (Thompson et al., 1994; Tamura et al., 2007). Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA using Kimura's DNA substitution model (Kimura et al.,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). The 16S rRNA partial sequences determined in this study were deposited in the Genbank database.

2.3. Decolorizing assays

Decolorizing assays were performed in Erlenmeyer flasks containing 350 mL of MMR (pH 7) supplemented with 100 mg L^{-1} of the dyes. Before starting the experiment, the bacteria were grown individually in 250 ml flasks containing 100 ml of MMR containing no dye. The flasks were incubated with stirring at 150 rpm for 24 hs at 30ºC, and 1% of inoculum added. The samples were incubated under microaerobic conditions at 30°C for 168 hs or until no color was observed.

Decolorization was measured in a UV-visible spectrophotometer (Shimadzu 2101) and the percentage decolorized was calculated. The samples were then frozen and freeze dried (FTS System model Dura-Dry MP).

2.4. The detection of aromatic amines

The aromatic amines in the solid phase were determined using a modified method of Marik et al (2003). After incubation, samples were taken under microaerobic conditions, frozen and freeze dried (FTS System model Dura-Dry MP). A mixture of samples treated by bacteria for each dye (5mg) was dissolved in 5 mL of a 0.4% solution of chloranil in dimethylformamide (DMF) and heated at 100ºC for 5 min. The absorption was measured in a Helios α Unicam UV-Vis spectrophotometer at 560 nm. A calibration curve was prepared using aniline-2-sulphonic acid as a model product of azo dye reduction, and the sample amine concentration was calculated in mM. The control was the MMR medium containing no dye, treated with the bacteria under microaerobic conditions, and the absorption at 560 nm was subtracted from the biodegraded samples containing the dyes.

2.5. Polymerization reaction using laccase

Polymerization of the aromatic amines generated by bacterial decolorization of azo dyes was evaluated using laccase as the polymerization enzyme. Freeze dried samples from the different bacteria for a particular dye were mixed together (3.0 g L^{-1}) and buffered with 0.1 M sodium acetate, pH 5.0 and incubated alone and with 500 µL of Novozyme 51003 *Myceliophthora thermophila* laccase at 45ºC for 72 h, with agitation at 100 rpm, and the amine and polymerizatin products produced by the laccase treatment were monitored by UV-Vis analysis and Photon Correlation Spectroscopy (PCS). The spectra over the range from 200-800 nm was determined in a J&M Tidas UV/Visible spectrophotometer equipped with a diode-array recorder (J&M Analytische Mess und Regeltechnik GmbH, Germany) before and after treatment for 0, 24 and 72 hs. The same experiment was performed with the non- biodegraded azo dyes. Aniline-2 sulphonic acid (ASA) (10mM) was used separately as a model product of azo dye reduction. The reaction was carried out three times.

2.6. Photon Correlation Spectroscopy (PCS) and Chemical Oxygen Demand (COD)

The size distribution of polymerization products with and without laccase was determined at pH 5.0 (acetate buffer) at 45ºC via (PCS) analysis (Malvern zetasizer NS). Prior to the PCS measurements, the buffer was filtered through 0.2 μm Millipore membrane filters and the samples through 0.45 μm Millipore membrane filters directly into the cuvette.

To verify the reduction of organic compounds in the samples after the laccase treatment, the COD was monitored according to the bio-available ASTM 1252 standard guidelines after centrifugation (20.000 x g for 15 min) of the samples obtained after 0, 1 and 72 h of laccase treatment (ASTM, 1994).

3. Results

3.1. Strain isolation and characterization

The strains VN-1, VN-15 and VN-38 were identified based on 16S rRNA gene sequence analysis. The partial sequences determined in this study were deposited in the Genbank database under the accession numbers FJ598006 (*Microbacterium* sp. strain VN-1), FJ598007 (*Brevibacterium* sp. strain VN-15) and FJ598008 (*Leucobacter albus* strain VN-38). Figure 1 show the phylogenetic tree constructed using the neighbour joining method with the Mega 4.0 program. Strain VN-1 was identified as *Microbacterium* sp., supporting values of 97% and 98% on the boot strap, similar to strain types *M. resistens*, *M.thalassium* and *M. oxydans.* Strain VN-15 was phylogenetically positioned in the genus *Brevibacterium*. The nucleotide alignment of this strain supported bootstrap values of 98 and 99%, showing similarity to different *Brevibacterium* strains including the sequences of the strains *B. linens*, *B. permense, B. epidermidis* and *B. Iodinum.* The 16S rRNA gene sequence of the VN-38 strain supported bootstrap values of 99%, showing similarity to type strain *Leucobacter albus.* The *Klebsiella* sp. strain VN-31 was previously identified according to Franciscon-Elisangela et al, (2009b).

