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STUDY OF AZO DYE DEGRADATION BY ISOLATED BACTERIA CULTURES FROM ACTIVE SLUDGE OF A TEXTILE INDUSTRY

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STUDY OF AZO DYE DEGRADATION BY ISOLATED BACTERIA CULTURES FROM ACTIVE SLUDGE OF THE TEXTILE INDUSTRY

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Grazielly Maria Didier de Vasconcelos

STUDY OF AZO DYE DEGRADATION BY ISOLATED BACTERIA CULTURES FROM ACTIVE SLUDGE OF THE TEXTILE INDUSTRY

O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Prof. Cristiano José de Andrade, Dr.
Orientador

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RESUMO

Os efeitos da água residual contendo corante azo no meio ambiente pode ser devastador. Assim, ele precisa ser tratado adequadamente antes de seu descarte - geralmente em corpos d'água. O lodo ativado é uma associação entre muitos (micro) organismos em uma comunidade, composta por bactérias aeróbias e anaeróbias e fungos que degradam o corante, por meio de descoloração e mineralização. No entanto, faltam informações sobre espécies degradantes específicas e suas interações, o que poderia melhorar significativamente os tratamentos de águas residuais contendo corantes azo. Portanto, o objetivo deste estudo foi avaliar a comunidade bacteriana de uma amostra de lodo ativado real de indústria têxtil, por meio do isolamento e identificação de cepas de bactérias degradadoras de corante azo. A dessorção/ionização a laser assistida por matriz, seguida de espectrometria de massas por tempo de voo (MALDI-TOF MS), foi a técnica utilizada para identificar uma ampla gama de espécies de bactérias incluindo aeróbia (*Lysinibaciullus fusiformis*) e anaeróbia facultativa (*Escherichia coli*). Dados preliminares indicaram os maiores potenciais de degradação de corante azo Reactive Red 141 (RR-141) por *Bacillus thuringiensis* e *Kosakonia radicincitans*. É importante notar que este é o primeiro relato sobre descoloração por *K. radicincitans*. Brain Heart Infusion (BHI), glicose, e RR-141 foram usados como fontes de carbono. No entanto, apenas nos meios de BHI e glicose detectou-se atividade de descoloração, indicando que o RR-141 não demonstrou ser uma boa fonte de carbono para desenvolvimento bacteriológico. Ambas as cepas exibiram capacidade de descoloração, atingindo 43% de descoloração em BHI por *B. thuringiensis*, e 21% em meio mineral com glicose por *K. radicincitans*. O rendimento acima de 40% foi alcançado aplicando-os simultaneamente no mesmo meio reacional, em condições não otimizadas. Frente à falta de dados sobre o microbioma de lodo ativado e a sua influência para avanços na área de biorremediação de corantes azo de efluentes têxteis, este trabalho destaca a técnica de MALDI-TOF-MS para identificação de espécies bacterianas isoladas de lodo ativado de efluente real de uma indústria têxtil. Além de avaliar a capacidade de descoloração destas com o corante azo RR-141, incluindo a *Kosakonia radicincitans* - maneira inédita.

Palavras-chave: Lodo ativado; indústria têxtil; microbioma; biodegradação, corante azo.

RESUMO EXPANDIDO

Introdução

Com o aumento da demanda global por têxteis, têm-se os impactos ambientais potenciais de sua produção. A indústria têxtil gera, inerentemente, um alto volume de efluente tóxico, principalmente devido aos banhos químicos e séries de enxágues. Os compostos que mais chamam atenção no que tange aos efluentes têxteis são os corantes. Dentre uma ampla classificação, o grupo dos corantes de nitrogênio, ou corantes azo, é o mais utilizado na indústria têxtil.

Convencionalmente, os principais processos para tratar (descolorir e degradar) efluentes coloridos, com eficiência, são baseados em métodos físico-químicos, como adsorção, coagulação e oxidação com ozônio. Porém, os processos de biodegradação de corante azos de efluente da indústria têxtil veem se destacando por apresentar algumas vantagens quando comparados aos métodos físico-químicos, como custo competitivo, produtos finais não tóxicos, bons rendimentos, menor necessidade de consumo de água, e por apresentar uma abordagem mais ambientalmente favorável.

Dentre essas técnicas, a biodegradação de corantes pelo processo de lodo ativado vem chamando atenção pela sua eficiência e seu baixo custo, com relação à implantação e operação. Entretanto, a identificação do seu microbioma, ou seja, dos microrganismos que o compõe, é de grande relevância para o entendimento mais profundo da descoloração do efluente (potencial de otimização de degradação e mecanismos). O estudo da atividade de descoloração por bactérias isoladas ainda é a rota preferida e visto como uma oportunidade para compreender mais profundamente os mecanismos de degradação, possibilitando a melhoria dos rendimentos de descoloração. Por outro lado, a avaliação de microrganismos em consórcio pode fornecer condições de melhor desenvolvimento e ação degradante/descolorante para as culturas, quando comparadas com suas performances isoladas.

Análises de identificação do microbioma são, em sua maioria, baseadas em técnicas caras que envolvem vários experimentos e procedimentos analíticos, por exemplo, extração, purificação, separação (sequenciamento do gene 16S rRNA e 18S rRNA), buscando compreender as características fenotípicas complexas, moleculares e morfológicas. Esses métodos, porém, são caros e geralmente não fornecem informações sobre a fisiologia microbiana. Nesse contexto, a técnica de dessorção/ionização a laser assistida por matriz seguida de espectrometria de massas por tempo de voo (MALDI-TOF MS) vem ganhando destaque, devido à sua rapidez, simplicidade de procedimentos e confiabilidade dos resultados. A MALDI-TOF MS é usada para identificação de microrganismos em hospitais, laboratórios clínicos e até em indústrias alimentícias. Quando comparada às metodologias moleculares (baseadas no DNA bacteriano), ela requer menor quantidade de material biológico e envolve protocolos de preparação mais simples, tendo, portanto, grande potencial de trazer resultados promissores se utilizado para a identificação do microbioma de lodo ativado.

Portanto, este estudo teve como objetivo isolar e identificar cepas bacterianas que são capazes de descolorir o corante azo RR-141 de lodo ativado de uma planta de tratamento de efluente têxtil real. As duas cepas mais promissoras foram utilizadas e investigadas quanto aos seus desempenhos cinéticos, capacidades de descoloração por espectrofotometria UV-VIS, eficiências de degradação por HPLC-MS, fitotoxicidade e atividade enzimática. A microscopia eletrônica de varredura da amostra de lodo ativado também foi executada, para investigação da sua microbiota.

Objetivo geral

Contextualizar o cenário de biodegradação de corantes azo por bactérias isoladas, com especificidade sobre aquelas isoladas de amostra de lodo ativado e avaliadas em relação aos seus potenciais de descoloração e degradação de corante azo, em diferentes fontes de carbono, avaliando desempenho em consórcio, a fitotoxicidade pré- e pós-tratamento, e a atividade enzimática.

Objetivos específicos

- Descrever os impactos ambientais potenciais de águas residuais contendo corante azo da indústria têxtil;
- Explorar a identificação do microbioma de lodo ativado obtido de uma indústria têxtil;
- Apresentar uma visão geral de culturas isoladas identificadas no contexto da degradação de corantes têxteis;
- Realizar um estudo do potencial de descoloração de culturas isoladas e em consórcio;
- Avaliar a degradação de corantes têxteis através das análises de HPLC-MS, fitotoxicidade, atividade enzimática.

Metodologia

Foi realizada amostragem de lodo ativado do tanque de decantação secundária de uma estação de tratamento de indústria têxtil localizada em Blumenau-SC, Brasil. A fração sólida foi separada e observada em microscópio eletrônico de varredura (HITACHI TM3030). O isolamento das diferentes culturas foi executado para sua posterior identificação pelo espectrômetro de massa MALDI-TOF-MS (Bruker Daltonics, Alemanha). O potencial de descoloração do corante azo foi avaliado por plaqueamento em estrias e por sistema aquoso em frascos Erlenmeyer sob agitação, com verificação periódica durante 7 dias, por percepção visual e medidas espectrométricas, respectivamente. As cepas selecionadas foram então avaliadas quanto ao desempenho em diferentes meios de cultura e fontes de carbono, tendo sua cinética também estudada. Ensaio de fitotoxicidade foi realizado com sementes de alface para verificar a toxicidade da solução antes e após o tratamento biológico. A degradação/mineralização completa e a atividade enzimática também foram investigadas por análise de HPLC-MS e ensaios em placas de Petri, respectivamente.

Resultados e discussão

A análise das micrografias de microscopia eletrônica de varredura do lodo ativado indicou a presença de uma matriz de biofilme aderida, muito provavelmente de bactérias floculantes. Algumas características importantes estão relacionadas à estrutura e função do biofilme, por exemplo, compostos orgânicos como substâncias poliméricas extracelulares que desempenham um papel significativo na modificação de superfície (carga, hidrofobicidade) para dar condições adequadas para a conexão bacteriana e ajudar em seus processos metabólicos. MALDI-TOF-MS foi apresentado como uma boa ferramenta para identificação rápida do microbioma. *Bacillus cereus, Klebsiella oxytoca, Bacillus thuringiensis, Kosakonia cowanii, Lysinibacillus fusiformis, Acinetobacter*

baumannii, Kosakonia radicincitans e *Escherichia coli* foram as espécies detectadas. Entre eles, *Kosakonia* sp. é a cepa menos explorada para descoloração de corante azo.

A triagem de culturas bacterianas descolorantes em meio sólido revelou colônias de *K. oxytoca*, *B. thuringiensis*, *K. cowanii* e *K. radicincitans* com maior potencial para descoloração. No entanto, a investigação em sistema aquoso forneceu resultados que foram utilizados para escolher o *B. thuringiensis* (> 40% de rendimento de descoloração) e *K. radicincitans* (> 20% de rendimento de descoloração) para estudos posteriores.

A análise de preferência da fonte de carbono e o estudo cinético revelaram o não desenvolvimento no meio cuja fonte de carbono era apenas o corante azo. Além disso, diferentes preferências de meios foram detectadas para cada cultura bacteriana analisada. O estudo cinético revelou níveis de descoloração de 21% em meio mineral com glicose por *K. radicincitans* e 43% em BHI por *B. thuringiensis*. O uso de ambas as culturas em consórcio no meio reacional mineral com glicose promoveu aproximadamente 40% de descoloração, indicando o potencial uso de culturas bacterianas mistas como agente de biorremediação para remoção econômica de corante de efluente têxtil.

O ensaio de fitotoxicidade e as análises HPLC-MS sugeriram que a degradação não foi completa, favorecendo uma possível adsorção de corante à biomassa. Além disso, uma ação enzimática potencial foi inferida pela formação de halo translúcido observada, que pode estar associada à produção de enzima extracelular.

Considerações finais

Os resultados obtidos neste trabalho compõem uma importante contribuição para a biodegradação de corantes azo por culturas bacterianas isoladas de lodo ativado de indústria têxtil, incluindo a avaliação inédita da *Kosakonia radicincitans*.

Palavras-chave: Lodo ativado; indústria têxtil; microbioma; biodegradação, corante azo.

ABSTRACT

The effluent from the textile industry is a complex mixture of recalcitrant molecules that can harm the environment and human health. Biological treatments are usually applied for this type of wastewater, mainly activated sludge, due to its high efficiency and low implementation and operation costs. However, the activated sludge microbiome is rarely well-known due to the variability of its origin. Some studies revealed the most usual presence of *Acidobacteria*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Proteobacteria*, and *Streptococcus* in activated sludges, and *Bacillus Pseudomonas* are highlighted for bacterial dye degradation. Consequently, the process is not performed on its optimum conditions (yield of treatment). In this sense, this review aims to contextualize the potential environmental impacts of azo dye-containing wastewater from the textile industry; evaluate its toxicity; explore the identification of activated sludge microbiome; and then highlights the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a novel, rapid and accurate strategy for the identification of activated sludge microbiome (potential to enhance treatment yield).

Keywords: activated sludge; textile industry; microbiome; biodegradation; azo dye.

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ABS	Absorbance
AOPs	Advanced oxidation processes
BHI	Brain Heart Infusion
BOD	Biological oxygen demand
CFU	Colony formed unit
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
GC-MS	Gas chromatography–mass spectrometry
HPLC-MS	High Performance Liquid Chromatografy - Mass Spectrometry
MBRs	Membrane bioreactors
MFCs	Microbial fuel cells
MLAs	Machine learning algorithms
MS	Mineral salt media without glucose
MSG	Mineral salt media with glucose
OD600	Optical density at 600 nm
RNA	Ribonucleic acid
RR-141	Reactive Red 141
SS-ABR	Submerged anaerobic deflector reactor with sponges
TOC	Total organic carbon
TW	Tap water
UASB	Upflow Anaerobic Sludge Blanket
λmáx	Maximum absorption wavelength

SUMÁRIO

CONCEPTUAL DIAGRAM

STUDY OF AZO DYE DEGRADATION BY ISOLATED BACTERIA CULTURES FROM ACTIVE SLUDGE OF A TEXTILE INDUSTRY

What?
• Investigation of dye degradation potential by isolated bacterial cultures from a textile effluent treatment plant.
Why?
• The world consumption of dyes has been increasing, mainly due to the textile industry (colourization of fibres). A massive amount of wastewater is generated, and its incorrect disposal into the environment harms aquatic life. • There is a growing interest in studying azo dye remediation by biological pathways due to its efficiency; non-toxic end products; good yields; less need for water consumption, a more environmentally friendly approach; and the low-cost concerning implementation and operation. • Among these techniques, the biodegradation of dyes by the activated sludge process has been attracting attention due to its efficiency and low cost with implantation and operation. • The study of the activated sludge microbiome for remediation of textile dyes requires a deeper understanding of which species promote the discolouration of the effluent, and it is not widely explored. • Isolated cultures assays are the preferred route and are seen as an opportunity for a deeper understanding of degradation mechanisms, improving decolourization yields.
Hypotheses
• Does the MALDI-TOF MS an efficient technique to identify activated sludge microbiome? • Do the decolourization assays reveal promising bacterial strains for azo dye decolourization? • Can the strains realize the degradation and/or the mineralization of the azo dye? • Can the final products and effluent toxicity de compatible with the proper discharge into the environment without causing damages?

CHAPTER 1

This chapter presents a brief introduction to the research developed and its general and specific objectives.

1 STUDY OF AZO DYE DEGRADATION BY ISOLATED BACTERIA CULTURES FROM ACTIVE SLUDGE OF A TEXTILE INDUSTRY

1.1 INTRODUCTION

With the increase in the global demand for textiles, there are potential impacts on their production. The textile industry inherently generates a high volume of toxic effluent, mainly due to chemical baths and rinsing series. The compounds that draw the most attention when dealing with textile effluents are dyes. Among a broad classification, the group of azo dyes, is the most used in the textile industry.

Conventionally, the main processes to efficiently treat (discolour and degrade) coloured effluents are based on physicochemical methods such as adsorption, coagulation, and oxidation with ozone. However, the biodegradation processes of azo dyes in effluent from the textile industry have been highlighted for presenting some advantages compared to physicochemical methods. Specific cost; non-toxic end products; suitable accessories; less need for water consumption; and a more environmentally friendly approach are some of the pros biological degradation.

Among these techniques, the biodegradation of dyes by the activated sludge process has been attracting attention due to its efficiency and the low-cost concerning implantation and operation. However, the study of its microbiome, that is, of the microorganisms that compose it, is of great generation for a deeper understanding of the discolouration of the effluent (degradation potential and mechanisms). The study of decolourization activity by fertilizers is still a preferred route and seen as an opportunity for a deeper understanding of degradation, enabling an improvement in decolourization adjustments. On the other hand, an evaluation of microorganisms in the consortium can provide the conditions for better development and action of cultures compared with their defined performances.

Microbiome identification analyses are mainly based on expensive techniques involving several experiments and analytical procedures, such as extraction, purification, separation (16S rRNA and 18S rRNA gene sequencing), and understanding the complex, molecular phenotypic, and morphological characteristics. These methods, however, are expensive and generally do not provide information about microbial physiology. In this context, the matrix-assisted laser desorption/ionization technique followed by time-of-flight mass spectrometry (MALDI-TOF MS) has gained prominence due to its speed, simplicity of procedures, and reliability of results. MALDI-TOF MS is used to identify microorganisms in hospitals, clinical laboratories, and even food industries. Compared to molecular methodologies (based on bacterial DNA), it requires less biological material and involves simpler preparation protocols. Therefore, it has excellent potential to bring promising results if used to identify the activated sludge microbiome.

Therefore, this study aimed to isolate and identify bacterial strains capable of discolouring the azo dye RR-141 from activated sludge from a real textile wastewater treatment plant. The two most promising strains were used to investigate kinetic performance, decolourization capacity by UV-VIS spectrophotometry, two-way HPLC-MS analysis, phytotoxicity, and identification of enzymatic activity. A scanning electron microscopy of the activated sludge sample was also performed.

1.1.1 Objectives

1.1.1.1 General Objective

Contextualize the scenario of biodegradation of azo dyes by isolated bacteria, with specificity on isolated bacteria and evaluate their potential for discolouration and degradation of an azo dye in different carbon sources, evaluating intercropping performance, pre- and post-treatment phytotoxicity, and enzymatic activity.

1.1.1.2 Specific Objectives

- Describe the potential environmental impacts of wastewater containing azo dye from the textile industry;
- Explore the identification of the activated sludge microbiome obtained from a textile industry;
- Present an overview of isolated cultures identified in the context of textile dye degradation;
- Assessment of textile dye degradation (HPLC-MS, phytotoxicity, enzyme active).

CHAPTER 2

In this section, a book chapter about the fundamental concepts of dye-containing textile wastewater treatments, including the most usual textile wastewater treatments and focusing on microbial and enzymatic approaches, the trends (modern technology) is discussed. This review chapter is linked to the book “Current Biological approaches in Dye Wastewater Treatment” and was sent to the editor in September 2021.

2 FUNDAMENTAL CONCEPTS OF DYE-CONTAINING TEXTILE WASTEWATER TREATMENTS: MICROBIAL AND ENZYMATIC APPROACHES

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Abstract

The world consumption of dyes has been increasing, mainly due to the textile industry (colourization of fibres). The textile industry generates a massive amount of wastewater. The incorrect disposal of coloured effluent into the environment leads to the derangement of aquatic life. Several techniques have been applied to reduce this impact, including adsorption, coagulation, filtration, among others that, on the one hand, are efficient; on the other hand, require additional management (e.g., a large volume of sludge). In this sense, specific biological pathways for dye degradation and wastewater discolouration have drawn attention to the industry since they can increase wastewater treatment yields. Therefore, this chapter describes the fundamental concepts of dye-containing textile wastewater treatments, particularly microbial and enzymatic approaches, including the most usual textile wastewater treatments and their trends (modern technology).