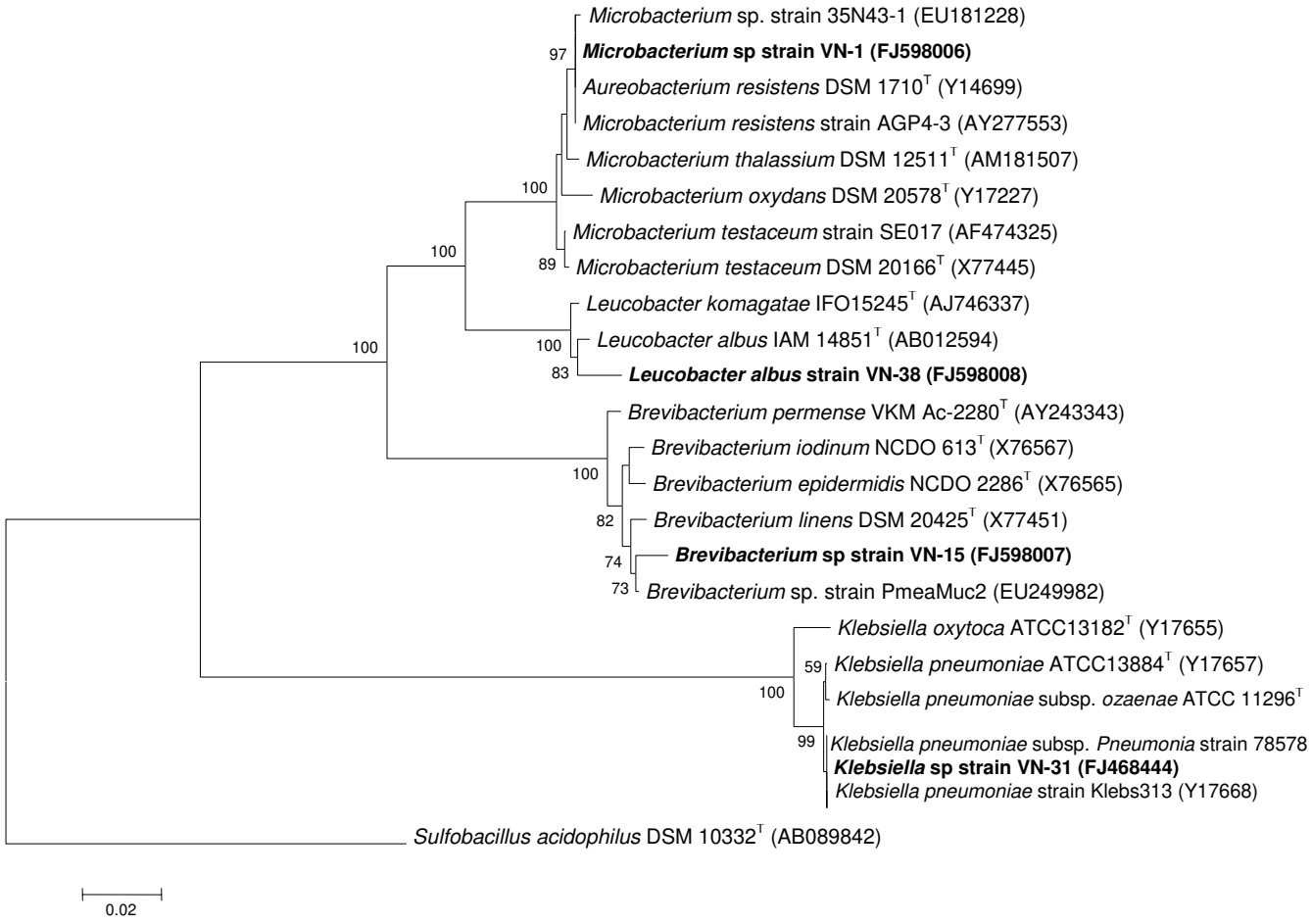

Figure 1. Phylogenetic tree for the strains VN-1, VN-15, VN-31 and VN-38 for the partial sequences based on 16S rDNA. Sulfobacillus acidophilus DSM 10332T was used as the out-group and the accession numbers are in parenthesis.

3.2. Decolorizing assays

The bacterial strains were tested for their ability to decolorize four azo dyes (CI Reactive Yellow 107, CI Reactive Red 198, CI Reactive Black 5 and CI Direct Blue 71) under microaerobic conditions. Complete decolorization (>95%) of the azo dyes was achieved (Table 1) by all the bacteria. The decolorizing time showed a relationship with the chemical structure of the dyes. RY107 and RR198 are both monoazo dyes and showed relatively short destaining times 72 hs and 120 hs respectively. The highly substituted diazo RB5 dye and the triazo DB71 dye showed longer destaining times 168 hs.

Table 1. Decolorization (%) ± S.D* and decolorization time (hs) of the samples containing azo dyes treated with bacteria under microaerophilic conditions.

	Decolorization (%)				Decolorization time (hs)			
Dyes	**RY107**	**RB5**	**RR198**	**DB71**	**RY107**	**RB5**	**RR198**	**DB71**
Strain								
VN-1	96 ± 0.2	96 ± 0.6	96,8 ± 0.2	91±0.4	72±4	168±5	120±3	168±6
VN-15	95 ± 0.2	97,6 ± 0.2	95 ± 0.3	95±0.7	96±4	168±8	120±4	168±5
VN-31	100 ± 0.1	94 ± 0.3	98 ± 0.3	94±0.2	72±3	120±6	72±3	144±5
VN-38	100 ± 0.1	100 ± 0.3	98 ± 0.3	97±0.2	48±3	168±6	144±3	168±8

S.D*: Standard Deviation

3.3. Detection of aromatic amines

The production of aromatic amines from azo dyes is indicative of the reduction of the azo bond. All the decolorized dye media showed the presence of aromatic amines after the bacterial treatment, 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 2). The concentrations of aromatic amines determined were in accordance with the dye structure and the decolorizing time. RY107 and RR198 are both monoazo dyes and showed relatively short decolorizing times and higher amine

concentrations (0.48 and 0.38 mM), respectively. The highly substituted diazo dye RB5 showed a longer decolorizing time and the lowest amine concentration (0.26 mM).

Table 2. Aromatic Amines concentration (mM) ± S.D in the mixed solution containing the azo dyes biodegraded by bacteria under microaerophilic conditions.

	Aromatic amines concentration (mM)
Dyes	
RY107	0.48± 0.1
RB5	0.26± 0.03
RR198	0.38±0.1
DB71	n.d.

n.d. – not detected

3.4. Polymerization reaction using laccase

The feasibility of oxidative coupling between aromatic amines mediated by oxidoreductive enzymes for use in remediation of environmental pollution has been described (Kalyani et al., 2009). Moreover recently, the one-step, simple and environmentally friendly enzymatic synthesis of polymers derived from aromatic amines has been developed (Vasileva et al., 2007).

Polymerization the aromatic amines generated by bacterial decolorization dye was evaluated using laccase as the oxidative enzyme. The experiments were carried out with stirring at pH 5,0 and 45°C, and the products produced by the laccase treatment were monitored by UV-Vis analysis and Photon Correlation Spectroscopy (PCS). Figure 2 shows the UV spectra of the azo dyes before and after bacterial and laccase treatment.

After biodegradation of the four azo dyes by bacteria under microaerobic conditions, the absorbance peaks in the visible region disappeared, indicating their decolorization. The decrease in the typical absorption peak of the hydrogenated azo bond structure (Ar---NH---NH---Ar') at 245 nm for all the dyes, indicated disruption of the azo bonds. The formation of aromatic amines after bacterial

treatment under microarobic conditions indicates the azo bonds were reduced (Table 2). The decrease in absorbance of the peak at 320 nm, which is related to the naphthalene rings, from the UV spectra for RB5, RR198 and DB71, and the formation of a new peak at 280 nm for all dyes, suggested an increase in the concentration of single aromatic amines (Bollag and Myers, 1992; Feng et al., 2000; Mutambanengwe et al., 2007).