Keywords: Azo dyes • Bioremediation • Bacterial • Fungal • Microalgae • Genetically modified organisms • Combined treatments systems • Biofilms • Resource recovery strategy • Machine learning

2.1 INTRODUCTION

Since the first commercially successful synthesis, dyes have been massively applied for the chemical, food, textile, paper, and other industries (Saratale et al., 2011).

Dyes can be classified taking into account their chromophore group (e.g., anthraquinone, azo, indigo, nitrated, nitro, phthalein, triphenyl) and/or their fibre interaction (e.g., acid, azoic, direct, disperse, reactive, sulfur, vat) (Benkhaya et al. 2017). Azo-dyes (N=N-) represent an environmental concern due to their high demand and toxicity, including carcinogenic (Bafana et al. 2011).

The textile industries are the most dye-consumers that inherently produce many effluents with high biochemical oxygen demand, turbidity, and low degradability since 10 to 15% of dyes do not remain on textile fibres (Verma

et al. 2012; El Bouraie and El Din 2016). These dyes are recalcitrant molecules that are harmful to the environment (Baban et al., 2003). Thus, biological, physical, and chemical treatments, including activated sludge, oxidation, ozonation, membrane filtration, coagulation, and their combinations, are extensively applied as textile wastewaters (Ahmad et al. 2019; Camargo et al. 2019; Li et al. 2019c; Herrera-González et al. 2019). However, there are drawbacks for each approach, such as high costs, inefficient, and generation of secondary polluters (e.g., high volume of sludge).

Regarding remediation of dye-contaminated water bodies, the biological processes by bacteria, fungi, algae, oxidative enzymes, and their combinations have been drawing attention since they are efficient and eco-friendly, operates at mild, among others (El et al. 2016; Uppala et al. 2019; Ooi et al. 2007; Gou et al. 2009; Bilal et al. 2019). In this sense, phycoremediation and genetically modified organism oxidoreductase producers are promising biological treatment alternatives. The integrated systems are advanced oxidation processes or membrane bioreactors (anoxic bioreactors, aerated bioreactors, UV-unit, and granular activated carbon filters).

Therefore, it is essential to discuss the treatment methods for treating dye-contaminated wastewater properly. In this context, this chapter describes the fundamental concepts of dye-containing textile wastewater, exposing the nature of dye, its classification, and global market with a specific approach of azo dye. Furthermore, some discussions about environmental effects when improperly discharged, the conventional textile wastewater treatments technologies, particularly microbial and enzymatic methods, including the most usual textile wastewater treatments and their trends (modern technology).

2.2 CHEMICAL STRUCTURE OF DYES

Dyes and pigments are chemical compounds widely used to impart colour to different substrates (fabric, fibre, leather, plastic, paper, etc.) (Chequer et al. 2013a; Varjani et al. 2021). Colour is described as the qualitative perception of light discriminated by the eyes and brain (Loe 2017; Xuan et al. 2021). According to The Ecological and Toxicological Association of Dyes and Organic Pigment Manufacturers (2021), dyes are intensely coloured or fluorescent soluble organic substances, which provides colour to a substrate by absorption of light. These compounds absorb light radiation within the range of the visible region spectrum (380 to 750 nm) whereas they reflect or diffuse that radiation (Benkhaya et al. 2020).

Synthetic dyes are complex organic substances coloured fluorescent (Gürses et al. 2016). On the other hand, pigments are solid or particle structures composed of organic or inorganic moiety. They can be coloured red, colourless (opaque), or fluorescent. It is worth noting that the last one is chemically unstable and shows low water solubility (Berradi et al. 2019; Pavithra et al. 2019). In a broader sense, the difference between these compounds lies precisely in how they interact with the substrate.

Dyes consist of two main components, chromophore and auxochrome (Temesgen et al. 2018; Berradi et al. 2019). The chromophore is an unsaturated atomic group, in which the arrangement of single and double bonds allows the absorption of light; that is, the chromophore group is responsible for adding colour to the substrate; examples are: sulfide (-C=S), azo (-N=N-), carbonyl (-C=O), and nitro ($-NO_2$) (Rawat et al. 2016; Temesgen et al. 2018; Berradi et al. 2019). On the other hand, the auxochromic group contains many functional groups, such as sulfonic ($-SO_3H$), amine ($-NH_2$), hydroxyl (-OH), and carboxylic (-COOH), which are substituents bound to the chromophore. They improve the colour of the material (fibre) and affect the solubility in water by either donating (auxochrome) or

receiving (antiauxochrome) electrons (Holkar et al. 2016; Rehman et al. 2020). Additionally, the third component of the dye corresponds to the matrix, composed of benzene, anthracene, perylene rings, among others (Temesgen et al. 2018; Berradi et al. 2019; Benkhaya et al. 2020).

2.3 GLOBAL DYE MARKET

Dyes have been used since pre-history, such as colour surfaces, objects, and fabrics (Cardon 2007). Initially, pigment production was limited to natural sources as plants, minerals, and insects (Ferreira et al. 2004; Zerin et al. 2020). Later, the first dye synthesis breakthrough was made in 1743 by Barth (indigo carmine) (de Keijzer et al. 2012). However, in 1856, William Perkin revolutionized the dye industry by accidentally finding a Mauveine dye chemical route and synthesizing (aniline purple, CI 50245) (Abel 2012; Hagan and Poulin 2021). Perkin's discovery was crucial to accelerating synthetic production of dyes at a global scale (Tamburini et al. 2021). Synthetic dyes were introduced into the market and applied to other industrial areas, such as paper, food, pharmaceutical, and others (Tkaczyk et al. 2020).

Since the invention of the first synthetic dye, it is estimated that more than 10,000 synthetic organic dyes became commercially available, with an annual production surplus of 700 thousand metric tons (Rawat et al. 2016; Katheresan et al. 2018; Sharma et al. 2021). In the early 20th century, Europe was responsible for the global production of dyes. Asian countries, particularly China and India, are the largest dye producers worldwide (Tkaczyk et al. 2020).

2.4 CLASSIFICATION OF TEXTILE DYES

Since there are many synthetic dyes and formulations, a systematic classification is essential to enhance the textile industry.

2.4.1 Chromophore structure and colour index (CI)

Several textile dyes can be classified according to their source, solubility, chemical structure, and application, among others (Tamburini et al. 2021). The chemical structure-based classification should be correlated to chromophore groups as azo dyes, anthraquinone, nitro, xanthenes, and arylmethane (Ali 2010; Benkhaya et al. 2020). Board 1 shows some widely used synthetic organic dyes.

In this sense, the solubility-based classification is often used since it is low cost and, highly related to application, since different textile substrates are evaluated (Berradi et al. 2019; Javaid et al. 2021; Varjani et al. 2021), for instance, water-soluble dyes are acidic, basic, reactive and direct (Hassan and Carr 2018; Varjani et al. 2021), whereas insoluble dyes can be classified as vat, dispersed, sulfur and azoic (Burkinshaw and Lagonika 2006; Hassan and Carr 2018; Katheresan et al. 2018; Sharma et al. 2021).

Alternatively, textile dyes can be classified according to the Colour Index (C.I.) (Wich 1977), in which each dye is assigned a Generic Colour Index Name based on the application class, colour, and identification code (Gupta 2009). The C.I. Number is five digits code, as shown in Board 1 (Gupta 2009; Benkhaya et al. 2020).

2.4.2 Azo-based textile dyes

Azo compounds contain at least one nitrogen-nitrogen double bond (-N=N-). The nitrogen is bound to aromatic ring groups, such as benzene, naphthalene, and heterocyclic rings (Chung 2016; Besegatto et al. 2021b; Didier de Vasconcelos et al. 2021). However, many chemical structural configurations are possible. As the number of azo bonds increases in the same molecular structure, they can be named Diazo, Triazo and Polyazo (Benkhaya et al. 2020a). Azo dyes are brightly coloured compounds, highly chemically stable, including sunlight exposure, biodegradation and, wash fading (Chung 2016, Vikrant et al. 2018). These dyes can be synthesized from the diazotization of aromatic amines and coupling reaction with electron-rich nucleophiles (Shankarling et al. 2017).

2.5 GLOBAL AZO DYE MARKET

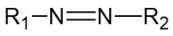
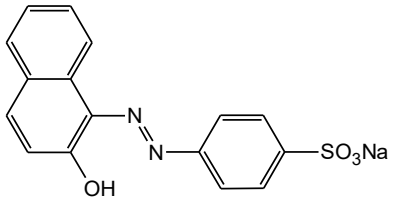
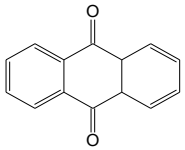
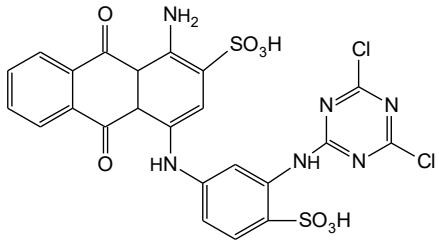
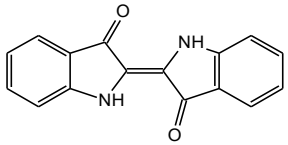
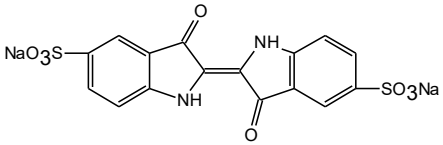
Azo dyes correspond to 70% out of the total synthetic dye market (Ali 2010; Balapure et al. 2015), in which the textile industry uses 80%, that is, 56% out of total (Oros et al. 2004; Singh et al. 2015; Sarkar et al. 2017; Tkaczyk et al. 2020; Varjani et al. 2021).

2.6 EFFECTS OF AZO DYES ON ENVIRONMENTAL

The dyeing processes are inherently related to dye (concentration, type, etc.), the textile material (shape, type, etc.), and the colour desired (Barker 2009; Chakraborty 2014). Most dyes are soluble organic molecules that adhere to fibres, yarns, or fabrics to impart colour; thus, dyes have to be highly stable to many factors such as surface-active agents (soaps and detergents), light exposure, among others (Chakraborty 2014; Mahapatra 2016) (Mahapatra 2016, Chakraborty 2014). In general, the dyeing procedure can be carried out in exhaust dyeing (batch), continuous or semi-continuous processes, involving a sequential mechanism that includes migration, adsorption, and diffusion (Clark 2011; Chequer et al. 2013b; Chakraborty 2014). Initially, the dye is transported from the dyebath onto the surface of the fibre, followed by the dye molecule's adsorption at the surface of the fibre. Finally, the dyes are diffused from the surface to the interior of the fibre by fixation (Chakraborty 2014).

The textile industry consumes substantial amounts of water, especially by dyeing and printing processes, which, in turn, are responsible for approximately 25% out of total water consumption (Arslan et al. 2016). Furthermore, water is also required for washing dyed textile material, generating high volumes of coloured effluents (Bisschops and Spanjers 2003; Patel and Vashi 2015; Arslan et al. 2016; Yaseen and Scholz 2019). It is worth noting that dyes and many other chemicals such as enzymes, minerals, and organic acids, alkalis, surfactants, salts, and oxidizing agents are required in dyeing processes (Patel and Vashi 2015). Hence, textile wastewaters are highly complex, recalcitrant, and unfeasible to treat by conventional methods (Arslan et al. 2016; Hassan and Carr 2018).

Board 1: Synthetic organic dyes.

Chromophore		Chemical structure	C.I. Name C.I. Number	Name	References
Azo			C.I. Acid Orange 7 C.I. 15510	Acid Orange II	(Muthirulan et al. 2014)
Anthraquinone			C.I. Reactive Blue 4 C.I. 61205	Procion Blue MX-R	(Nakamura et al. 2019)
Indigo			C.I. Acid Blue 74 C.I. 73015	Indigo carmine	(Aleboyeh et al. 2003)

The textile industries can be responsible for massive environmental impacts (Board 2) since their effluents are composed of enzymes, mineral and organic acids, alkalis, surfactants, salts, oxidizing agents, and dyes at high concentrations (Yaseen and Scholz 2019, Patel and Vashi 2015). The wastewater generates in the dyeing mill comprises the most significant amount of the total wastewater of the textile industry. This wastewater comes from the dye preparation, spent dye bath, and washing processes (Patel and Vashi 2015). It is estimated that over 7 x 10^5 tons of synthetic dyes are produced annually worldwide, and during the colouration process of the textile industry, up to 3.10^5 tons of this amount are lost to wastewater (Chequer et al. 2013b; Neifar et al. 2019). According to Samsami et al. (2020), the textile industry is responsible for 54% of the total discharge of dyes into the environment (Samsami et al. 2020).

The dye wastewaters are intensely coloured, possess high biological oxygen demand (BOD), high dissolved solids (DS), low suspended solids (SS), high salt content, alkalinity, and low concentration of heavy metals (Patel and Vashi 2015).

Many dyes are easily visible (naked eye) even at low concentrations. Thus, when incorrect disposal into the water bodies, they will become visually unpleasant and affect aquatic life (Yagub et al. 2014; Arslan et al. 2016; Neifar et al. 2019).

Textile dyes are recalcitrant compounds. Thus, they can reach the aqueous ecosystem as pollutants. In addition, dyes can harm water quality in terms of total organic carbon (TOC), chemical oxygen demand (COD), and BOD (Dafale et al. 2010; Vikrant et al. 2018). Furthermore, some dyes, as azo-type textile dyes, can be bioaccumulated in the food chain (biomagnification) (Dafale et al. 2010; Lellis et al. 2019). Many synthetic textile dyes and intermediate metabolic products can have direct and indirect toxic effects, mainly because of mutagenic and carcinogenic agents. (Kadirvelu et al. 2003; Yagub et al. 2014). Thus, azo dyes can be correlated to animal and human diseases such as allergies, tumors, cancers, dysfunction of the liver, reproductive system, kidney, brain, and central nervous system, and suppression of the immune system (Kadirvelu et al. 2000; Kadirvelu et al. 2003; Neifar et al. 2019; Aruna et al. 2021). In this sense, it is worth noting that the toxicity of azo dyes is mainly related to aromatic amines that can be biodegradation products (Neifar et al. 2019).

Despite some azo dyes (for example, Acid Orange 7 and Reactive Red 195) have been reported as non-toxic (Chung 2016; Rawat et al. 2016; Rawat et al. 2018), most of them are environmentally hazardous substances (Sharma et al. 2021). It is worth noting that these azo dyes can reach the drinking water supply systems (Rawat et al. 2016). As already mentioned, the textile industry is the major azo dye polluter (Kalyani et al. 2009; Besegatto et al. 2021a). In this sense, it is estimated that between 15 to 50% of azo dyes eluates (do not adsorb) from textile fibres (Kakarndee and Nanan 2018; Lellis et al. 2019).

The incorrect disposal of azo dye-containing azo is visually unpleasant (Lellis et al. 2019), decreases penetration of sunlight, and, consequently, affects the aquatic biota, leading to lower oxygen levels (Yaseen and Scholz 2016; Albahnasawi et al. 2020; Selvaraj et al. 2021).

The synthetic origin and molecular arrangement of azo dyes lead to high chemical stability, including sunlight exposure and biodegradation (Pandey et al. 2007; Garcia-segura et al. 2011; Duarte Baumer et al. 2018). Therefore, in recent years, the contamination of these substances and their degradation products have been intensively investigated (Sun et al. 2017b; Brüschweiler and Merlot 2017; Berradi et al. 2019; Rovira and Domingo 2019; Varjani et al. 2021; Didier de Vasconcelos et al. 2021).

The accumulation of azo dyes in plants (Yaseen and Scholz 2016), animals (Mansour et al. 2010), water (Rajaguru et al. 2002; Vacchi et al. 2017), and soil (Solís et al. 2012) can be absorbed (oral or inhalation) by human beings and, consequently, impact their health as blood disorders, colic, allergies, and skin irritation (Sen et al. 2016; Lellis et al. 2019). It is also known that prolonged exposure to these substances can induce carcinogenic and mutagenic effects in animal cells (Mansour et al. 2010; Parrott et al. 2016), including humans (Rafii et al. 1997; Balakrishnan et al. 2016; Rawat et al. 2016; Sen et al. 2016). Furthermore, some azo dyes reveal mutagenic potential at the chromosomal level (Rafii et al. 1997; Chequer et al. 2009), inducing DNA and RNA damage.

Board 2: Azo dyes and their adverse environmental and health impacts.

C.I. Name of dye	Effects	References
Acid Orange 7	Molecular, cellular, and organism level toxicity; chromosomal abnormalities and reduced mitotic index in *Allium cepa* bioassay.	(Rawat et al. 2018)
Direct Blue 15	Mutation in microalgae, cladocerans, and zebrafish embryos	(Martínez-jerónimo 2019)
Disperse Orange 1	Mutation in human lymphocyte and human hepatoma cells;	(Chequer et al. 2009)
Disperse Blue 291	Mutation in mouse bone marrow cells; DNA fragmentation, genotoxic and mutagenic in a human hepatoma cell line	(Tsuboy et al. 2007; Fernandes et al. 2019)
Disperse Red 1	Chromosome aberrations and primary DNA damage in liver	(Fernandes et al. 2018)

Therefore, the commercial relevance of azo dyes is undeniable. However, their toxicity and environmental impact should be carefully evaluated.

2.7 TEXTILE WASTEWATER CONVENTIONAL TREATMENTS

During dyeing and printing processes, the fixation of reactive dyes on fabrics forms a covalent bond between the dye molecule containing the reactive electrolytic group and proteic fibre (Queiroz et al. 2019). However, the linkage is insufficient - emphasizing the azo-type textile dyes, which, around 15-50%, do not bind to the fabric - and are released into wastewater (Rehman et al. 2018). About 1000-3000 m^3 of sewage is generated per day to produce 12-20 tons/day of a fabric product (Rehman et al. 2018; Ilyas et al. 2019). Table 1 illustrates the consumption of water, chemicals, and energy for 1 kg coloured fabric. In this sense, the textile industry is recognized among all industrial sectors as the most polluters due to the volume of water discharged and the environmental load generated (e.g., high consumption of energy and chemicals, CO_2 emissions, and effluent production with a high rate of impurities) (Alkaya and Demirer 2014). The effluent composition of various stages of a textile manufacturing industry is shown in Board 3. The high concentrations of BOD, COD, colour, pH, and metals hamper the treatment of these wastewaters.

Table 1: Global inventory for the dyeing process (functional unit: 1 kg of coloured fabric) (Parisi et al. 2015).

System unit	Input	Conventional process
Pre-treatment	Water	20 L
	Solvent	120 kg
	Energy	0.13 kWh
Dyeing	Water	20 L
	Solvent	20.98 g
	Dyestuff	10 g
	Auxiliary	25 g
	Energy	3.82 kWh
Washing	Water	10 L
	Washing agents	5 g
	Energy	0.16 kWh
Drying	Energy	0.04 kWh
	Gas	0.27 kWh

Different physical-chemical and biological treatments, such as separation and concentration, decomposition, degradation, and exchange, are used in textile manufacturing. Therefore, it is necessary to combine different treatment processes to enable textile wastewater disposal. In summary, three main treatment processes guide textile wastewater remediation: primary, secondary, and tertiary (Figure 1). According to Islam and Mostafa (2019), suspended solid waste, excessive oil, and granular materials present in the effluent are removed by primary treatment. In the secondary treatment process under aerobic or anaerobic conditions, there is a reduction in BOD, phenol, oil contents, and colour control in the effluents. Electrodialysis, reverse osmosis, and ion exchange - briefly described below – are examples of tertiary treatments.

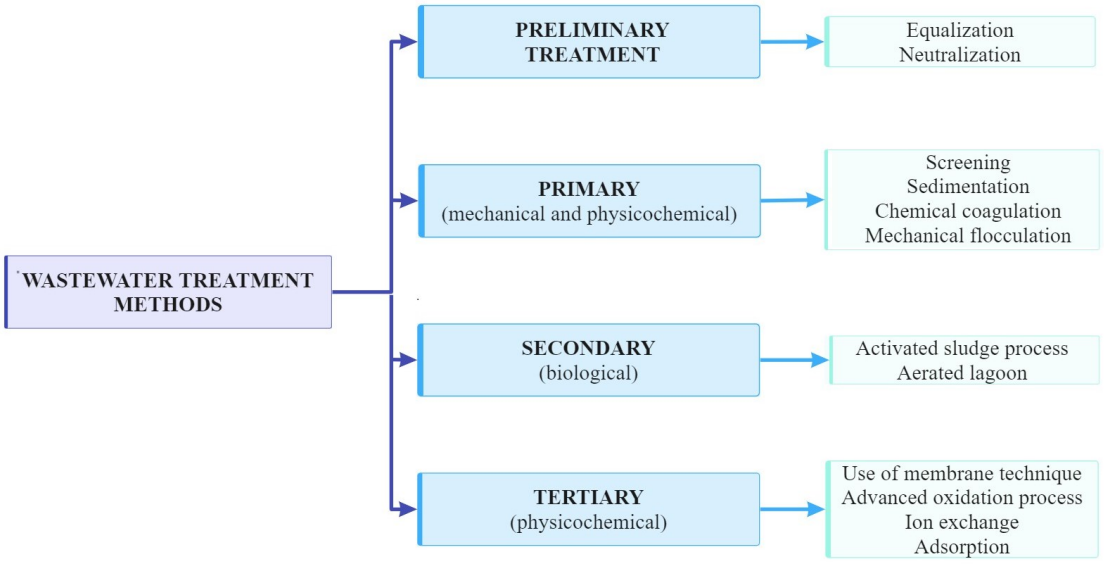


Figure 1: Classification of wastewater treatments.

2.7.1 Primary treatment

Before primary treatment, the textile manufacturing industry effluent processing, pre-treatment, or preliminary treatment must be applied to equalize the effluent, to homogenize it in terms of pollution load, pH, and temperature (Kumar et al. 2012). Then, primary treatments based on screening, sedimentation, and flotation processes are used to remove sedimentable organic and inorganic solids.

After primary treatment, fine suspended particles and colloidal particles are not removed efficiently; therefore, mechanical flocculation and chemical coagulation processes are effectively employed. These processes have the function of destabilizing colloidal particles by adding coagulating agents (e.g., lime [$Ca(OH)_2$], alum, ferrous sulphate ($FeSO_4$), ferric chloride ($FeCl_3$), among others), which have a high charge/mass ratio with colloidal particles significantly increasing their size. This factor contributes to sedimentation, and the effluent can then be processed through a flocculation and settling tank (Kumar et al. 2012). Treating textile wastewater with coagulating agents helps to reduce colour (insoluble dyes, 70-90%), suspended solids (50-75%), BOD (25-50%), COD (50-60%), and oils and grease (65%). Although, the addition of a coagulating agent to the wastewater stream increases the cost of this process and the sludge formed requires further treatment (Kumar et al. 2008).

Board 3: Major textile polluters at stages of manufacturing (Verma et al. 2012).

Process	Constituents	Wastewater characteristics/Typical concentrations
Sizing	Yarn waste and unused starch-based sizes	High BOD and medium COD.
Desizing	Enzymes, starch, waxes, and ammonia	BOD (34-50% of total), high COD, and temperature 70-80 ºC.
Scouring	Disinfectants and insecticides residues, NaOH, surfactants, and soaps	Oily fats, BOD (30% of total), high pH, temperature 70-80 ºC, and dark colour.
Bleaching	H_2O_2, Adsorbable Organic Halogen, NaOCl, and organics	High pH and TDS.
Mercerisation	NaOH	Low BOD (less than 1% of total), TDS, and oil and grease.
Dyeing	Colour, metals, salts, acidity/alkalinity, and formaldehyde	High toxicity, BOD (6% of total), high dissolved solids, and high pH
Printing	Urea, solvents, colour, and metals	High toxicity, high COD, high BOD, high dissolved solids, high pH, and intense colour
Finishing	Chlorinated compounds, resins, spent solvents, softeners, waxes, and acetate	Low alkalinity, low BOD, and high toxicity

2.7.2 Secondary treatment

In secondary treatment, biodegradation has become a promising method for treating textile effluents compared to physicochemical processes. Besides the lower cost of the process, it is unnecessary to use chemical products, and the sludge produced - a result of cell proliferation - has a low content of chemical compounds (Rai et al. 2005). In this process, dissolved organic compounds, remaining colloidal particles, and the colour present in wastewater are removed and/or reused to stabilize organic matter due to the presence of microorganisms (Joshi et al. 2004). The activated sludge system stands out as one of the most commonly used treatment methods and is divided into two categories: aerobic and anaerobic treatment processes.

The aerobic process removes the biodegradable components from the effluent (e.g., carbohydrates and readily degradable compounds). In other words, in this process occurs the: (I) coagulation and flocculation of colloidal matter, (II) oxidation of organic matter dissolved in carbon dioxide, and (III) degradation of nitrogenous organic matter into ammonia, which is then converted into nitrite and eventually into nitrate. However, after this process, more complex xenobiotic compounds, such as dyes and surfactants, remain in the effluent (Kumar et al. 2012; Queiroz et al. 2019)

In anaerobic treatment, factors such as pH, temperature, waste load, absence of oxygen, and toxic material directly influence the efficiency of the process, which is mainly used to digest activated sludge (Kumar et al. 2012).

Most dyes are generally recalcitrant to aerobic degradation but can undergo reductive discolouration under anaerobic conditions (Rai et al., 2005). In a treatment plant, the two treatment systems are usually used together. The anaerobic system is followed by an aerobic system, producing better results, for example, the colour reduction is significantly more significant than using the isolated aerobic process (88% vs. 28%), as well as the reduction of Total Organic Carbon (79-90%) (Joshi et al. 2004; Kumar et al. 2012; Yaseen and Scholz 2019). It is essential to mention that textile effluent contains significant amounts of non-biodegradable chemicals (e.g., amino benzene sulfonic or naphthyl amine sulfonic acids and heavy metals) (Kumar et al. 2012; Santoro et al. 2014; Queiroz et al. 2019). Since these conventional treatment systems are not very effective in removing pollutants, an efficient tertiary treatment process is needed.

2.7.3 Tertiary treatment

Environmental quality standards for the release of textile effluents into surface water bodies demand tertiary treatment. In addition to conventional treatments, textile effluents have tertiary processes to remove specific contaminants (e.g., various types of dyes such Congo Red, Methyl Orange, Methylene blue, C.I. Direct red 80, C.I. Reactive Red 17, C.I. Direct Yellow 50, among others), as well as complete removal of solids and organic matter, reduce colour or degrade recalcitrant compounds, reduce nutrients (e.g., ammonia and phosphorus) and assist in effluent disinfection (Kumar et al. 2012; Queiroz et al. 2019).

The most common tertiary treatment solutions are removing residual colour organic compounds by adsorption and removal of dissolved solids by membrane filtration. Wastewater is also treated with ozone or another oxidizing agent to destroy many contaminants and minimize textile effluent disposal problems (Kumar et al. 2012).

2.8 AEROBIC AND ANAEROBIC MICROBIAL DEGRADATION OF DYES: BACTERIAL, FUNGAL, AND MICROALGAE

Biological treatments have several advantages, such as implementation and operation, low costs, and high yields (relatively). It can be carried out under aerobic and/or anaerobic conditions, reaching the complete mineralization a (Bhatia et al. 2017; Crini and Lichtfouse 2019; Varjani et al. 2020).

Aerobic and anaerobic bacteria, fungi, yeasts, and algae can be used for dye degradation. These microorganisms play essential roles in environmental maintenance since they are responsible for metabolizing organic, inorganic, natural, or xenobiotic compounds (Gao et al. 2018). The degradation of dyes can be carried out by pure cultures or microbial consortiums (Ebrahimi et al. 2019). Mandal, Dasgupta, and Datta (2010) reported that microbial consortiums present higher degradation yields when compared to pure cultures due to synergistic metabolic actions and higher stability (temperature and pH).

2.8.1 Aerobic microbial degradation of dyes

The required oxygen for aerobic microbial degradation can be supplied by atmospheric air (dissolution), and/or pure oxygen, and/or biochemical sources. Conventional aerobic treatment systems are compacted bed reactors, biological filters, aerobic stabilization ponds, and activated sludge (Samuchiwal et al. 2021; Behera et al. 2021).

The activated sludge system is the most widely used aerobic treatment for effluents in the textile industry. It shows shorter hydraulic holding time and higher operational flexibility when compared to anaerobic treatments. However, it generates high biomass volumes (sludge) containing (adsorption) residual compounds that are resistant to biodegradation (Manai et al. 2016).

The microorganisms commonly used for the decolourization of dyes in aerobic conditions are fungi and bacteria. Bacteria can grow faster than fungi; however, the more efficient microorganisms at degrading synthetic dyes are white-rot fungi. White-rot fungi include *Polyporus sanguineus*, *Daedalea flavida*, *Dichomitus squalens*, and *Irpex flavus* (Balamurugan et al. 2011; Naresh et al. 2013; Behera et al. 2021).

Kodam et al. (2005) realized a study showing the use of a bacterium isolated from the textile effluent to degrade azo sulfonated dyes. In this study, the researchers isolated and purified a pure culture called KMK 48 from the sludge collected in a textile dyeing industry located in India. The pure culture KMK 48 was efficiently degraded Reactive Red 2, Reactive Red 141, Reactive Orange 4, Reactive Orange7, and Reactive Violet 5.

Balamurugan, Thirumarimurugan, and Kannadasan (2011) evaluated the colour degradation of dye-containing textile effluent and the reduction of COD through anaerobic digestion using *Halomonas variabilis* and *Halomonas glacier*. Experiments were carried out at 30 ºC in a CO_2 incubator. The maximum degradation was reached after 144h. Nevertheless, the aerobic treatment did not achieve a high yield (Behera et al. 2021).

Subramanian, Ramesh, and Kalaiselvam (2014) studied the degradation of triphenylmethane dye malachite green by litter decomposing fungi. In mangrove regions, the authors isolated and identified fungi (*A. flavus*,

A. niger, *A. terreus*, *F. oxysporum*, *Penicillium* sp.). Among the isolated microorganisms, *Aspergillus flavus* showed greater decolourization capacity (83%) after 9 days.

The aerobic fungal and bacterial degradation of textile effluents has several advantages: low cost and high yields (mineralization). Nevertheless, white-rot fungi present disadvantages of other fungi, such as prolonged growth phase and retention times (up to 14 days) (Balamurugan et al. 2011; Behera et al. 2021).

2.8.2 Anaerobic microbial degradation of dyes

The main advantage of anaerobic biological processes is the wide variety of microorganisms in the anaerobic consortium. In addition, the anaerobic processes generate biogas (energy source) (Şen and Demirer 2003; Behera et al. 2021).

Dye degradation under anaerobic conditions is a combination of biological and chemical mechanisms. Anaerobic biological treatment involves an oxidation-reduction reaction with hydrogen, allowing the azo dye and other water-soluble dyes to be discoloured. The decolourization of azo dyes under anaerobic conditions occurs with a redox potential < 50 mV. The dye acts as an electron receptor. The double bond (-N=N-) is broken and promotes the formation of an intermediate hydrazo, which has the bond (-N-NH-), which undergoes a reductive cleavage and produces amines. The chemical contribution to reducing and reducing azo dyes is attributed to biogenic reducers such as sulfide, cysteine, ascorbate, and Fe^{2+} (Popli and Patel 2015; Bhatia et al. 2017; Gao et al. 2018).

$$R_1 - N = N - R_2 + 2e^- \rightarrow R_1 - HN - NH - R_2 \text{ (Intermediate hydrazo)}$$

$$R_1 - HN - NH - R_2 + 2e^- \rightarrow R_1 - NH_2 + R_2 - NH_2 \text{ (Aromatic amines)}$$

Conventional anaerobic treatment systems are anaerobic lagoons, septic tanks, anaerobic filters, and high-rate reactors, capable of receiving more significant amounts of organic load per volumetric units, such as UASB reactors (upflow anaerobic sludge blanket). The anaerobic filter and UASB show high colour removal efficiency (Xu et al. 2018).

Anaerobic degradation of azo dye Acid Orange 7 was performed using a continuous upflow packed bed reactor containing biological activated carbon. The high conversion rates of the azo dye (up to 99%) were obtained using a continuous upflow packed bed reactor containing biological activated carbon. The use of a continuous upflow packed bed reactor containing biological activated carbon has proved to be an effective and promising treatment system for the degradation of anaerobic azo dyes (Mezohegyi et al., 2007).

A new method to increase anthraquinone dye reactive blue 19 degradation using the anaerobic system has been proposed by Cai et al. (2021) using resuscitation-promoting factors (RPF), which has been proven to revitalize and stimulate bacterial growth. Resuscitation-promoting factors were efficient in the decolourization of anthraquinone dye reactive blue 19. Compared to the conventional methods, there was an increase in dye decolourization by an additional 20% with the addition of the RPF process. The addition of RPF also resulted in a greater decolourization efficiency of anthraquinone dye reactive blue 19 using microorganisms of the *Peptostreptococcaceae* family.

Nguyen et al. (2021) evaluated the potential of a submerged anaerobic deflector reactor with sponges (SS-ABR) to improve the processing performance of azo dye-containing wastewaters. The dye-degrading microorganisms present in the SS-ABR were *Clostridium* sp. and sulfate-reducing bacteria *Desulfomonile* sp. and *Desulfovibrio* sp. The efficiency of colour and COD removal were 65±3% and 83±2%. In addition, SS-ABR showed excellent stability.

2.8.3 Sequential Aerobic-anaerobic treatment

The combination of anaerobic-aerobic treatments using different microorganisms has shown promising results for the degradation of dyes present in textile effluents. Wastewater treatment indicates that aeration improves COD and is an essential complement to anaerobic colour removal (Shindhal et al. 2021; Ceretta et al. 2021).

You and Teng (2009) used a sequential batch anaerobic reactor combined with an anaerobic membrane bioreactor to treat azo dye (Reactive Black 5). In addition, sixty anaerobic bacteria degrading Reactive Black 5 dye were isolated from the sludge taken from the sequential batch anaerobic reactor. The COD removal was 92.3% and 5.2%, whereas the proper colour removal was 74.6%, and 9.1% used a sequential batch anaerobic reactor combined with an anaerobic membrane bioreactor. Five subspecies of *Lactococcus lactis* were sequenced and showed the ability to degrade 99% of Reactive Black 5 dye after 5.5 h. Twenty subspecies of *Lactobacillus casei* showed the ability to degrade more than 99% of Reactive Black 5 dye after 36h.

Naimabadi, Movahedian Attar, and Dhahsavani (2009) studied the decolourization and biological degradation of reactive Red 2 using an anaerobic-aerobic sequential process. An anaerobic deflector reactor on a laboratory scale and a fixed activated sludge reactor were used. The reactors were operated with different organic loads and hydraulic retention times. The experiments were carried out in continuous mode, and the effluent from the anaerobic deflector reactor was used as feed for the fixed activated sludge reactor. The removal efficiency of the COD was 54.5% using 1day-hydraulic retention in the anaerobic reactor. Regarding colour, the removal was 89.5%. The results demonstrated that the anaerobic/aerobic sequential system efficiently degraded the reactive azo dye Red 2.

2.9 PHYCOREMEDIATION

An exciting approach for decolourizing and removing dyes in textile effluents is the phycoremediation (algae) (Bhardwaj and Bharadvaja 2021).

Although some azo dyes are highly toxic to fish, they do not significantly inhibit algal growth. In this sense, *Chlorella vulgaris*, *Oscillatoria*, and *Chlorella pyrenoidosa* degrade azo dyes (Acuner and Dilek 2004). It is worth noting that algal biomass can also be used to produce methane and/or biodiesel.

The main advantage of phycoremediation is the algae metabolic versatility: (1) Photoautotrophic: the energy (ATP) is produced from light sources and CO_2 (photosynthesis); (2) Heterotrophic: the energy is produced from the oxidation of organic molecules; (3) Myxotrophic: photosynthesis and oxidation of organic molecules occur concurrently; and (4) Photoheterotrophic: the energy is produced from light, and

oxidation of organic molecules (Krishnamoorthy and Manickam 2021; Sarkar and Dey 2021; Bhardwaj and Bharadvaja 2021).

A *Cosmarium* species (green alga) was studied as a viable biomaterial for the biological treatment of triphenylmethane dye, Malachite Green (MG). The effects of temperature, pH, dye concentration, and algae concentration on dye decolourization were studied. The highest discolouration rates were at temperatures above 45 ºC. The ideal pH was 9. *Cosmarium* showed high efficiency of decolourization, reuse, and stability (Daneshvar et al. 2007).

El-sheekh, Gharieb, and Abou-el-souod (2009) evaluated the decolourization and removal of ethyl red, orange II, G-Red (FN-3G), basic cationic, and basic fuchsin using *Chlorella vulgaris*, *Lyngbya lagerlerimi*, *Nostoc lincki*, *Oscillatoria rubescens*, *Elkatothrix viridis*, and *Volvox aureus*. The removal efficiency ranged from ~ 4 to 95%. The basic cationic fuchsin and basic fuchsin dyes showed the greatest capacity for decolourization and removal by all tested algae. *Elkatothrix viridis* removed cationic basic fuchsin (91.6%) and basic fuchsin (90.7%) dyes after three days of incubation. The Basic Fuchsin dye removal rate was up to 95% using *Oscillatoria rubescens*. However, *Volvox aureus* removed only 5.02 and 3.25% of the orange II and G-Red dyes.

2.10 ENZYMATIC DEGRADATION OF DYES

Enzymes are well-known as effective biocatalysts that promote specific bioconversion of substances under mild conditions (Nunes Costa et al. 2020). Thus, considering the recalcitrant properties of dyes from the textile industry, the enzymatic process is a promising alternative in reducing pollutants from industrial wastewaters.