The UV-vis spectrum showed considerable changes after 72 h of laccase treatment of the aromatic amines. Immediately after the addition of laccase (0 h) three peaks at 250, 280 and 320 nm, were observed. These peaks are characteristic of the laccase spectrum and the peak at 320 nm suggests the presence of the type III binuclear Cu (II) pair (Eggert et al., 1996). The UV-vis spectrum at the end of the laccase treatment (72 hs) showed peaks at 330 and 360 nm for all the dyes except for DB 71, which only showed a shoulder at 360 nm. A general increase in absorbance throughout the entire visible spectra was also observed, due to the formation of coloured and precipitated products. The aromatic amines formed after decolorizing of the azo dyes are substrates for enzymatic oxidation, and when oxidized can undergo further reactions and couple between themselves, producing coupled products leading to a darkening of the solution and/or the formation of polymeric products (Zille et al., 2005).

After laccase treatment, the spectrum of the model compound aniline 2 sulphonic acid exhibited the peaks characteristic of the enzyme and also a new peak at 360 nm (see discussion above). In the visible region the spectrum showed an increase in the regions from 400-460 nm. Moreover after laccase treatment, a green colouration was observed, different from the colour of the solutions of the biodegraded dyes treated with laccase, which showed a light brown colouration.

The laccase enzyme in buffer, and the control runs in the absence of laccase, gave no evidence of the formation of coloured or precipitated products in the solutions.

To evaluate if the laccase from *Myceliophtora Thermophila* used in the present study was able to decolorize the azo dyes, the control dye solutions were treated with laccase in MMR. UV-vis spectroscopy was not significantly modified, and showed that the azo dyes were not decolorized by the laccase treatment.