The enzymatic degradation of dyes can be carried out by using crude and purified. Purified enzymes present high activity per dosage. However, the presence of redox mediators at low concentrations may significantly impact the feasibility of crude and purified approaches (Nguyen et al. 2016). The purification costs are also a relevant drawback when applying enzymes to the degradation of dyes. In this sense, enzyme immobilization is an attractive alternative since it eases recovery, protects the biocatalyst by enhancing its resistance, and maintains the enzyme's ability as a catalyst (Lima et al. 2017).

A wide range of enzymes has been used in general wastewater treatment. However, due to the recalcitrant characteristic of dyes, the oxidoreductase class is the most cited in the literature for the textile industry field. This class includes enzymes that require an oxidizing agent (e.g., hydrogen peroxide, chlorine, and potassium permanganate) to promote the catalysis of the reaction. Azoreductases, laccases, and peroxidases are examples of oxidoreductases commonly applied to dye degradation. In contrast, the first one is used specifically on the degradation of azo dyes, the last two present activity on several classes of dyes. In this way, this section brings a brief description related to these enzymes, their role in dye decomposition, and some relevant studies.

2.10.1 Laccases

Laccases are multicopper oxidase proteins (Singh et al. 2015). Laccases are oxidoreductases that play an essential role in biotechnological applications in the bioremediation field due to their non-specific oxidation

capability, no co-factors demand, and ability to use molecular oxygen as electron acceptor (Kalyani et al. 2012). Laccases have attracted relevant attention in the degradation of dyes from textiles wastewater (Husain 2006). When in the presence of redox mediators, laccases present higher activity (Ravikumar et al. 2013). Fungi or plants biosynthesize these enzymes; nevertheless, some bacteria are laccase producers (Claus 2003).

Laccases are related to removing hydrogen atoms from hydroxyl groups of ortho and para mono and polyphenolic substances and aromatic amines resulting in depolymerization, demethylation, or quinine synthesis (Gonçalves and Steiner 1996). Since laccases catalysis uses molecular oxygen as an electron acceptor, laccases do not show high specificity (when compared to other enzymes). Thus, laccases oxidize phenolic an-phenolic compound also a wide range of substances, including azo dye-containing textile wastewaters (D'Souza et al. 2006; Sharma et al. 2007). Laccases degrade azo dyes by a nonspecific free radical mechanism that forms phenolic products and inhibit potentially risky aromatic amine formation (Wong and Yu 1999). The reaction oxidation of aromatic pollutants, such as anilines and phenols, by laccases, yields the formation of phenoxy radicals from its original compounds; these reactions additionally develop a phenolic polymer on polymerization or, by further laccase action, produce quinine (Bollag et al. 1992.

The benefits of enzyme immobilization are well known by researchers also in the dye degradation field. Studies involving enzyme encapsulation on several supports for wastewater decolourization report enhances enzyme activity, thermal and pH stability, reusability, and resistance to metals and organic solvents (Jaiswal et al. 2016; Sun et al. 2017a; Bilal et al. 2018). Several researchers have reported the activity of laccases from a variety of sources on the degradation of recalcitrant industrial dyes used in the textile manufacturing, as reported in Table 2. (Zhuo et al. 2019) suggest possible pathways for the degradation of Malachite Green (MG) and Remazol Brilliant Blue R (RBBR) based on the intermediates generated. Regarding laccase-MG, there are two possible pathways: (I) laccase mediates successive demethylation, which results in the decrease of m/z value; and (II) MG is hydroxylated, then occurs the ring removal and the demethylation of the dye. Based on the intermediates of RBBR degradation, the proposed laccase-mediated pathway breaks the molecule into two sub-products followed by deamination, hydroxylation, and oxidation, finally opening the ring.

Sun et al. (2015) reported the immobilization of laccase in chitosan grafted polyacrylamide hydrogel and its application on the degradation of Acid Orange 7 (AO7, an azo dye) and Malachite Green (MG). First, the cationic hydrogel used as enzyme support promoted extra adsorption of the anionic AO7, which increased the substrate concentration for the encapsulated laccase and enhanced activity. For the second, the cationic MG did not interact with the surface of the hydrogel, and the degradation occurred exclusively due to enzyme activity. Jaiswal, Pandey, and Dwivedi (2016) developed an immobilized laccase in chitosan beads and tested its activity on indigo carmine. The results showed complete decolourization of 50 $\mu m.mL^{-1}$ solution within 8h, and the supported enzyme presented 40% of its initial activity after 3 cycles. The decrease in activity may be attributed to blocking some pores on the surface of chitosan by substrate oxidation products (Daâssi et al. 2014).

Table 2: Lacasse-based decolourization of dyes.

Form/Source	Dye	Elapsed Time (h)	Decolourization (%)	References
Encapsulated laccase/*Trametes versicolor*	Malachite Green	6.5	>90	(Sun et al. 2015)
	Acid Orange 7		>80	
Immobilized Laccase/Papaya	Indigo Carmine	8	100	(Jaiswal et al. 2016)
Laccase/recombinant *Yarrowia lipolytica*	Bromocresol Purple	1	43	(Darvishi et al. 2018)
	Safranin		54	
	Malachite Green		55	
	Kristal Violet		49	
	Bromothymol Blue		56	
	Nigrosine		53	
	Phenol Red		37	
Laccase/*Arthrospira maxima*	Reactive Blue 4	96	89	(Afreen et al. 2018)
	Remazol Brilliant blue R		49	
Immobilized Laccase/*Trichoderma harzianum*	Malachite Green	100	16	(Bagewadi et al. 2017)
	Methylene Blue	90	18	
	Congo Red	60	20	
	Reactive Brilliant Blue X-BR		60	
	Remazol Brilliant Blue R		61	
Immobilized Laccase/*Trametes pubescens*	Acid black 172	96	77	(Ma et al. 2018)
	Congo Red		69	
	Methylene Blue		37	
	Neutral Red		48	
	Indigo Blue		56	
	Naphthol Green B		65	
	Crystal Violet		40	
	Reactive Brilliant Blue X-BR		52	
	Remazol Brilliant Blue R		48	
Immobilized Laccase/*Trametes pubescens*	Congo Red	48	54	(Zheng et al. 2016)
	Acid Black 172		68	
	Methylene Blue		25	
	Neutral Red		44	
	Indigo Blue		45	
	Naphthol Green B		37	
	Direct fast Blue FBL 74190		56	
	Crystal Violet		20	
Immobilized Laccase/genetically modified *Aspergillus*	Direct Red 23	1	88	(Kashe et al. 2019)
	Acid Blue 92		48	

Table 2: (continued)

Laccase/*Streptomyces ipomoeae*	Acid Black 48	24	22	(Blánquez et al. 2019)
	Acid Orane 63		14	
	Reactive Black 5		94	
	Orange II		89	
	Tartrazine		21	
	Azure B		9	
	Indigo Carmine		98	
	Cresol Red		12	
	Malachite Green		91	
Laccase/*Pleurotus ostreatus*	Methyl Orange	24	73	(Zhuo et al. 2019)
	Bromophenol Blue		79	
	Remazol Brilliant blue R		85	

2.10.2 Peroxidases

Peroxidases are widely applied for decolourize textile effluents since they act on phenolic compounds (Ulson de Souza et al. 2007). Peroxidases are hemoproteins that use oxygen peroxide as a mediator to catalyze oxidative reactions of recalcitrant dye compounds yielding insoluble polymeric products (Chiong et al. 2016). Passardi et al. (2007) classified peroxidases into three classes according to their origins. Class I are intracellular peroxidases, including yeast cytochrome peroxidase, ascorbate peroxidase, and bacterial catalase peroxidases. Class II comprehends the secretory fungal peroxidases such as lignin and manganese peroxidases that are mainly related to lignin degradation. Class III are secretory plant peroxidases that present numerous functions such as degradation of H_2O_2 and additional toxic substances from chloroplasts and cytosol and auto-defense against wounding.

Huaiyan Sun et al. (2017) immobilized horseradish peroxidase in ZnO nanowires/macroporous SiO_2 composite and tested its activity on the decolourization of Acid Blue 113 and Acid Black 10 dyes. The solution concentration range of this study was between 30 and 50 $mg.L^{-1}$, and the colour removal was 95% for Acid Blue and 90% for Acid Black. Chiong et al. (2016) reported the application of soybean peroxidase and *Luffa acutangula* peroxidase to degrade azo dye methyl orange from liquid effluents. The first presented degradation of 81% within 1 h and the second a decolourization of 75% after 40 min. The results for crude peroxidases reveal their potential in enzymatic dye treatment.

Bilal et al. (2019) proposed a pathway for the degradation of Reactive Black 19 by horseradish peroxidase based on HPLC-MS analysis. The successive enzymatic action on the dye leads to the degradation of the chromophore group and the subsequent disappearance of the colour. Bilal et al. (2017) also reported a pathway for the degradation of azo dye Methyl Orange based on its UPLC-MS analysis after horseradish peroxidase activity. The decline in spectral shift followed by the absence of new peaks during enzymatic activity indicates the total degeneration of the dye by the cleavage of azo bonds. Ali et al. (2018) exposed a potential pathway for the degradation of Reactive Blue 4 by a ginger peroxidase. GC-MS was used to elucidate the oxidative reactions by the intermediates. The authors concluded that the dye was first

desulphonated, dechlorinated, and deaminated to generate less *m/z* value products; the following reactions lead to the cleavage of the chromophore group and consequent solution decolourization.

Evaluating the toxicity of compounds from enzymatic degradation of dyes is essential to verify if the products of the dye decomposition still present relevant risks for the environment. Some authors studied the toxicity of untreated and peroxidase treated textile industrial wastewater and reported exciting outcomes. Ali et al. (2018) report a reduction in genotoxicity after decolourizing Reactive Blue 4 by a single-strand break in DNA analysis. Maria et al. (2007) employed horseradish peroxidase on the degradation of Remazol Turquoise Blue G, and Lanaset Blue 2R concluded that the treated wastewater presented a more negligible toxicity effect on *Daphnia magna.* However, no change was detected towards *Artemia salina.*

Baumer et al. (2018) tested the viability of horseradish peroxidase on the degradation of Reactive Black 5 (azo), Reactive Blue 19 (anthraquinone), Reactive Red 239 (azo), and Reactive Blue 21 (phthalocyanine). They evaluated the toxicity of the treated wastewater by *Daphnia magna, Euglena gracilisalgae*, and *Vibrio fischeri*. The tests were conducted in batch mode, 125 mL of a dye concentration of 50 $mg.L^{-1}$ at 30 ^{o}C, and the decolourization attained at 30 min were 87, 96, 17, and 90 %, accordingly. The toxicity outcomes showed a significantly higher adverse effect on the organisms in the test after enzymatic treatment. The authors attribute these results to the formation of decolourization metabolites more toxic than the original dye molecule and suggest a next successive treatment step for wastewater cleaning. Table 3 presents several researchers who have reported the activity of peroxidases from various sources on the degradation of recalcitrant industrial dyes used in textile manufacturing.

Table 3: Peroxidase-based decolourization of dyes.

Form/Source	Dye	Elapsed Time (min)	Decolourization (%)	References
Immobilized Peroxidase/ Horseradish	Reactive Blue 21	30	90	(Duarte Baumer et al. 2018)
	Reactive Blue 19		96	
	Reactive Black		87	
	Reactive Red		17	
Immobilized Peroxidase/ Horseradish	Acid Blue	35	95	(Sun et al. 2017a)
	Acid Black		90	
Immobilized Peroxidase/ Horseradish	Methyl Orange	300	100	(Bilal et al. 2018)
Peroxidase/soybean	Methyl Orange	40	81	(Chiong et al. 2016)
Peroxidase/*Luffa acutangular*			75	
Immobilized Peroxidase/ Ginger	Reactive Blue 4	180	99	(Ali et al. 2018)
Lignin Peroxidase/*P. ostreatus* and *G. lucidum*	Remazol brilliant blue R	1440	100	(Oliveira et al. 2018)

Table 3: (continued)

Peroxidase/*Irpex Lacteus* F17	Reactive Blue 4	30	79	(Duan et al. 2018)
	Reactive Blue 5		74	
	Reactive Blue 19		78	
	Direct sky blue 5B		81	
	Reactive Black 5		60	
	Acid red 18		43	
	Reactive Violet 5		92	
	Methyl Orange		33	
	Direct yellow 8		3	
	Orange G		18	
	Orange yellow II		29	
	Orange yellow IV		63	
	Congo Red		0	
	Neutral Red		22	
	Malachite Green		85	
	Basic Fuchsin		45	

2.10.3 Azoreductase

Azoreductases are enzymes capable of catalyzing the cleavage reduction of azo groups (-N=N-). These enzymes are produced by bacteria that degrade the azo dyes. As an oxidoreductase, this enzyme can be classified according to its demand of cofactors in flavin-dependent and flavin-independent, and even further categorized as a function of the electron donor NADH or NADPH (Verma et al. 2019).

The demand for coenzyme factors is the main drawback for the azoreductase application in wastewater treatment. Thus, it is necessary to incorporate NAD(P)H recycling enzymes, such as glucose dehydrogenases and formate dehydrogenases (Dong et al. 2019). Azoreductases also act on degrading dyes from the azo group, such as Remazol Blue (Karatay et al. 2015), methyl orange (Verma et al. 2019), and even aminoanthraquinone dyes such as Dispersive Blue (Elfarash et al. 2017). In this sense, the importance of glucose 1-dehydrogenase presence in an integrated system with azoreductase and the improvement of this association on decomposing methyl red dye was reported by Yang et al. (2013).

Regarding azoreductase immobilization, silicas functionalized with amino and epoxy groups were applied to immobilize a novel azoreductase from *Rhodococcus opacus*. The enzymatic activity and storability increased significantly compared to the free enzyme (Qi et al. 2017). The association of enzymes may promote further degradation due to the increase in degradation routes possibility. Guo et al. (2021) employed a bacterium consortium to verify its ability to produce enzymes to decolourize saline water containing metanil yellow G, an azo dye used in paints. It reports that laccase, peroxidases, and azoreductase are responsible for the dye removal outcomes and the reduction of toxicity associated with the proposed degradation pathway.

The wide range of reactions enzymes can catalyze expands their application and makes them suitable in several fields. In general, the studies presented here show the feasibility of oxidoreductases on reducing dyes with recalcitrant characteristics to compounds that can easily be assimilated during conventional

biological treatment. Different approaches related to enzyme purification and immobilization have been tested worldwide to develop cost-effective systems. Nevertheless, further research is required to turn enzymatic systems into a viable treatment of real wastewaters.

2.11 NEW TRENDS ON MICROBIAL AND ENZYME DEGRADATION OF DYE-CONTAINING TEXTILE WASTEWATER TREATMENT

Regarding wastewater treatments, there are new approaches to traditional methods as dye adsorption and biofilms. In addition, there are emergent techniques as genetically modified organisms, combined treatment systems, membrane bioreactors, microbial fuel cells, support-based nanomaterials, and machine learning.

2.11.1 Dye adsorption

Adsorption is a well-known process for textile industry wastewater treatment, with activated carbon the most used adsorbent. Nevertheless, its high cost encourages the research of low-cost alternative adsorbents (Pavithra et al. 2019; Gadekar and Ahammed 2020; Ravenni et al. 2020; Ahmad et al. 2020). Activated sludge adsorbent is an exciting alternative and can be synthesized by centrifugation, sludge carbonization, pyrolysis, steam activation, H_2SO_4, and NaOH treatment (Pavithra et al. 2019).

Water treatment residuals in the dried form can also be applied as adsorbents on filtration column tests, as Gadekar and Ahammed (2020) explored for the decolourization of real textile dye wastewater. The adsorption process promoted a maximum colour removal of 36%, and the column operation obtained a decolourization rate in the range of 60–70%. Ravenni et al. (2020) developed a comparative study about the dye adsorption properties of waste chars from gasification of wood chips and pyrolysis of wastewater sludge with a commercial activated carbon. Sludge char performed maximum adsorption capacities of 13.4 and 8.4 $mg.g^{-1}$ for the anionic and cationic dye, respectively. Steam activation improved these values to 19.6 and 12.3 $mg.g^{-1}$. However, the sludge-based adsorbent has not achieved the efficiency of the commercial activated carbon and wood char. The removal of methylene blue was studied using cow dung biochar (CDB), domestic sludge biochar (SB) subject to slow pyrolysis at 500 °C, and rice husk biochar (RHB) as adsorbents. The dye removal efficiencies by CDB, RHB, and SB in a batch experiment were 97.0–99.0, 71.0–99.0, and 73.0–98.9%, respectively (Ahmad et al. 2020). The application of biomass adsorbent at an industrial scale presents some limitations such as the accessibility of adsorbents, adsorption sites, adsorbent stability, desorption rates at specific pH, and low adsorption, which should be improved to make this technology competitive (Li et al. 2019b; Zhou et al. 2019).

2.11.2 Genetically modified organisms (GMOs)

The variations of textile effluent composition led to the development of some alternatives to improve its remediation. Some molecular biology methodologies, such as cloning, directed evolution, gene recombination techniques, heterologous expression, metagenomics, random mutagenesis, rational design, and site-directed mutagenesis, are explored to enhance the treatment of effluents. The advances in genetic

engineering and molecular genetics enable virtual expression and clone of any gene in a suitable microbial host (Sen et al. 2016).

Laccase purified from *Pleurotus* sp. MAK-II was tested to diazo dye, congo red, anthraquinone, and remazol brilliant blue R decolourization. The enzyme presents high stability in the presence of violuric acid redox mediator (Manavalan et al. 2015). Liu et al. (2015) inserted the LacTT gene from *Thermus thermophilus* SG0.5JP17–16 into *Pichia pastoris,* promoting a more effective aptitude to degrade congo red, reactive black, reactive black WNN, and remazol brilliant blue R. A thermo-alkali-stable laccase gene purified from *Klebsiella pneumoniae* was cloned into *E. coli* to be applied for the remediation of azo phloxine, bromophenol blue, congo red, cotton blue, malachite green, mordant black 9, reactive brilliant blue X-BR, reactive brilliant blue K-GR, reactive brilliant blue KN-R, and reactive dark blue M-2GE from a textile industry wastewater (Liu et al. 2017). Undoubtedly, the main benefit of GMOs is the potential to accelerate decolourization efficiency. However, some disadvantages comprise cross-pollination and damaged environment due to horizontal gene transfer, reduced biodiversity, and uncertain long-term health effects (Kishor et al. 2021).