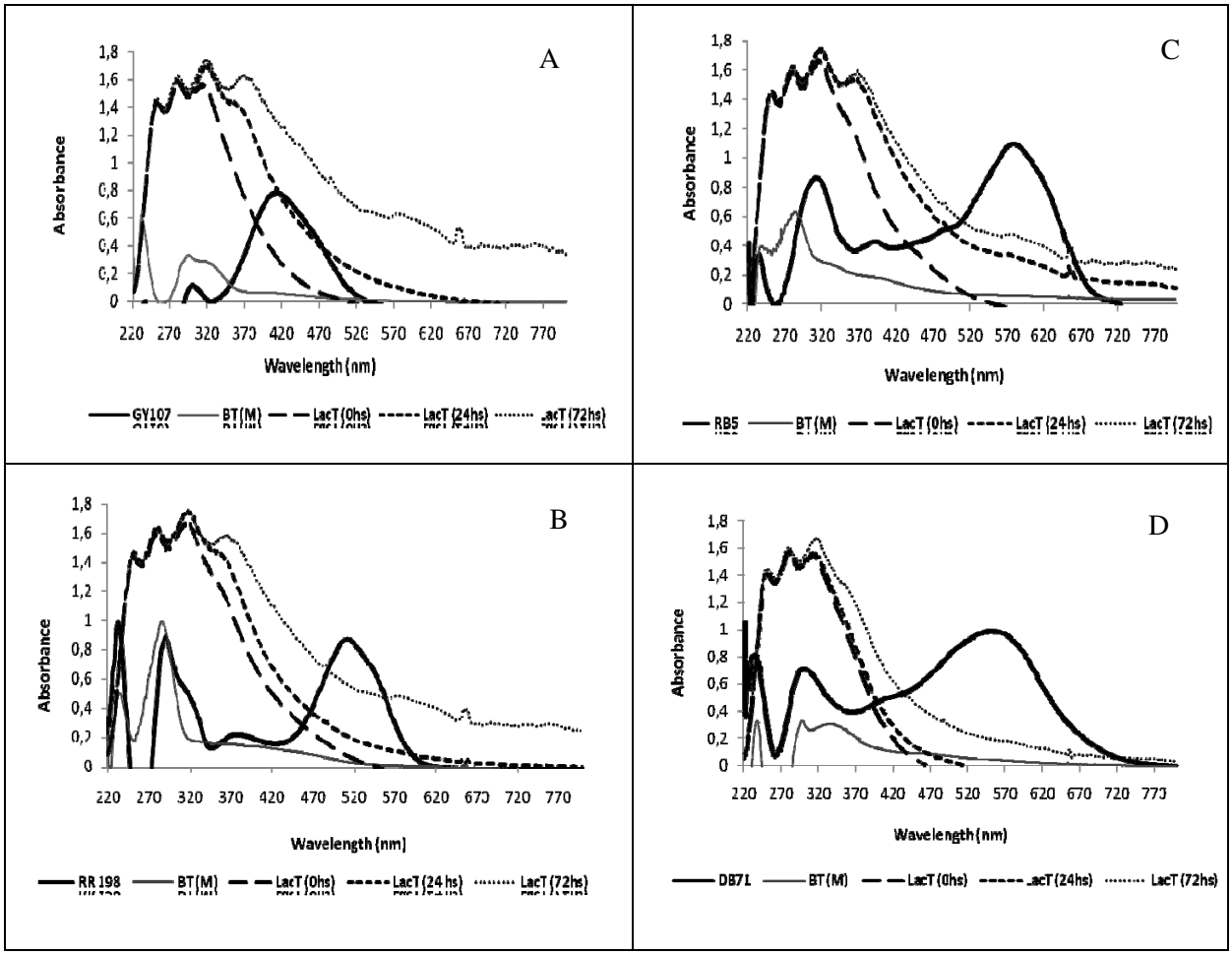

Figure 2. UV-Vis spectra of the azo dyes before (bold straight line) and after bacterial (straight line) and laccase (dashed and dotted line) treatments - A: RY107; B: RB5; C: RR198; D: DB71; BT (M): Bacterial treatment under microaerobic conditions; LacT: Laccase treatment after 0, 24 and 72 hs.

3.5. Photon Correlation Spectroscopy (PCS) and Chemical Oxygen Demand (COD).

The size of the products generated after polymerization by the enzyme laccase, were analyzed by the PCS technique using Malvern Zetasizer NS Instruments.The size profiles of the particles showed a significant increased after laccase treatment. The analysis showed that the particle size of the products formed in the solutions varied from 105 nm–483 nm (Table 3). The size of the particles from the monoazo dyes GY107 and RR 198 (483.9 and 356.3 nm) were much greater than those

from the diazo RB5 and triazo DB71 dyes (165.2 and 105.2 nm). The particle size for biodegraded DB71 and RB5, the large and more complex dyes, were higher than those in the solution containing the biodegradation products from the smaller and morev readily decolorized dyes GY107 and RR 198. After polymerization using the enzyme laccase, the slightly smaller sized dyes produced larger particle sizes, while the larger dyes produced smaller particle sizes, increasing by approximately half or less as compared to the initial size. These results are in agreement with the amount of aromatic amines formed, since the dyes GY107 and RR 198 produced a greater quantity of aromatic amines than RB5, confirming a relationship between the quantity of aromatic amines produced and the particle size.

Table 3 shows the reduction in COD in the solution after laccase treatment. The tests were carried out using the supernatants of the solutions imediately after 1 and 72 hs the addition of laccase. When the solutions were treated with laccase, the COD reduction was approximately 12-15% for RY 107 and RR 198 and 25% for RB5 and DB 71 after 72 hours as compared with the first hour of treatment.

Table 3. Photon Correlation Spectroscopy (nm) ± S.D and Chemical Oxygen Demand (%) ± S.D in the solution containing the azo dyes biodegraded by bacteria under microaerobic conditions.