2.11.3 Combined treatments systems

2.11.3.1 Bio-advanced oxidation process

Advanced oxidation processes (AOPs) combined with biological methods are known for their high efficiency for recalcitrant wastewater treatment and their promising application in industries. Biodegradation and photodegradation are the best methods for removing the pollutants in water, besides enhancing water pollutants. The application of biological processes can be applied either as a pre-treatment or as a post-treatment (Oller et al. 2011). Biological degradation as a first step acts on the biodegradable fraction of wastewater by a low-priced and more eco-friendly method, demanding less energy and chemical inputs for further degradation of the remaining contaminants by chemical approaches (Paździor et al. 2019), used only for the remediation of remained compounds resistant to biological oxidation (Ledakowicz et al. 2017). Thanavel et al. (2019) applied a combined system of biological and AOPs treatment to study the decolourization of Remazol Red, Reactive Black 5, and Reactive Red 180 by *Aeromonas hydrophila* SK16. The individual treatment was also analyzed, and a 72% decolourization rate was reported. However, the combination of AOPs treatment with biological treatment proved to be more effective than single wastewater treatment by achieving 100% decolourization.

Regarding non-biodegradable-coloured compounds, chemical processes can assist or induce their biodegradability. Nevertheless, it is also possible that the by-products of dye degradation from chemical oxidation negatively interfere with the metabolic pathways of microorganisms (Dias et al. 2020). In this context, the main objective of chemical pre-treatment is partial oxidation (Venkatesh et al. 2017). Shanmugam et al. (2019) explored the application of Fenton (H_2O_2 & Fe^{2+}) oxidation and posterior biological treatment for the biodegradation of effluent with Acid Blue 113 azo dye. The AOP reduced the dye concentration by 40%, promoting a maximum dye degradation of 85%, i.e., 45% by biodegradation. In this sense, Gott (2010) recommended chemical oxidation as pre-treatment according to the BOD5/COD ratio below 0.2.

2.11.3.2 Membrane bioreactors

Membrane bioreactors (MBRs) conjugate membrane filtration with a biological approach (Kurade et al. 2019). MBR is a simple, cost-effective, and reliable method with solid potential to remove high nutrient load, organic chemicals, and colouring pollutants (Rondon et al. 2015).

Membranes efficiently separate the macro-molecules and microorganisms, but the major drawback is the membrane fouling in the bioreactor. Enhanced membrane bioreactor formed by two anoxic bioreactors, aerated bioreactor, UV-unit, and a granular activated carbon filter obtained 95% of colour removal from a synthetic textile industry wastewater (Rondon et al. 2015). Sepehri and Sarrafzadeh (2018) tested and proved the efficiency of a nitrifying-enriched activated sludge to decrease membrane fouling in MBR. An enhancement of 2.5 times in the permeation performance was achieved with the nitrifiers community compared to the conventional activated sludge process, which, consequently, improved MBR yields production; compact nature; high degradation rate inorganic pollutants, nutrient removal, quality of treated effluent; lower sludge generation; reliability; and small footprint, when compared to conventional activated sludge process, are some of the advantages of MBR. Nevertheless, some disadvantages alert the requirement of more study and process improvements, such as aeration limitations, stress on sludge in external MBR, membrane fouling, and higher operation cost (Rondon et al. 2015).

2.11.3.3 Microbial fuel cells

Microbial fuel cells (MFCs) are a bio-electrochemical treatment system composed of various microorganisms used as catalysts to oxidize inorganic and organic compounds and generate electrons (Yuan et al. 2019; Sayed et al. 2020). Sulphate-reducing consortium in MFC anodic chamber was used for simultaneous dye degradation, electricity generation, and sulfate reduction, in which the dye removal yield achieved ≤ 85%. At stable operating conditions, the power generation of 258 ± 10 $mW.m^{-2}$ was achieved (Miran et al. 2018).

Yang et al. (2019) used bio-electrochemical systems to decrease the concentration of Reactive Black 5 (RB5) from 0.503 mM to 0.124 mM. The effect of circuit connection was investigated for the azo dye degradation and bioelectricity generation. Compared to the open circuit system, the closed-circuit system exhibited higher decolourization efficiency (96% of Acid Red 18, 67% of Acid Orange 7, and 60% of Congo Red). The voltage outputs were ranked in the decreasing order of AR18 > AO7 > CR. This method represents an advanced, fast treatment efficiency, novel, and sustainable approach for the efficient treatment of industrial wastewaters and the power generation with reduced CO_2 emissions. However, the high treatment cost and sludge generation are associated disadvantages (Oon et al. 2020).

2.11.3.4 Support-based nanomaterials

According to several pilot-scale, laboratory, and in situ water treatment studies, nanomaterials have been explored as a promising alternative. These materials drew attention since they have a wide range of applications as remediation, drinking, and wastewater treatment. Nanomaterials have remarkable properties due to their small size and consequent unique physicochemical effects, including increased adsorption

yields and photocatalysis reactivity (Dasgupta et al. 2017; Bouabidi et al. 2019). Suganya and Revathi (2016) immobilized *P. putida* and *B. licheniformis* on sodium alginate and polyacrylamide gel beads to explore the decolourization potential. According to the results, the sodium alginate support provided better conditions for the process when compared to polyacrylamide gel beads and free cells processes.

2.11.3.5 Biofilms

Biofilms are negatively charged layers of microbial conglomerations of one or more species trapped to abiotic or biotic surfaces by incorporation in the matrix of extracellular polymeric substances (EPSs) (Mohapatra et al. 2020). Compared to free bacterial usage, bacterial biofilms have some advantages, such as the ability to exchange nutrient and genetic materials, bigger tolerance to toxic compounds and different metabolic states, protection from the effects of environmental changes (Mohapatra et al. 2020). The dye adsorption mechanism by biofilms outcomes from two stages: the first is the dye molecules transport through the solution to the surface of the biofilm, followed by the adsorption of dye molecules to the active sites of biofilm (Sun et al. 2016). Moving bed biofilm reactor (MBBR) is one of the most explored bioreactors and unites the benefits of activated sludge and biofilm (Paździor et al. 2019). Due to the promotion of biomass growth on a moving support, some advantages can be observed, such as the absence of obstruction problems, decrease in hydraulic retention time, easier separation among liquid and solid phases, flexibility in operation, increase in biomass weight, less space only and environmental effects, low sludge production, superior biomass residence time, and superior capability to degrade complex compounds (Castro et al. 2020). MBBR may work in anaerobic or aerobic conditions and have been effectively used in dye decolourization and degradation studies with great yields of decolourization (Castro et al. 2020; Deng et al. 2020; Ong et al. 2020).

2.11.3.6 Dye industry waste and resource recovery strategy

With the proposal to reduce the amount of waste discharged from industries, its use in the resource recovery process as input material for recovering value-added products is being widely explored. Mishra et al. (2020) have performed the decolourization of mixed dyes reactive red 21 (RR21) and reactive orange 16 (RO16). The authors obtained 83% of colour removal using a microbial fuel cell from dye wastewater with bioenergy generation of 940.61 ± 5 $mW.m^{-2}$ power density with 790 ± 5 mV voltage output generation. A single-chamber microbial fuel cell with microalgal biocathodes (with 42% of the cathode surface covered by microalgal naturals) was designed for the simultaneous biodegradation of real dye textile wastewater and the generation of bioelectricity. It was observed that the atmospheric and diffused CO_2 promoted good algal growth rates and immobilization, indicating its operation at air-exposed conditions. The maximum volumetric power density achieved was 123.2 ± 27.5 $mW.m^{-3}$ highlighting it as a promising alternative to a Pt cathode (Logroño et al. 2017). Phytoremediation technology by microalgal strains viz. *Anabaena ambigua*, *Chlorella pyrenoidosa*, and *Scenedesmus abundans* using textile wastewater as a nutrient source were studied to generate electricity. The authors correlated the differences of various UV-Vis in the spectra obtained with the breakdown and formation of compounds and postulated the biodegradation for

wastewater remediation. Electrical conductivity and redox potential reduced to 2.0 mS and 157.86 ± 1.89 mV, respectively (Brar et al. 2019).

2.11.3.7 Machine learning

Due to their sensibility to environmental changes, algal systems in complex bioprocesses applications are not precisely controlled. Therefore, machine learning algorithms (MLAs) and dynamic models, control systems and real-time monitoring are a solution to preserve optimum conditions. Their application brought some exciting results for the optimizing study of algal system performance, which provides a better understanding of the biological processes when compared to the conventional kinetic or phenomenological models (Iratni and Chang 2019).

MLAs application leads to effective real-time monitoring, defect detection, optimization, and anticipation of uncertainties of complex environmental systems. Besides, disruptions or failures from leaking pipelines, malfunctioning of bioreactors, unexpected flow rate variations, organic loadings, and temperature are also situations that can be efficiently anticipated by integrating these algorithms with online sensors. Notwithstanding the MLAs application benefits, it is essential to consider that to implement these advanced models, sensors, data acquisition, transmission, and storage, have to promote knowledge at decision making to optimize their system performance. Increments could be performed by implementing multi-parameter sensors and the internet of things (IoT) for full-scale WWTPs, which give plant managers support to identify equipment faults, improve energy usage, and decrease greenhouse gas emissions (Sundui et al. 2021).

2.12 CONCLUSIONS

Regarding dye-containing textile wastewaters, they are often pumped into freshwater bodies or sea without using a treatment system, in particular developing countries. In this sense, biological processes are efficient and eco-friendly, operate at mild, among others. Anaerobic/aerobic sequential systems are highly efficient on azo dye Red2-degradation. In addition, phycoremediation, and GMO oxidoreductases producers, particularly azoreductases, laccases, and peroxidases, are promising alternatives. Despite the problematic operating control, the integrated systems as AOPs-*Aeromonas hydrophila* or MBRs (anoxic bioreactors, aerated bioreactor, UV-unit, and granular activated carbon filter) have remarkable potential to remove such high nutrient load, organic chemicals, and dyes. Finally, microbial fuel cells can be associated with wastewater treatments.

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CHAPTER 3

In this chapter, a literature review about the potential environmental impacts of azo dye-containing wastewater from the textile industry, including toxicity; activated sludge, and its potential use in dye-degradation reaction by the isolation and identification of the inherent microbiome; and the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a novel, rapid and accurate strategy for the identification of activated sludge microbiome (potential to enhance treatment yield). This review chapter was submitted to the World Journal of Microbiology and Biotechnology and accepted on 30 April 2021, https://doi.org/10.1007/s11274-021-03067-6.

3 BIODEGRADATION OF AZO DYE-CONTAINING WASTEWATER BY ACTIVATED SLUDGE: A CRITICAL REVIEW

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Abstract

The effluent from the textile industry is a complex mixture of recalcitrant molecules that can harm the environment and human health. Biological treatments are usually applied for this wastewater, particularly activated sludge, due to its high efficiency, and low implementation and operation costs. However, the activated sludge microbiome is rarely well-known. In general, activated sludges are composed of *Acidobacteria*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Proteobacteria*, and *Streptococcus*, in which *Bacillus* and *Pseudomonas* are highlighted for bacterial dye degradation. Consequently, the process is not carried out under optimum conditions (treatment yield). Therefore, this review aims to contextualize the potential environmental impacts of azo dye-containing wastewater from the textile industry, including toxicity, activated sludge microbiome identification, in particular using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a novel, rapid and accurate strategy for the identification of activated sludge microbiome (potential to enhance treatment yield).

Keywords Activated sludge · Textile industry · Microbiome · Biodegradation · Azo dye

3.1 INTRODUCTION

The high fluctuations in composition and other parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), colour, pH, and salinity during textile processing contribute to the complexity of textile wastewater treatment (Senthilkumar et al. 2011; Farias et al. 2017). The textile industry generates, inherently, a high volume of toxic effluent, mainly due to the chemical baths and rinsing series (Harane and Adivarekar 2017). According to Leão (2002), 150 L of water are necessary to produce 1 kg of fabric, in which 132 L (88%) are wastewaters.

The dyeing stage leads to a large amount of wastewater with high levels of COD (from the chemical used in the process), BOD (due to the direct discharge of wastewater in water sources which promotes a rapid depletion of dis- solved oxygen), pH, and dyes (Holkar et al. 2016; Swati and Faruqui 2018). The incorrect disposal of textile industry effluents into water bodies interferes with the penetration of light. Hence, it harms the photosynthetic activity of water body communities (Elisangela et al. 2009). Thus, the textile industry effluent has to be properly treated before disposal into water bodies. In this sense, conventional textile industry treatments include coagulation/flocculation, biological, membrane, and advanced oxidation processes (Vajnhandl and Valh 2014; Yukseler et al. 2017). Among these techniques, the dye biodegradation by the activated sludge process is drawing attention due to its low cost (implementation and

operation) and high efficiency (Haddad et al. 2018). Regarding activated sludge, the effectiveness of COD and BOD reduction can reach up to 90% (Pereira et al. 2010; Waghmode et al. 2019).

Activated sludges are composed of microbial communities. However, there are few reports on their characterization (El et al. 2016; Zhu et al. 2018; Cao et al. 2019). The evaluation of activated sludges as microbial consortia is essential to comprehend the interactions among the microbial com- munity and optimize biodegradation (Köchling et al. 2017). Nevertheless, experiments of dye-biodegradation by isolated strains from activated sludges makes easier the elucidation of biodegradation mechanisms (Khehra et al. 2005).

Therefore, this review describes the main biodegradation aspects of textile effluent. Then, it correlates the MALDI-TOF MS as a promising methodology for identifying activated sludge microbiome and, consequently, improving the treatment yield.

3.2 POTENTIAL IMPACTS OF AZO DYES

Azo dyes are chemically composed of aromatic groups and azo chromophore (-N=N-). They are highly water-soluble. It is worth noting that azo dyes are widely applied by textile industry corresponding ≥ 50% out of the worldwide dye production (Brüschweiler and Merlot 2017). However, they hamper conventional textile wastewater treatment plants due to their recalcitrance.

It is usually observed a very low degradation rate of azo dyes at primary and secondary treatment stages due to their recalcitrant behaviour, which is related to their synthetic origin and chemical structure, providing them high resistance to photo-oxidation, biological activity, and other environmental conditions. The molecular arrangement and size of these substances contribute negatively to their biodegradation since a steric effect hinders the enzymatic access necessary to start the degradation (Rittmann 2018). Therefore, the accumulation of azo dyes can occur in sediments, soils, and contaminate the drinking water supply system (Salter-Blanc et al. 2016; Xiang et al. 2016; Mullai et al. 2017). The colour reduction ≈ 50% (azo dyes) from coloured wastewater is considered adequate. However, the toxicity factor must also be considered, particularly due to the aromatic amines (Xiang et al. 2016; Brüschweiler and Merlot 2017). Heavy metals, salts, and sulfides are potentially microbial inhibitors of biological treatment system. Thus, it must be also evalu- ated (Sarayu and Sandhya 2012).

It is known that the chromophore azo groups present in anionic and nonionic dyes suffer reductive cleavage producing highly toxic aromatic amines (Table 4): R-N = N-R′ + 4ē + 4H+ → R-NH2 + R′-NH2 (Xiang et al. 2016). The chemically reduced form of dyes was already found in sediments of aquatic bodies. These molecules are carcinogenic, since they can be oxidized to N-hydroxylamines. Thus, the nitrenium ion generated can bind with cellular macromolecules as DNA, proteins, and RNA (Ford and Griffin 1992; Sari and Simarani 2019). Nevertheless, an activating metabolism varies according to the balance between numerous competing steps, the bioavailability of the reactive metabolite, and nutritional habits. Moreover, possible differences in individual susceptibility influence the higher complexity of the metabolic pathway, polymorphisms of enzymes associated with the metabolism of aromatic amines, and equilibrium between activating and inactivating steps (Gregory 2007; Neumann 2010).

Kumar et al. (2019) carried out the optimal process of decolourization of Acid Black 24 azo dye by *Bacillus pseudomycoides*. The authors also evaluated the genotoxicity and phytotoxicity of the degraded dye.

Genotoxicity assay was carried out using Allium cepa (onion), evaluating the DNA damage in cells treated with dye solution performed by single-cell gel electrophoresis method. The results were obtained by comparing before and after decolourization. The untreated dye sample presented a genotoxic effect on the root cells of *Allium cepa*. Phytotoxicity experiments were also performed on seeds of *Vigna radiata* and *Sorghum vulgare* at 25 °C. After 48 h was observed the number of full seeds germinated, and with five days of incubation was calculate the length of plumule (cm) and radicle of seedlings (cm) and germination percentage. The control and treated sample results presented similar values, which indicate the production of non-toxic metabolites correlated to high degradation yields.

On the other hand, according to Waghmode et al. (2019) higher phytotoxicity was observed with Phaseolus mungo and Sorghum vulgare after a sequential photocatalytic and biological treatment consisting of ZnO as the photocatalyst and a microbial consortium of *Brevibaccilus laterosporus* and *Galactomyces geotrichum*. Through HR-MS and GC–MS analysis, degradation products were analyzed after bacterial treatment. Many products with a fragmentation pattern inferring asymmetrical/symmetrical cleavage in azo bonds were found, probably due to the demethylation and desulfonation mechanisms. It was also observed that the produced metabolites in the activated sludge can be correlated to microbial species (Tabasum et al. 2019).

The toxicity of a treated textile effluent was analyzed by Carvalho et al. (2020), using *Vibrio fischeri* as a toxicity indicator, monitoring cell luminescence inhibition (30 min). The dilution factor (effluent) required to not affect bacteria metabolism was also used as a comparison parameter, in which the acute toxicity is correlated to 50% reduction in the bacteria bioluminescence. In the conventional up-flow anaerobic sludge blanket (UASB) effluent, it was obtained a dilution factor between 14 and 16, which can be associated with the presence of aromatic amines, (strong toxicity levels). Whereas the results of UASB effluent micro aerated on top indicated that aromatic amines were converted into nontoxic compounds.

Aromatic amines from the azo-dye degradation have mutagenic effects in *Salmonella* and *Mammalian*. Methyl Red, which is naturally mutagenic, has already been associated with N,N-dimethylphenylenediamine (DMPD) formation, a toxic and mutagenic aromatic amine (Wong and Yu 1999). Consequently, methodical optimization studies simultaneously with metabolite toxicity testing should be implemented for each system, considering dye, microbe, enzyme, or mediator (Sen et al. 2016).

Daily large amounts of residual water containing a high concentration of azo dyes, between 100 and 250 mg L^{-1}, are incorrectly discharged into water bodies (Garcia-Segura and Brillas 2016). To mitigate the impact of azo dyes (colour), their chemical bonds must be broken within the chromophores group structures (Ghosh et al. 2017). Aerobic biodegradation of dyes through the activated sludge process is recognized as an economical and efficient technology. Nevertheless, some drawbacks can cause serious environmental impact, in particular, due to the large amounts of sludge that are inherently generated (Lv et al. 2013). In addition, most dyes and chemicals from the textile process have a low rate of biodegradability. Deraniyagala (2017) reported that the treatment of textile industrial wastewater by activated sludge produces 2000 tons of dangerous sludge, considering a daily flow rate of 4000 m^3 of wastewater. Thus, the activated sludge treatment should be improved, constantly. Regarding biodegradation investigations of

Table 4: Reports about the decolourization capability of bacteria from textile-activated sludge.