	Photon correlation spectroscopy (nm)		Chemical Oxygen Demand (%)
Biodegraded dyes	Before laccase	After laccase	After laccase
RY107 (M)	76.91± 1.5	483.9±5.6	12.3 ± 2
RB5 (M)	86.33± 1.3	165.2±4.8	24.8 ± 4
RR198 (M)	79.64± 1.5	356.3±6.3	15.6 ± 2
DB71 (M)	87.03± 1.9	105.2± 5.9	25.0 ± 3

4. Discussion

The bacterial strains VN-1, VN-15, VN-31 and VN-38 used in this study were isolated from raw effluents from a textile industry in Itatiba-Brazil, and selected based on their ability to destain four azo dyes (100 mg L^{-1} of each) in MMR in this study. Although these bacteria showed considerable dye degradation ability, little or no literature regarding dye decolorizing using he genera associated with these isolates these isolates is available. Previous studies have shown that the strains of *Microbacterium sp, Brevibacterium* and *Leucobacter sp* are able to degrade a range of aromatic and PAHs (Junfeng et al.,2008; Rodrigo et al.,2008; Stefan et al.,1994; Chiara et al.,2004). Both the azo dyes and aromatic hydrocarbons are characterized by the presence of aromatic structures such as the benzene, toluene and naphthalene rings. In agreement with the present findings, Wong et al. isolated five bacteria from dye-contaminated sludge and found that two bacteria, identified as *Klebsiella ssp. and Klebsiella pneumonae*, showed destaining ability with respect to the dye Methyl Red (Wong and Yuen, 1996).

The bacterial strains tested in this study for their ability to destain four azo dyes achivied complete destaining (>95%) of the azo dyes.

The destaining time showed a relationship with the chemical structure of the dyes. RY107 and RR198 are both monoazo dyes and showed relatively short destaining times. The increase in degradation time for RR198 was probably due to the triazine group, whose degradation is more recalcitrant than that of the benzene and naphthalene rings (Guillard et al., 2003). The chemical structures of the dyes greatly influenced their destaining rates, and the destaining efficiency was limited to a few azo dye structures (Chivukula and Renganathan, 1995; Pasti-Grigsby et al., 1992). Dyes with simple structures and low molecular weights usually exhibited higher rates of color removal, whereas color removal was less effective with highly substituted, high molecular weight dyes (Chen et al., 2003). It is therefore likely that this is the reason, the highly substituted diazo RB5 dye and the triazo DB71 dye showed longer decolorizing times. Moreover, steric hindrance by substitution at the *ortho* and *ortho-para* positions of the azo bond decreased the decolorizing performance. In addition, further substitution in the proximity of the azo linkages has been shown to decrease the efficience of the biodecolorization (Chung-Chuan and Bor-Yann, 2008). It has also been

reported that the presence of sulphonates in reactive dye structures could result in low levels of colour removal. However, this was not applicable to the azo dyes used in this study, which exhibited high levels of colour removal, independent of the number of sulphonate groups in the dye structure, reinforcing the idea that steric hindrance and the number of azo bonds are responsible for the different destaining times (Hitz et al.,1978).

The reductive cleavage of the –N=N– bond is the initial step in the bacterial degradation of azo dyes (Zimmermann et al., 1982). Decolorizing of azo dyes occurs under aerobic, anaerobic and microaerobic conditions. There are few bacteria that are able to grow on azo compounds as the sole carbon source. These bacteria reductively cleave the azo bonds and use the amines as their source of carbon and energy (Zimmermann et al., 1982; Kulla et al., 1983). Reduction under anaerobic or microaerobic conditions is the most frequently reported form of azo dye degradation, where the dye probably acts as an electon acceptor in the electron transport chain. Recent studies have also shown that the addition of redox mediators to anaerobically incubated cultures of various taxonomically different bacterial species could result in significantly increased reduction rates of azo dyes (Rau et al., 2002). Pure bacterial strains, including *Pseudomonas luteola, Sphingomonas* sp., *Staphylococcus arlettae* and *Klebsiella sp* have been described as being capable of reducing azo dyes under anaerobic and microaerobic conditions (Kudlich et al.,1997; Chung-Chuan and Bor-Yann, 2008; Franciscon et al.,2009 a,b). Recent reports showed that the monoazo dye (Acid Orange 7) was rapidly destained by anaerobic cultures, and the presence of aniline (metabolite from the reductive degradation of azo dyes) was observed (Carvalho et al., 2008). Also, in a pathway proposed for the biodegradation of Reactive Red 2 by *Pseudomonas* sp. SUK1, the aromatic amine aniline was one of the intermediate products originating from the biodegradation reaction (Kalyani et al., 2009).

After biodegradation of the four azo dyes by bacteria under microaerobic conditions, the absorbance peaks in the visible region disappeared. Moreover, the decrease in the typical absorption peak of the hydrogenated azo bond structure (Ar---NH---NH---Ar') at 245 nm for all the dyes indicated disruption of the azo bonds (Feng et al., 2000). The decrease in absorbance of the peak at 320 nm, which is related to the naphthalene rings, from the UV spectra for RB5, RR198 and DB71, and the formation of a new peak at 280 nm for all dyes, suggested an increase in the concentration of single aromatic amines (Bollag and Myers, 1992; Mutambanengwe et al., 2007).

The UV-vis spectrum showed considerable changes following 72 hs of laccase addition in the biodegraded solutions containing aromatic amines. A general increase in absorbance throughout the entire visible spectra was also observed, due to the formation of coloured and precipitated products, which were possibly oligomers and polymers. The formation of oligomeric or polymeric products as a result of coupling reactions between intermediates of the dye degradation caused by the laccase oxidative process, have been described earlier (Moldes et al.,2004 ;Kandelbauer et al.,2004). In addition the absorption band at 360 nm has been extensively described and ascribed to electron transitions in the aromatic π- π* rings, on the basis of earlier studies on enzymatic polymerization of aromatic amines such as aniline (Streltsov et al.,2008). The peak at 330 nm shown in the UV-vis spectra can be attributed to a reduction by O_2 caused by laccase, and probably involves changes in the coordination geometry of at least one of the copper sites in the enzyme molecule (Michel et al., 1980). An earlier study reported that when O_2 reacted with partially reduced laccase, extra absorbance at 330 nm was observed (Andreasson and Reinhammar, 1976). Confirming the present findings a recent study about biotransformation of the azo dye Sudan Orange during enzymatic treatment with laccase showed that the peak originally giving maximum absorption for the dye decreased, and concomitantly an increase in the absorption intensities at 325 and 530 was observed and associated with the generation of biotransformation products such as an extensive array of oligomeric products (Pereira et al., 2009).

The above methods provided for enzymatic polymerization within the reaction mixture, including a monomer that could be aniline, a template and an enzyme. When aniline is the monomer, oxidation will produce a free radical from the *ortho* or *para* positions in the benzene ring. The oxidized monomers then undergo a coupling reaction to form a polymer with each other. The templates facilitate polymerization, and could be an azo polymer, protein, azo or sulphonated compound (Samuelson et al., 2004). In the present study, no template was used, but the components of the medium, including azo compounds with a sulphonated group, could facilitate the coupling reactions. Therefore after polymerization using the enzyme laccase, the slightly smaller sized dyes produced larger particle sizes, while the larger dyes produced smaller particle sizes and only increased by approximately half or less as compared to the initial size.

When the solutions were treated with laccase, the COD reduction was approximately 15 % for all dyes, after 72 hours as compared with the first hour of treatment. These results are associated with the formation of highly insoluble products. At the start of the laccase treatment (1 h) these polymeric products had not been generated significantly, large particle sized products were obtained only after for 72 hs, which could be removed from the samples in the form of a precipitate after centrifugation (Pereira et al., 2009).

5. Conclusion

In conclusion the bacterial strains VN-1, VN-15, VN-31 and VN-38, isolated from the textile effluent and identified by 16S DNA gene analysis, were able to completely decolorize the azo dyes under microarobic conditions. The formation of amines during the bacterial treatment was confirmed by direct measurement and by the UV-Vis analysis. The laccase polymerization reaction of the resulting aromatic amines was investigated. It was observed that after long periods of time laccase catalyzed polymerization of the aromatic amines present in the solutions. The products generated precipitated spontaneously from the solution and acquired some colour, as shown by the UV-Vis analysis. The particle size was also significantly higher after laccase treatment. In addition the laccase demonstrated the ability to reduce the COD of the solutions.

The most effective treatments with respect to colour removal for the textile effluents were the anaerobic and microaerobic processes. Unfortunately they produce carcinogenic aromatic amines and are thus inadequate for the removal of organic matter. The present study showed the possibility of removing the aromatic amines using enzymatic polymerization with laccase. In addition, the possibility of obtaining polymers from the aromatic amines produced during the treatment of the effluent, in a simple and environmentally friendly way opens the possibility of developing economic ways for reusing the effluent.