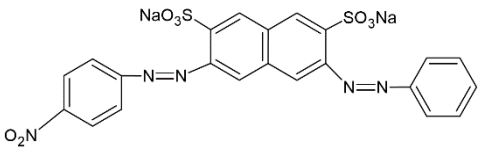
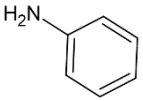
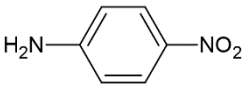
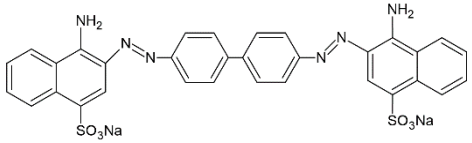
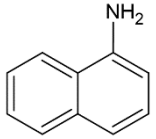
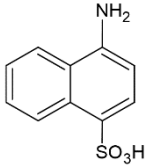
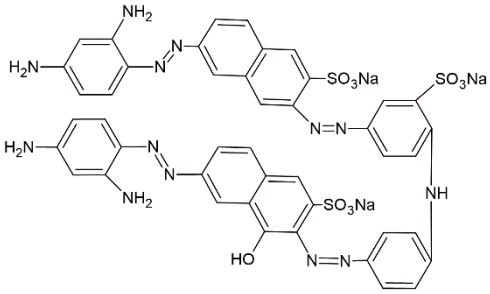
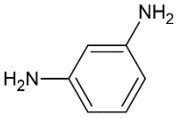
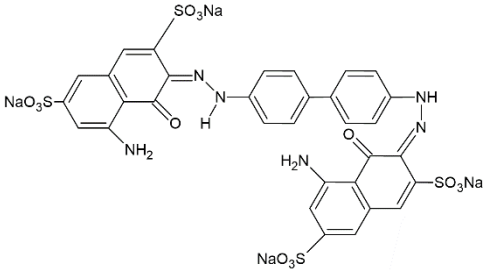
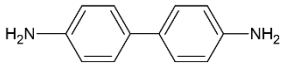
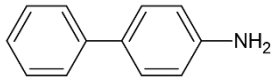
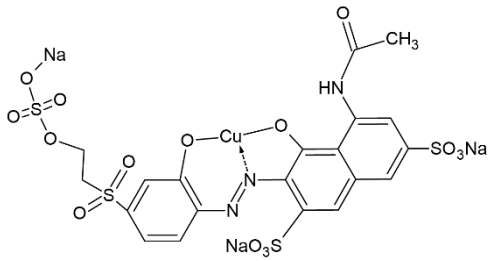
Dye	Corresponding aromatic amines	Toxicity/ecotoxicity of aromatic amines	Microbiome	Degradation system	Decolourization rate	Reference
Amino Black (AB) 	Aniline  4-nitro-aminobenzene 	Naphthylamine has an occupational carcinogen potential and can be absorbed into the body by ingestion, inhalation, and through the skin. It is toxic to aquatic life with long-lasting effects. 4-nitro-aminobenzene is expected to have high mobility in soil.	*Bacteroides* and *Lactococcus*	Decolourization reaction by anaerobic condition, and mineralization process under aerobic environment	85.1% 85.2%	Zhu et al. (2018), Tomioka et al. (2015), Varanasi (1989)
Congo Red (CR) 	Naphthylamine  4-aminonaphthalene-1-sulfonate 					
Direct Black 22 (DB22) 	Benzene-1,3-diamine 	Suspected carcinogen behavior. According to its structure, there is a high adsorption potential with suspended solids and sediment in the water.	*Methanosaeta*, *Syntrophus, Trichococcus, Brevundimonas,* and *Ornatilinea*	Two up-flow UASB reactors, one with aeration in the upper part	79%	Carvalho et al. (2020)

Table 4: (continued)

Direct Blue 2B (DB2) 	Benzidine  4-aminobiphenyl 	Exposures to 4-aminobiphenyl and benzidine in the textile dye industries have a historic on cancer of the urinary bladder in humans. Moreover, they can induce neoplasms at multiple organ sites in laboratory animals.	*Proteobacteria, Firmicutes,* and *Acidobacteria*	Anaerobic, temperature 38.70°C, pH 7.57, NaCl concentration 20.10 $g.L^{-1}$	89.2%	Cao et al. (2019), Choudhary (1996) and Airoldi (2002)
Reactive Violet 5 (RV5) 	No aromatic amine formation occurs	It is not applied.	*Acidithiobacillus, Acidocella*, *Streptococcus* *Trichosporon*, *Aspergillus,* and *Clostridium*	Activated sludge treatment and partial Fenton's oxidation	86.1 %	Meerbergen et al. (2017), (Chung and Chen 2009)

textile effluents, usually they are outlined to enhance the activated sludge microbiome degradation, biosorption (sequestration of dyes from solution by chelation, complexation, precipitation, or ionic interactions), and mineralization (complete oxidation of dyes to H_2O, CO_2, and other inorganic compounds) by varying physicochemical parameters such as pH, temperature, carbon and nitrogen sources, dye concentration, inoculum size, among others (Chen et al. 2003; Moosvi et al. 2005; Kapdan and Erten 2007; Pandey et al. 2007; Khalid et al. 2008; Dhanve et al. 2008; Mullai et al. 2017). However, there are new technologies as MALDI-TOF MS that can assist to reach higher treatment yields.

3.3 DYE ADSORPTION PROCESS AND TEXTILE SLUDGE

Adsorption is a wide used wastewater treatment technology, also applied to the textile industry (Ho and McKay 2003; Jain et al. 2003). Intermolecular attraction forces between adsorbate and adsorbent lead to mass transfer, in which the accumulation of contaminants occurs at the interface between phases: gas–liquid, gas–solid, liquid–liquid, or liquid–solid interface (Reisch 1996; Dąbrowski 2001). In this sense, the molecular structure of the adsorbent, medium pH, solute solubility, and temperature significantly affect the adsorption process (Foust et al. 1980).

Regarding adsorption of dyes, there are four main steps: (i) the dye movement from the crude solution to the liquid film or the interface of the adsorbent solid; (ii) its diffusion through the liquid film to the external sites of adsorption; (iii) its inner diffusion through the adsorbent solid pores or capillaries, and; (iv) its adsorption at the available places of the capillary surfaces or walls (Reynolds and Richards 1996).

The most usually used adsorbent for textile wastewater treatment and colour removal is the activated carbon. However, its high cost is promoting the development of low-cost alternative adsorbents (Aksu 2001; Calvo 2001; Wang and Hu 2007; Ju et al. 2008; Smith et al. 2009; Rafatullah et al. 2010). In this sense, waste materials, such as activated sludge, are an interesting alternative since large quantities of sludge are inherently produced (Smith et al. 2009).

The sludge-based adsorbent can be produced by the carbonization of sludge, centrifugation, H_2SO_4 treatment, NaOH treatment, pyrolysis, steam activation. It is worth noting that microbial membranes of sludge affect the adsorbent properties since microbial membranes are negatively charged surfaces (Pavithra et al. 2019). Thus, the control mechanisms of dye adsorption include chelation, complexation, ion exchange, and surface adsorption (Crini 2006; Wang and Hu 2007; Sadhasivam et al. 2007).

The decolourization efficiency of an alternative adsorbent from textile effluent sludge was tested by Vasques et al. (2011) on Reactive Orange 16 (RO16), Reactive Red 2 (RR2), and Reactive Red 141 (RR141) dyes. The absorbent was submitted to a thermal activation at 500 °C followed by the chemical activation with acetic acid. At 25 ºC – equilibrium - it was observed the complete removal of RO16 and RR2. Regarding RR141 and RO16, the adsorption capacity was enhanced with NaCl and Na_2SO_4, respectively.

Sludge from the textile industry was also used as a low- cost adsorbent for Reactive Red 2 dye. A sequential thermal (500 °C for 70 min) and chemical (H_2SO_4, 25 °C for 3 h) treatment were evaluated. The kinetic experiments, the pseudo-second-order model, were performed in batch mode. The adsorption isotherm model was evaluated under different temperature and pH conditions. Maximum adsorption 213.9 $mg.g^{-1}$ was obtained with pH 2 and 25 °C (Sonai et al. 2016).

Autoclaved bio-sludge was tested for disperse dye adsorption in sequencing batch reactor (SBR) systems with and without granular activated carbon (GAC–SBR) using textile wastewater (TWW) collected from a central wastewater treatment plant in a textile factory in Thailand; and synthetic textile wastewater (STWW). The GAC–SBR system presented more effectiveness compared with SBR in treating TWW, resulting in a dye decolourization rate of 93.0 ± 1.1%, under the organic loading of 0.18 kg $BOD5.m^{-3}$ (Sirianuntapiboon and Srisornsak 2007).

Haddad et al. (2018) highlighted the optimization of aerobic biodegradation efficiency to reduce the residual adsorbed dye in the final waste sludge. Laboratory and pilot-scale investigations were carried out. The process at pilot-scale was tested under different hydraulic retention times (HRT) of 2–5 days and sludge recycling rates (SRR) of 220–680 $m^3.day^{-1}$, which achieved the optimal result at HRT of 5 days and a SRR of 0.22 with dye biodegradation efficiency of 5%. These best conditions applied at full-scale reduced the amount of the discharged dyes (89%) significantly.

Water treatment residuals (WTR) in the dried form were used as adsorbents in filtration column tests for the colour removal from a real textile dye wastewater. The process presented a maximum colour removal of 36% in the adsorption process and a decolourization rate in the range of 60–70% in column operation, which generally shows a greater removal. The authors defended the use of WTR as a primary treatment for textile wastewater decolourization (Gadekar and Ahammed 2020).

There are some limitations to applying biomass at an industrial scale, including the accessibility of adsorbents, adsorption sites, adsorbent stability, low adsorption, and desorption rates at specific pH and other environmental factors such as ions and salts. These factors should be improved to make this technology competitive (Li et al. 2019a; Zhou et al. 2019). Regarding emerging advanced technologies used for dye adsorption, the most promising are: magnetic nanoparticles; metal/nonmetal-doped nanostructures; ceramic and modified nanoclays; and carbonaceous nano-materials such as single and multiwalled carbon nanotubes, carbon quantum dots, and expanded graphite and graphene nanosheets (Fraga et al. 2021). These technologies also present an environmental clean-up perspective and have been attempted to achieve high rates of colour removal efficiency and low cost (implementation and operation) (Anand et al. 2020; Nayak et al. 2020).

Therefore, regarding the dye biodegradation by bacteria culture, the biological adsorption phenomenon interferes on decolourization results, making it difficult to understand biological degradation in details, since adsorption occurs at the same time (Ghosh et al. 2017; Wang et al. 2020). In this sense, Kiayi et al. (2019) investigated this factor through a biosorption test, based on spectrophotometric visualization of the solution from the suspension of bacterial pellets in methanol and water. However, no adsorption interference was detected. Corso and Maganha De Almeida (2009) evaluated the adsorption contribution using different concentrations of isolated biomass (autoclaved and non-autoclaved) to inoculate on dye solution. After 120 h, it was measured absorbances of the supernatants, which revealed high levels of decolourization index. The identification of adsorption in biodecolourization processes was also pointed by Asad et al. (2007) through the gradual decrease of adsorption peaks identified in a decolourization. Besides this verification, the authors made an association between live and inactivated cells, inferring that inactivated cells cannot decolourize an aqueous system by the adsorption process.

The adsorption process is possible due to the cell sur- face composition from active functional groups (amine, carboxyl, hydroxyl, phosphate, and sulfhydryl) for dye binding (Kapoor et al. 1999; Corso and Maganha De Almeida 2009). Nevertheless, it also depends on the concentration of dye (Ghosh et al. 2017). The presence of these functional groups on the cellular wall provides a negative charge that attracts positively charged molecules as cationic azo dyes or with positively charged groups (e.g. basic red 29, and basic blue 41) (Srivastava and Thakur 2006; Congeevaram et al. 2007). Therefore, the adsorption must be considered, carefully, in biological treatments.

3.4 ACTIVATED SLUDGE MICROBIOME

Activated sludge is an association among many organisms in a community, mostly composed of aerobic and anaerobic bacteria. Some bacterial species can flocculate, which favours sedimentation (Paździor et al. 2019). In addition, they can reach high rates of decolourization and mineralization, which leads to low toxic sludge generation and a cost-effective process. Species belonging to the genera *Aeromonas*, *Bacillus*, *Proteus*, and *Pseudomonas* are some of the widely investigated bacteria for dye degradation (Mullai et al. 2017).

The azo dyes biodegradation by bacteria generally requires a combination of two stages. First, an anaerobic step responsible for discolouration when azo bonds are broken in the presence of redox mediators through the azo reductase enzyme (Klepacz-Smółka et al. 2010). Then, an aerobic phase promotes the efficient removal of organic compounds. Since the decolourization by pure cultures is associated with the development of aromatic amines (toxic compounds), mixed cultures (Table 4) are often used due to their synergistic metabolisms. This synergy promotes the conversion of toxic intermediates into nontoxic by-products (Yang et al. 2012; Patel 2013; Lotito et al. 2014; Manekar et al. 2014; Ali et al. 2016; Mullai et al. 2017).

Carvalho et al. (2020) evaluated two up-flow anaerobic sludge blanket (UASB) reactors R1 and R2 (with aeration in the upper part), as a comparative system to remove tetra-azo dye Direct Black 22 (DB22). The discolouration and COD removal efficiencies for both reactors were similar (67 e 72% for R1 and 59 e 78% for R2), pointing to no consider- able influence of oxygen in R2. DNA extraction (Power Kit Soil®—MO Bio laboratories, Carlsbad-CA, USA), quantification (NanoDrop2000 spectrophotometer—Thermo Sci- entific, USA), storage at − 20 °C, and Illumina MiSeq with the universal primer 515 F paired with 907R for Archaea and Bacteria domains, with 20,000 reads and 2 × 300 bp. Microbiome identification of the sludge bed of both reactors was carried out (sequencing), which were similar to each other, that is, *Methanosaeta*, *Syntrophus*, and *Trichococcus* genera. Sequences with less than 150 bp and ambiguous base calls were not considered, and the Operational Taxonomic Units (OTUs) were defined by clustering at 97% similarity. The authors proved that higher salinity in some zones of the reactors promoted some alterations on the microbial community and the association between putative genera *Brevundimonas* and *Ornatilinea* and aromatic amine microaerobic removal. An investigation of the metagenomic of activated sludge from the common effluent plant of Chennai (India), used in the textile effluent treatment process with mixed azo dyes, was conducted by (Krishnaswamy et al. 2020). The nanopore sequencing was carried out with PCR amplification and barcoding of the sample from the acclimatized sludge used to treat synthetic textile wastewater treatment. After the obtaining and purification of the activated sludge sample, it was amplified

with PCR and barcoded. Then, adapters were connected to the amplified fragments constructed a library, which was sequenced using nanopore sequences. The fragments of 16 s rRNA genes were computed, and in the diversity of the organisms was found Actinobacteria, Proteobacteria (abundantly), and Terrabacteria. The Proteobacteria phylum were represented by the *Acidithiobacilia*, *Burkholderiales*, *Betaproteobacteria*, *Neiseriales*, *Nitrosomonadales*, and *Rhodocyclales* genera.

Cao et al. (2019) isolated and developed an indigenous bacteria consortium from a sludge sample of a dying factory for characterizing the active functional microbial communities involved in the degradation of a sulfonated azo dye, Direct Blue 2B (DB2), in a simple batch reactor. The decolourization potential of isolated and combined cultures was analyzed under different temperatures, pHs, dye, and NaCl concentrations, operation modes (static, and agitated). The study obtained 90.74% of maximum decolourization of 100 $mg.L^{-1}$ DB2 at 48 h, static condition, with 38.7 °C of culture temperature; initial pH was 7.57, and initial NaCl concentration was 20.10 $g.L^{-1}$ predicted by the quadratic model.

To identify the main microorganisms from activated sludge responsible for the degradation of Congo red (CR) and Amino Black (AB) dyes, Zhu et al. (2018) proposed a combined model. Besides identifying the species directly involved in azo dye degradation, the study aimed to reveal the relationship between azo dye degradation and microorganisms through DNA extraction, polymerase chain reaction (PCR), and Illumina Sequencing Analysis, with a multiple linear regression model. The reactions of transformation in each of the six reactor compartments were investigated, and it was verified that degradation intermediates present in each compartment were affecting the microbial communities differently. Concerning the functional species and decolourization process, *Bacteroides* and *Lactococcus* exhibited significant correlations with the azo bond with the t-value of the corresponding regression coefficient larger than 2.0. The study highlighted the occurrence of the decolourization process by anaerobic condition and mineralization under an aerobic environment. The microbial community was significantly affected by the structures of azo dyes and, consequently, their intermediates.

Zhang et al. (2018) investigated activated sludge samples from three typical Chinese municipal wastewater treatment plants: domestic sewage, fine chemical industry, and textile dyeing wastewater. Microbial DNA was extracted by the liquid-nitrogen grinding pretreatment method; metagenomic sequencing and bioinformatic analysis were executed to understand their metabolic potentials. The dominant phyla in every sample included Proteobacteria (12.3–58.5%), Acidobacteria (1.8–35.1%), Chloroflexi (2.8–37.7%). However, were also found in all samples Bacteroidia (0.7–19.2%), Actinobacteria (0.7–6.8%), TM7 (0.1–5.2%), Synergistetes (0.02–5.6%) and Thermi (0.03–7.89%). In the textile dyeing industry wastewater, Nitrospirae (48.68%) and Acidobacteria (34.82%) were prevailing in the oxidation ditch.

A synergic effect of activated sludge treatment and partial Fenton's oxidation for decolourization of azo dye Reactive Violet 5 (RV5) was observed. Pretreatment with Fenton's reagent in 500 $mg.L^{-1}$ RV5 aqueous solutions promoted 52.9, 83.9, and 91.3% of colour removal within 60 min to H2O2 concentrations of 1.0, 1.5, and 2.0 mM, respectively. Then biological treatment removed 70.2%, on average, of the residual RV5 concentration. An activated sludge microbial community analysis was realized through Genomic DNA using the Power Soil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA). Several of the most abundant bacteria were *Acidithiobacillus*, *Acidocella*, and *Streptococcus*, that presented azo dye reducing abilities. The study also revealed that exposure to RV5 modified a highly-specialized community

with degrading activity to azo dye, including *Aspergillus*, *Clostridium*, and *Trichosporon* species (Meerbergen et al. 2017).

Thus, the wide variety of activated sludge microbiomes mentioned above is directly associated with the complexity of each textile effluent, the structures of the azo dyes, their intermediates, and treatment conditions. However, the microbial community affects directly the decolourisation yield. Thus, it should be investigated deeply.