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8. Conclusões Gerais

O principal objetivo desta tese foi estudar os mecanismos de biodegradação dos azocorantes por bactérias e investigar as melhores condições de degradação, bem como a investigação de metabólitos formados após utilização dos corantes pelas bactérias e redução na toxicidade.

De um modo geral todas as bactérias isoladas contribuiram eficientemente na degradação dos azocorantes.

As melhores condições encontradas para descoloração (30-35ºC com adição de 100 mg L^{-1} de azocorantes, condições microaeróbias ou estáticas e adição de fontes de carbono como fonte de energia) foram utilizadas para todas as linhagens (capitulo 1, 2, 3 e 4). Nenhum dos microrganismos testados foram capazes de descolorir os azocorantes como única fonte de carbono, indicando que estas bactérias necessitam de fontes externas de carbono para alçancar a descoloração dos azocorantes através de uma reação cometabólica, onde outros compostos são utilizados para degradar moléculas mais complexas.

A fonte de carbono utilizada demostrou ser específica para cada bactéria. No primeiro estudo (capitulo1), com *Staphylococcus arlettae*, o extrato de levedura foi a fonte de carbono e nitrogênio essencial para ocorrer a degradação. Os estudos seguintes com *Klebsiella* sp e *Brevibacterium* sp (capitulo 2 e 3) mostraram que apenas piruvato de sódio foi suficiente para ocorrer um eficiente degradação dos corantes. A linhagem *Brevibacterium* sp mostrou significante atividade de Tirosinase, confirmando que a biodegradação de azocorantes por bacterias é um mecanismo específico dependente da linhagem, meio de cultura utilizado e estrutura química dos azocorantes.

Houve a formação de aminas aromáticas após tratamento microaerofílico ou estático como foi confirmado por medida direta e por análises de Espectrofmetria de Infravermelho com Transformadas de Fourier.

Constatou-se que, após agitação o qual proporcionou um processo oxidativo, houve ganhos significativos na biodegradação dos substratos presentes no meio, bem como na diminuição da concentração de aminas aromáticas, que nestas condições, foram mais susceptiveis á ação dos microrganismos. A diminuição na concentração destes compostos após tratamento aeróbio permitiu a total redução da toxicidade como confirmado pelas análises ecotoxicológicas com o microcustácio

Daphnia magna. O estágio aeróbio foi de extrema importância para uma parcial mineralização dos produtos de degradação dos corantes e dos metabólitos de meio.

Este trabalho também abordou a possibilidade de polimerização das aminas aromáticas resultantes da degradação microaeróbia ou estática, usando a enzima lacase (capítulo 4). Os produtos gerados após adição de lacase precipitaram na solução e estas adquiriram novamente alguma coloração, como confirmado por análises de UV-Vis. Os tamanhos das partículas analisadas por Correlação de Espectroscopia de Fóton foram significativamente maiores após a adição de lacase, que ainda mostrou habilidade de reduzir a DQO das soluções. A utilização da lacase após tratamento microaeróbio ou estático tem como vantagem, além da remoção física destas aminas do efluente final, a possibilidade de reuso destas estruturas poliméricas dependendo das características formadas por elas, podendo até serem novamente reutilizados para colorir outros materiais de aplicação menos nobres.

Finalmente, foi possível concluir que um sistema formado por um tratamento microaeróbio ou estático seguido de um tratamento aeróbio é uma alternativa promissora para tratamento de efluentes contendo azocorantes, com a simplicidade de apenas mudar as condições de oxigenação sem elevar os custos do tratamento. Um sistema de tratamento com lacase, também poderia ser acoplado antes da fase aeróbia, para diminuir o consumo de energia e insumos, pois este poderia auxiliar na remoção das aminas aromáticas por precipitação e também reduziria a carga orgânica a ser tratada no sistema seguinte, com ganhos para o meio ambiente.

Trabalhos futuros poderão ser focados no estudo das estruturas formadas após polimerização de efluentes contendo aminas aromáticas para avaliar a possibilidade de reuso destes compostos formados.