3.5 SCREENING OF BACTERIA FROM ACTIVATED SLUDGE; RAPID IDENTIFICATION BY MALDI-TOF

Usually, microbial identification is carried out using multiple experiments and analytical procedures, for instance, extraction, purification, separation (e.g. through 16S rRNA and 18S rRNA gene sequencing), complex phenotypic, molecular, and morphological characteristics. These methods are costly and often do not provide information on microbial physiology (Padliya and Wood 2004; Kemptner et al. 2009; Kim et al. 2010; Singhal et al. 2015).

In this sense, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a technique for a wide range of chemical identification. It can also be used for rapid microbial identification (protein pattern overlay) (Murugaiyan et al. 2012). Generally speaking, a reference library (e.g. MALDI Biotyper) composed of proteomic signals (spectrum) of known microorganisms is used. The spectrum of an unknown sample is instantly matched against the reference library to identify microorganisms by their molecular fingerprint. Its comprehensive database of pathogenic microorganisms, rapid process, relatively higher accuracy, sensitivity, and economy in terms of labour and costs involved lead to advances over other microbial identification methods prevalent in clinical diagnosis. To date, there are some limits to the applicability of MALDI-TOF MS in the area of microbial ecology research due to the deficiency of data on non-clinical microorganisms. In other words, the reference library should be expanded to all microbial species as soon as possible (Singhal et al. 2015; Rahi et al. 2016).

Nevertheless, this technique is becoming increasingly fundamental for microbial characterization and identification, describing new species due to its ability to distinguish at the species level (Lang et al. 2015; Patil et al. 2015; Tong et al. 2015). MALDI-TOF MS technique was already used in a wide range of application, for instance, to distinguish bacterial species of the *Rhizobiaceae* family (Ferreira et al. 2011), to identify bacterial species from the human gut (Lagier et al. 2012), to detect pathogenic bacteria (food security assessment) (Bier et al. 2017; Fröhling et al. 2018), to make faster the urinary tract infection identification (Li et al. 2019b), to identify marine bacterial symbionts (Dieckmann et al. 2005; Vidal et al. 2020).

In the context of the textile industry, the MALDI-TOF MS analysis was already used to obtain mass spectra of bacterial proteins from cotton cloth samples contaminated with *Shigella flexneri*, *Escherichia coli*, and *Aeromonas hydrophila*, which are species that could cause illness through the faecal-oral routes. The authors confirmed the technique as a rapid method with a high potential for detecting biomarker proteins recovered directly from clothing samples (Holland et al. 2000).

A *Bacillus* sp. isolated from sediments of distillery unit was found to overproduce laccase with enormous potential for decolourization of various recalcitrant dyes. The enzyme peptide sequences were obtained

with MALDI–TOF MS, the spectra were analyzed using MASCOT software (Matrix Science) and compared with the NCBI database for placement of enzyme with known sequences. About decolourization tests, after enzymatic action, there was around 73% decolourization of dye (trypan blue) and 62% of BBR along with the precipitation of dye contents (Kaushik and Thakur 2013). A study developed by Afreen et al. (2017) showed the use of MALDI-TOF MS to bacterial enzyme identification by peptide mass fingerprinting. The enzyme was obtained from *Spirulina platensis* CFTRI, purified, and used in the decolourization of anthraquinone dye Reactive blue 4 (96%) within 4 h.

In this context, MALDI-TOF MS analysis could bring promising results when used to identify activated sludge microbiome. As subtly explored by Mulinari et al. (2020), who used MALDI-TOF MS analysis for species identification of activated sludge, which showed the presence of both types of microorganisms: aerobics (e.g. *Lysinibaciullus fusiformis*) and facultative anaerobic (e.g. *Escherichia coli* and *Kosakonia cowanii*). The MALDI-TOF MS-based biotyping is a remarkable resource. Due to the speed, accuracy, and sensitivity, the wastewater treatment plant can operate with fine adjustments to enhance the biodegradation, for instance, correlates specific dyes with microbial changes. In addition, the MALDI-TOF MS data can be used for more drastic changes, for example, after microbial isolation and identification, the best azo-degrading species could be immobilized and then added into the treatment plant, or it could be growth (ex-situ), then periodically inoculated into the treatment plant.

3.6 STATE-OF-THE-ART AND PERSPECTIVES

The textile industry produces large volumes of recalcitrant effluents, including azo dyes that negatively affect water bodies and their biological activity. The biodegradation of textile industry effluent, in particular azo dyes, by activated sludge stands out due to its high yields of decolourization. The genera *Pseudomonas*, *Bacillus*, *Proteobacteria*, *Clostridium*, *Acidobacteria*, and *Streptococcus* are usually found in activated sludges. However, there are unknown microbial species that should be investigated, besides the seasonality and complexity of textile wastewater composition change activated sludge microbiome, inherently. Thus, the evaluation of isolated cultures from activated sludge can provide insights and significantly enhance biodegradation yield. In this sense, MALDI-TOF MS, a rapid with high accuracy and sensibility technique for microbial identification, is a potential strategy to enhance the biodegradation of azo dye-containing wastewater from the textile industry, in particular identifying microbial species that degrade azo dyes.

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Author contributions

GM and CJ carried out the literature review, designed the study, and wrote the manuscript. JM, AA, DO, and SM proofread the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors have no conflict of interest to declare.

Research involving human and/or animal rights

This article does not contain any studies with human participants or animals performed by any of the authors.

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CHAPTER 4

In this chapter, the methodologies and results concerning bacterial strains isolation and identification from the activated sludge of textile effluent treatment process; the screening to verify their ability to decolourize Reactive Red 141; the kinetic, toxicity, and degradation performance were studied and carefully described. This study was written in an article format to be further submitted.

4 SCREENING ACTIVATED SLUDGE MICROBIOME FOR AZO DYE-CONTAINING WASTEWATER TREATMENTS

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ABSTRACT

The effects of azo dye-containing wastewater on the environment are devastating. Thus, it needs to be properly treated before its disposal - usually into water bodies. Activated sludge is an association among many (micro)organisms in a community, composed of aerobic and anaerobic bacteria and fungi dye-degrading (decolourization and mineralization). Nevertheless, there is a lack of information on specific degrading species and their interaction (microbiome). This information can significantly enhance the azo dye-containing wastewater treatments. Therefore, this study aimed to evaluate the bacterial community of an activated sludge sample from the real textile industry by isolating and identifying the Reactive Red 141-degrading bacteria strains. A wide range of bacteria species was identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), including aerobic (*Lysinibaciullus fusiformis*) and facultative anaerobic (*Escherichia coli*). Preliminary data indicated the azo-degrading potential of *Bacillus thuringiensis* and *Kosakonia radicincitans*. It is worth noting that this is the first report on the potential use of *K. radicincitans* for decolourization. Brain Heart Infusion (BHI), glucose, and RR-141 were used as carbon sources. However, only BHI and glucose systems lead to decolourization activity, indicating that RR-141 cannot be used as a carbon source. Both strains exhibited decolourization ability, reaching 43% decolourization in BHI by *B. thuringiensis*, and 21% in mineral medium with glucose by *K. radicincitans.* A yield above 40% was achieved by applying them simultaneously at the same reaction medium, at non-optimal conditions. Regarding phytotoxicity, the degradation result solutions did not promote the germination of the seeds, which can be associated with the formation of toxic aromatic amines. The HPLC-MS analyses proved that the decolourization process was carried out; however, very likely, due to microbial adsorption.

Keywords: biodegradation; reactive red 141; MALDI-TOF MS; toxicity; *Bacillus thuringiensis*; *Kosakonia radicincitans*.

4.1 INTRODUCTION

Global textile trade is expected to grow 4.4% between 2021 and 2028 (Grand View Research 2021). This continuous growth of textile products manufacture is connected with a wastewater production rate above 130 L per kg of produced fabric (Didier de Vasconcelos et al. 2021), which comprehends a diversity of contaminants and toxic constituents such as dyes, resulting in an effluent with high biological oxygen demand (BOD), chemical oxygen demand (COD), intensive colour, pH, salinity, and total organic carbon (TOC) (Lade et al. 2012; Bilińska et al. 2016).

Azo dyes are recalcitrant and highly water-soluble compounds widely used by the textile industry. They are chemically compost of nitrogen-to-nitrogen double bond (-N=N-) (Paździor et al. 2019). The incorrect disposal of textile effluents impacts the environment holistically, for instance, reducing the oxygen concentration in water bodies, as well as the sunlight penetration, with consequent reduction of photosynthetic activity of aquatic algae and plants, the formation of aromatic amines - potentially carcinogenic, mutagenic, among others (Paździor et al. 2017; Zhu et al. 2018).

Usually, the textile industry effluent treatment is composed of physical-chemical steps (coagulation/flocculation and decantation) followed by biological processes, mainly activated sludge (Manekar et al. 2014). In this sense, the activated sludge approach inherently generates approximately 500 kg of sludge per m3 of wastewater (Deraniyagala 2017) and does not act on azo dyes (Chung and Stevens Jr. 1993). In this context, the microbiome of activated sludge from wastewater treatment plants are being explored to identify isolated species with dye decolourization potential (Zhu et al. 2018; Zhang et al. 2018; Li et al. 2019a). These habitats suggest a significantly adapted bacterial group with a wide variety of azo dye degrading activity species, supporting the potential to find new and better azo dye decolourizing bacteria (Meerbergen et al. 2018). Exploring activated sludges as microbial consortia is a vital perspective to optimize biodegradation. However, the study of isolated strains from activated sludges enables the biodegradation pathways elucidation. MALDI-TOF-MS is highlighted as a promising methodology for activated sludge microbiome identification with consequently treatment yield enhancement (Didier de Vasconcelos et al. 2021).

Therefore, this study aimed to isolate and identify bacterial strains capable of decolourizing RR-141 azo dye from activated sludge of a treatment textile wastewater plant. The two most promising strains were used to investigate kinetic performance, decolourization ability through UV-VIS spectrophotometry, possible pathways by HPLC-MS analysis, phytotoxicity, and enzyme activity identification. The scanning electron microscope of the activated sludge sample was also executed.

4.2 MATERIAL AND METHODS

4.2.1 Chemicals

Reactive Red 141 (RR-141/RHE7B), acetonitrile (CH_3CN, Sigma-Aldrich), brain heart infusion (BHI, KASVI), dibasic potassium phosphate (K_2HPO_4, VETEC, anhydrous), ethanol (C_2H_6O, Sigma-Aldrich), magnesium sulfate ($MgSO_4 \cdot 7H_2O$, NUCLEAR), sodium chloride (NaCl, VETEC), formic acid (CH_2O_2, BIOTEC), α-cyano-4-hydroxycinnamic acid ($C_{10}H_7NO_3$, Sigma-Aldrich), trifluoroacetic acid (CF_3COOH,

ÊXODO CIENTÍFICA), barium chloride ($BaCl_2 \cdot 2H_2O$, DINÂMICA), methanol (CH_3OH, UV-IR-HPLC-HPLC isocratic Panreac), calcium chloride ($CaCl_2 \cdot 2H_2O$, NEON), magnesium sulfate ($MgSO_4 \cdot 7H_2O$, VETEC), sodium bicarbonate (NaHCO3, NUCLEAR), potassium chloride (KCl, LAFAN), dipotassium phosphate (K_2HPO_4, Sigma-Aldrich), monopotassium phosphate (KH_2PO_4, NUCLEAR), ammonium sulfate ($(NH_4)2SO_4$, NUCLEAR), glucose ($C_6H_{12}O_6$, MERCK) were used as chemicals, without any pre-treatment.

4.2.2 Scanning electron microscope (SEM)

Activated sludge from the secondary settling tank of a textile industry treatment plant located at Blumenau-SC, Brazil, was collected and transported to the laboratory at room temperature. The solid fraction of an activated sludge sample was separated from the liquid medium through centrifugation at 10,000 *g* for 5 min. A Pasteur pipette extracted the aqueous phase, and the remaining portion was forwarded to be observed in a scanning electron microscope (HITACHI TM3030).

4.2.3 Bacterial isolation

Homogenized biological sludge (4 $g.L^{-1}$) was added in a 37 $g.L^{-1}$ of Brain Heart Infusion (BHI) solution and incubated at 30 °C and 150 rpm for 24 h. Posteriorly, the samples were diluted, using a dilution factor of 10^{-6}, in a NaCl 0.85% solution. From this solution, 1 mL of each diluted sample was spread in BHI agar and grown at 30 °C for 24 h in an incubator. The isolated and different colonies were transferred to BHI agar tubes and grown at 30 °C for 24 h.

4.2.4 Microbiome identification

The incubated cells (Petri plate - BHI at 30 °C for 24 h) were transferred, with a pipette tip, to a 1.5 mL screw-cap extraction tube (Eppendorf, Germany) and wholly mixed with 0.3 mL of double-distilled water. Absolute ethanol (0.9 mL) was added, cautiously mixed, and the tubes were centrifuged for 2 min at 20,000 *g*. The supernatant was rejected. The precipitate was air-dried, mixed thoroughly with 50 µL of formic acid (70%), and, subsequently, 50 µL of acetonitrile. The mixture was submitted to centrifugation (20,000 *g*, 2 min). The supernatant (1 µL) was dried at room temperature on a ground steel MALDI target plate. The samples received an extra layer of 2 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid and dried at room temperature (Marklein et al. 2009). An UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) performed the mass spectrometry analysis at the linear positive ion mode. Mass spectra were obtained in a range from 2 to 20 kDa with ions generated by the irradiation of smartbeam using a frequency of 2000 Hz, PIE 100 ns, 7 kV lens (Alves et al. 2016). For the first and second ion sources, the voltages were 25 kV and 23 kV, respectively. MALDI Biotyper CA System software (Bruker Daltonics, Germany) was used to identify bacteria with cut-off values higher than 1.7 for species identification (Han et al. 2015).

4.2.5 Screening of dye-decolourizing in solid medium

The azo dye decolourization potential was evaluated by the streak plate method. Preculture broth (100 μL) of each culture was streaked on a solid medium of BHI (37 $g.L^{-1}$) and RR-141 (60 $mg.L^{-1}$) and incubated at 30 °C (Kiayi et al. 2019).

4.2.6 Screening of dye-decolourizing in the aqueous system

RR-141 (30 $mg.L^{-1}$) was added to BHI (37 $g.L^{-1}$) and, separately, mineral salt media (NaCl 5$g.L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.1 $g.L^{-1}$, K_2HPO_4 10 $mg.L^{-1}$, KH_2PO_4 1$g.L^{-1}$, and $(NH_4)_2SO_4$ 2$g.L^{-1}$) with 3$g.L^{-1}$ of glucose (MSG) aqueous solutions and inoculated with 10% (v/v) preculture ($\simeq 1\times10^9$ $cells.mL^{-1}$) into 250 mL Erlenmeyer flasks - working volume 120 mL - and then incubated at 30 °C, and 100 rpm during 7 days. Each sample was centrifuged at 10,000 *g* for 10 min, and its supernatant was analyzed by a UV/Vis spectrometer (Femto Cirrus 80) at $\lambda_{máx}$ = 516 nm. The removal of colour was obtained using the following equation: Decolourization (%) = $(ABS_0 - ABS_f) / ABS_0 * 100\%$, where ABS_f is the sample absorbance after 7 days, and ABS_0 is the initial system absorbance. Based on the absorbance reduction, strains that revealed the highest decolourizing potential were selected for further carrying out tests.

4.2.7 Carbon sources and kinetic study

Considering the two strains that better removed the colour, *B. thuringiensis* and *K. radicincitans,* Erlenmeyer experiments (in triplicate) were executed to confirm their azo dye decolourizing capability, evaluate differences in their decolourization rate and their performance isolated and at consortium study. The consortium was standardized in the same work volume, considering 10% (v/v) of inoculum (CFU $\simeq 1\times10^9$ $cells.mL^{-1}$ to each bacteria species). The bacteria decolourization kinetics were evaluated in three culture media: BHI, mineral salt media without glucose (MS, NaCl 5$g.L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.1 $g.L^{-1}$, K_2HPO_4 10 $mg.L^{-1}$, KH_2PO_4 1$g.L^{-1}$, and $(NH_4)_2SO_4$ 2$g.L^{-1}$), and mineral salt media with 3$g.L^{-1}$ of glucose (MSG). The pH, optical density, and decolourization (516 nm) were measured from 0 to 120 h.

4.2.8 Phytotoxicity assay

Lactuca sativa seeds were used for uniform and rapid germination (dos Santos et al. 2019). Before inoculation, crop seeds were cleaned, and surface sterilized with 99% ethanol solution for 5 min and then washed several times using sterilized distilled water. Sterile Petri dishes (Ø 90 mm) were covered with qualitative filter paper (Unifil®, 80 $g.m^{-2}$), previously sterilized. In each plate, 3 mL of the solution to be tested were added, and 10 seeds, equally spaced, on the filter paper. Tap water (TW) was used as positive control (Peduto et al. 2019). The Petri dishes were sealed and incubated (TECNAL TE-371, type BOD) at 30 °C. The germination and growth rate were analyzed daily for 7 days. The experiment was carried out in duplicate.

4.2.9 Biodegradation analysis

HPLC coupled to mass spectrometry detection (HPLC–MS) was performed with a C18 column (Shimpack XR-ODS 50 x 2.0 mm I.D.). The samples were prepared by precipitation with $BaCl_2$ followed by filtration. The eluents A (ultrapure water containing 1% formic acid) and B (methanol) served as mobile phase in an isocratic mode (30% A and 70% B). The samples were eluted at a flow rate of 0.05 $mL.min^{-1}$ and monitored at 370 nm. Nitrogen was used as the nebulizing gas (1.50 $mL.min^{-1}$), heated sheath gas, and drying gas (3 $L.min^{-1}$, 250 °C).

4.2.10 Enzyme detection

Isolated colonies of each bacterial culture were inoculated (needle) in Petri dishes containing BHI agar and RR-141 (60 $mg.L^{-1}$) and then incubated at 30 °C for 24 h. The halo formation diameters were calculated considering the difference between the total and the CFU diameter. The diameters were the result of two perpendicular axes measurement average (Barros et al. 2013).

4.3 RESULTS AND DISCUSSION

4.3.1 Scanning electron microscope (SEM)

The analysis of SEM micrographs of activated sludge indicated the presence of a biofilm matrix adhered, very likely flocculating bacteria. Some essential characteristics are related to the structure and function of biofilm, for example, organics compounds as extracellular polymeric substances (EPS), which play a significant role in modifying surface (charge, hydrophobicity) to give suitable conditions for bacterial connection (Holbrook et al. 2006; Tansel et al. 2006; Naz et al. 2013). Large amounts of EPS and different bacterial species can be found in biofilms (Weber et al. 2007). EPS is defined as a complex combination of high molecular weight microbial biopolymers. Its composition is based on humic substances, lipids, polysaccharides, proteins, and uronic acids. Its liquid anionic composition enables effective sequestration of positively charged species such as some dyes (Mohapatra et al. 2020). There are two primary forms of EPS: as a capsule bounded, covalently, to the cell surface, or as slime polysaccharides roughly associated with the cell surface, as detected in Figure 2. The EPS synthesis has been predictable in certain bacterial cultures (Bala Subramanian et al. 2010). Gram-negative bacteria, such as *E. coli*, have lipopolysaccharides as a significant component of the outer membrane, which strongly influences the microorganism adhesive aspect (Fletcher 1996). *Klebsiella* spp. can produce an adhesive slime (Bala Subramanian et al. 2010).
Biofilm formation may favour coloured effluent treatment. However, it is essential to highlight that a species that can form biofilm is not necessarily azo-degrading.

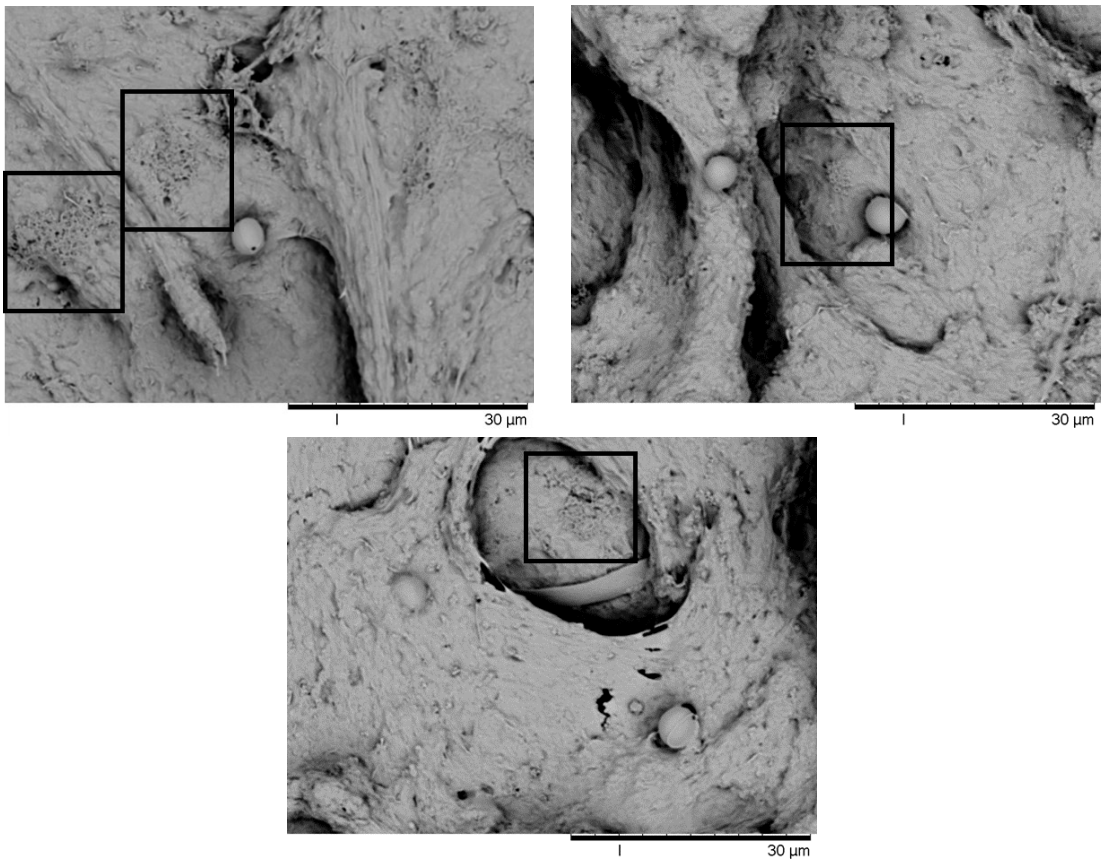

Figure 2: Scanning electron microscope of the activated sludge - biofilm formation.

4.3.2 Microbiome identification (MALDI-TOF-MS) and maintenance

The majority of microorganisms present in the biological sludge, used as the microbial source, are presented in Table 5. A documental database using the keywords/booleans "strain name" AND "decolourization" (Table 5) was carried out to identify scientific trends in the biological dye remediation context.

The most abundant strain present in the activated sludge is *Bacillus thuringiensis*, which presents a moderate number of reports associated with decolourization than *E. coli* and *B. cereus* species (widely used). Some detected bacteria, such as *Kosakonia* sp., have subtle correlations with decolourization; it should be further evaluated.

The isolated bacteria were maintained on BHI agar slants and 1.5 mL microcentrifuge tubes (Eppendorf, Germany) with a cryopreservative liquid (BHI Broth with glycerol, 2:8) and preserved at 20 °C for further assays.

Acinetobacter baumannii, *Klebsiella oxytoca*, and *Escherichia coli* are Gram-negative bacteria widely related to dye decolourization. *A. baumannii* aerobically decolourized two textile azo dyes - Reactive Blue and Reactive Black 5 - with 90% and 87% efficiency after 48 h (Sreedharan et al. 2021), and were also tested to decolourize Reactive black 5, Reactive blue 19, Reactive red 120, and Reactive red 198 reaching yields above 96% (Unnikrishnan et al. 2018; Ameenudeen et al. 2021). *K. oxytoca* promoted the highest decolourization potential of 69.68% for vat brown dye (Adebajo et al. 2017) and achieved simultaneous decolourization (83.8% within 24 h) and biohydrogen production (2.47 $mL.h^{-1}$) (Yu et al. 2017). Balraj et al. (2016) used *E. coli* to biodegrade methylene blue. The authors reported that 92.9% of dye removal. *E.*

coli spp. can also be applied simultaneously with other microorganisms such as *Pseudomonas putida* (Hilda Josephine and Sekar 2014)*, Enterobacter asburiae*, *E. ludwigii*, and *B. thuringiensis* with an excellent yield of over 96% (Haque et al. 2021).

Table 5: Microorganisms identified in the biological sludge and its associated studies with biodecolourization theme, detected by different journal directories.

Strain	Identification code	Score	DOAJ*	JSTOR*	Science Direct*	Scopus*	Springer*	Google Scholar*
Bacillus cereus	1	2.16	0	1	92	7	335	11,600
Klebsiella oxytoca	2	1.74	0	0	8	1	57	605
Bacillus thuringiensis	3	2.21	0	1	31	2	112	1,190
Kosakonia cowanii	4	1.96	0	0	1	0	1	11
Lysinibacillus fusiformis	5	1.81	0	0	4	1	17	198
Acinetobacter baumannii	6	2.16	0	0	10	1	43	676
Kosakonia radicincitans	7	1.86	0	0	0	0	0	7
Escherichia coli	8	1.75	7	1	318	29	1028	17,200

*Journals directories

Abo-State et al. (2017) evaluated the potential of some bacterial strains to decolourize the congo red azo dye. The results highlighted the Gram-positive *B. cereus* with the best yield (96.92%) obtained. *E. coli* was also used for Eriochrome Black T decolourization, in which it reached 92.34% of dye removal at optimal conditions (Uppala et al. 2019). *Bacillus thuringiensis*, a facultative anaerobic and Gram-positive bacterium, is promising dye-degrading species. However, there are only a few reports on its dye-degrading properties, including Remazol Black B, Reactive yellow 42, Reactive red 58, Reactive blue 13, and a real dyehouse effluent which achieved yields between 80 and 95% of decolourization (Olukanni et al. 2013; Joshi et al. 2014).

The Gram-positive bacterium *Lysinibacillus fusiformis* was used in a recent study to explore its discolouration capability over methyl red which provided the highest decolourizing of 96% (Sari and Simarani 2019). *Kosakonia radicincitans* and *Kosakonia cowanii* belong to the new genus *Kosakonia*, in which some strains were isolated from plants. However, some strains are being related to facultative human pathogens (Mertschnigg et al. 2020). *K. cowanii*, isolated from soil samples, was investigated in a consortium with *Pseudomonas seleniipraecipitans* for congo red decolourization, with a maximum percentage of 78.5% obtained (Krishnamoorthy et al. 2018). It is worth noting that there is no report on decolourization by *K. radicincitans*.

Individual assays were performed to determine which cultures have the most significant ability to degrade azo dyes and the necessity of consortia (synergistic effects) and enzyme study. With this, it was possible to select the best species and perform assays using one or a consortium of microorganisms for the azo dye degradation. This understanding is an opportunity to develop textile wastewater treatments and increase yields using strains with higher decolourization aptitude.

4.3.3 Screening of dye-decolourizing in solid medium

In biological processes, the bioavailability of enzymes interferes directly with the dye transformation, which can be performed extracellularly and intracellularly. Nevertheless, the most effective strategy involves extracellular degradation (Kandelbauer and Guebitz 2005); since azo dyes have complex structures, their diffusion through cell membranes is complex. This test is strictly related to extracellular enzyme production and action. The visual analysis of plates (Figure 3) indicated that *K. oxytoca*, *B. thuringiensis, K. cowanii,* and *K. radicincitans* colonies have the highest potential for decolourizing RR-141 (60 mg.L^{-1}) after 168 h of incubation at 30 °C. Moreover, the decolouration occurred primarily during 48 h of incubation. A similar trend was observed by Kiayi et al. (2019). The solid-plate test promoted total decolourization of carmoisine (50 mg/l) within 4 days by *S. cerevisiae* colonies, with no visual changes in the fifth and sixth days.

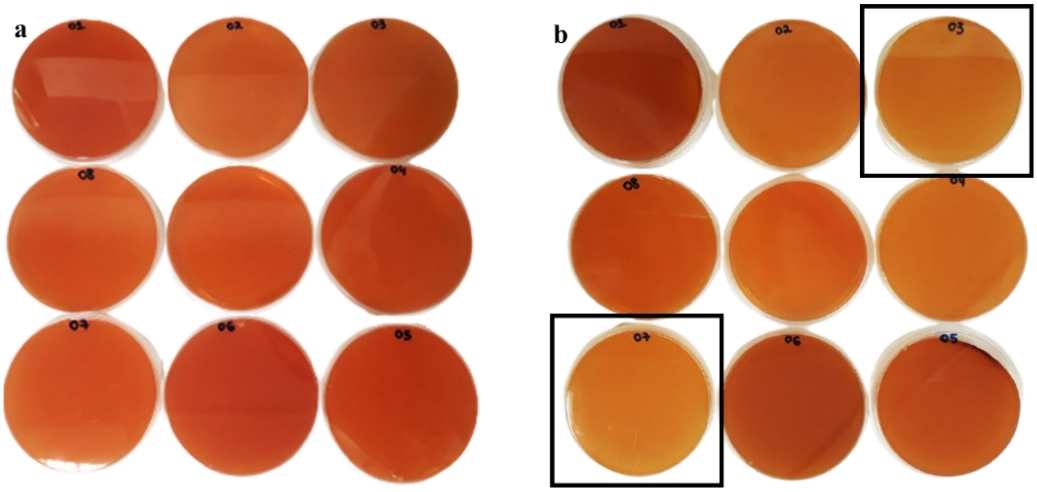

Figure 3: Decolourization potential study at (a) t = 0 h and (b) t = 168 h and the highest decolourized Petri dishes (highlighted).

4.3.4 Screening of dye-decolourizing in the aqueous system

Considering SEM micrographs of activated sludge, biofilm-producing bacteria is an important factor, since biofilm is an excellent means to retain microorganisms and improve their performance in environmental biotechnologies. Proteins and carbohydrates from EPS allow binds between the microbial biomasses and substrates, favouring their activities. In this context, it is worth noting that the diffusion effects are essential to reach the high yields of biodegradability. Considering that no biofilm was produced as an alternative to enhance the degradation process, the dye diffusion to the microorganism is hampered in this experiment. The BHI and MSG media were used to comprehend the preferred route, concerning the performances of the strains at a rich and turbid medium (BHI) and a less supplemented and more translucent medium (spectrophotometric evaluation). The analysis of results confirmed *B. thuringiensis* (Figure 4) as promising dye-degrading species. It also indicated that *K. radicincitans*, *B. cereus,* and *A. baumannii* as potential

strains for the RR-141 degradation. Added to the solid medium assay results and considering the number of reports related to each species (Table 5), *B. thuringiensis* (B) and *K. radicincitans* (K) were chosen for further studies.

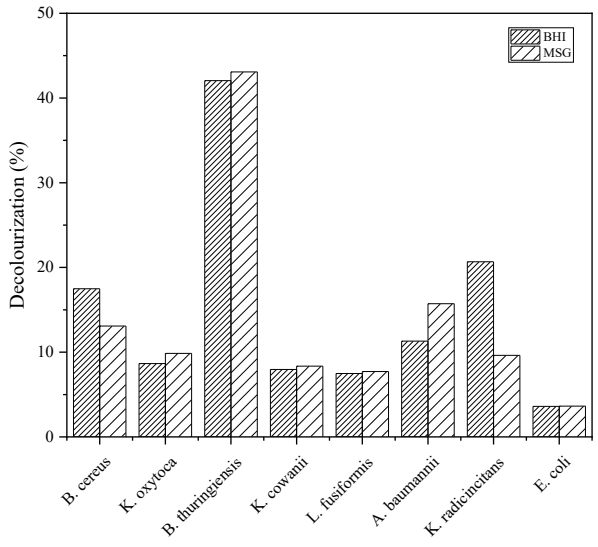

Figure 4: Decolourization yields of each isolated culture in BHI and MSG media.

4.3.5 Carbon sources and dye-degrading kinetic by *B. thuringiensis* or *K. radicincitans* - enzymatic or metabolic degradation

Considering the hypothesis of media composition influence in the degradation process, BHI, MSG, and MS were used to evaluate the kinetic and behaviour of each bacterial culture about carbon source, enzyme production, and spectrophotometric interferences.

Cell growth expressed by the optical density at 600 nm (OD600) plus pH revealed the bacteria growth at, as expected, the BHI as the most appropriate medium for the development of both strains. No relevant modification was detected in the MS medium (absence of carbon source, except azo-dye), which could indicate the lack of ability of the cultures to use the dye as a primary carbon source (Figure 5). At the microbial level, the azo dye biodegradation mechanism is controlled by enzymes (Saratale et al. 2007). Considering the results from solid-state media, enzyme interference can be regarded as in decolourization processes. Azoreductases, laccases, and peroxidases are the enzymes frequently related to azo dye decolourization (Shah et al. 2016; Mullai et al. 2017; Chandanshive et al. 2018; Chen et al. 2018). Azoreductase action is through reduction mechanisms mediated by a flavoprotein in the microbial electron transport chain (Misal and Gawai 2018), which converts azo dyes into colourless products (aromatic amines) (Pandey et al. 2007). Laccases action can be by direct or indirect oxidation (Khlifi et al. 2010) through a nonspecific free radical mechanism that results in phenolic products and avoids the formation of aromatic amines (Wong and Yu 1999). The peroxidase mechanism comprises the phenolic group oxidation and the production of a radical close to the azo linkages (Chivukula and Renganathan 1995). Enzyme action comprehension and identification at each metabolism stage are essential to improve the degradation process and are indicated for further studies. From the kinetic analysis, *Bacillus* strain had a better performance at

mineral salt media (38%), while *K. radicincitans* achieved the highest yield at BHI medium (26%). Visual decolourization perception at the final point confirmed no changes at MS medium, besides highlighted the better performance of *B. thuringiensis* at both media (Figure 6).

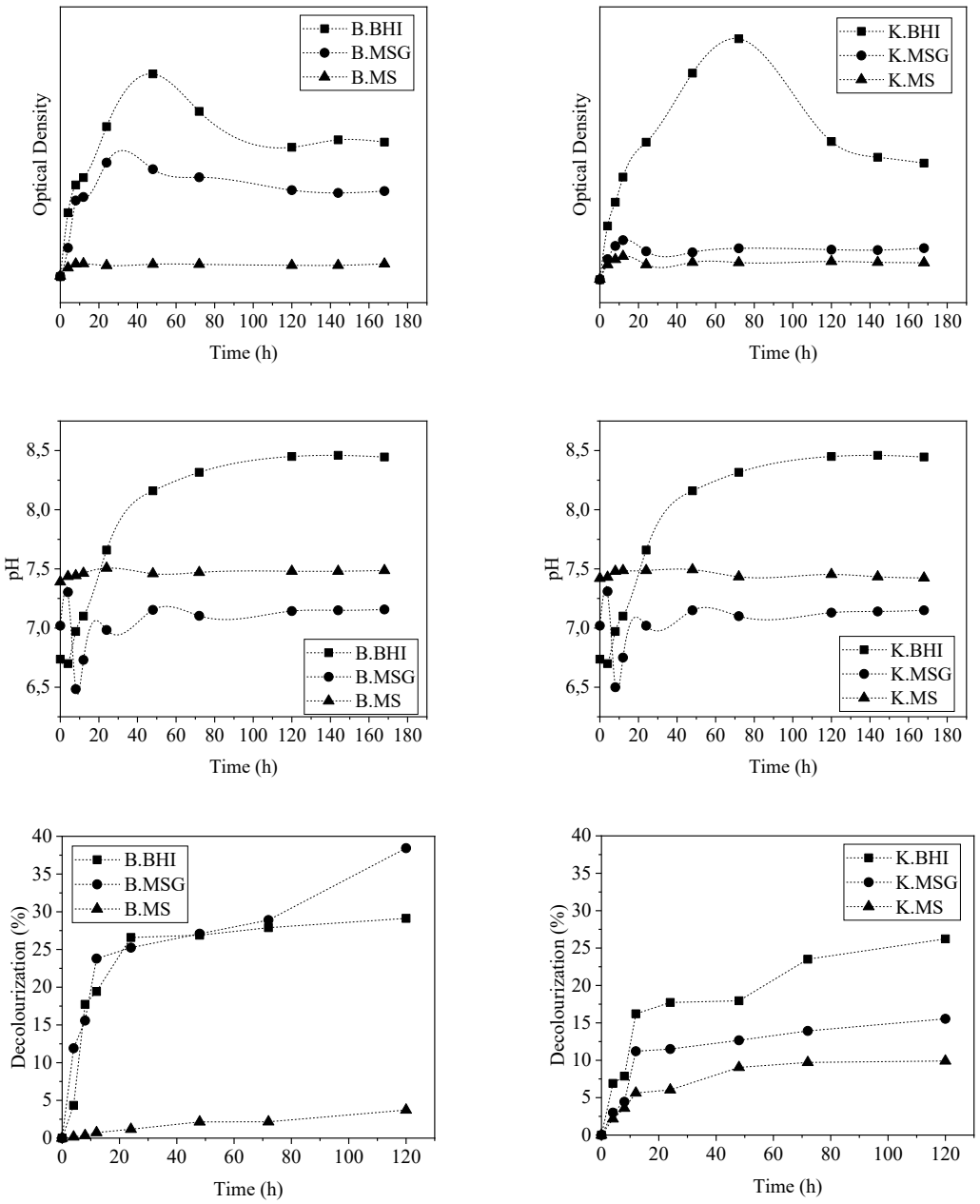

Figure 5: Growth rate, pH and decolourization rate of the systems with *Bacillus thuringiensis* and *Kosakonia radicincitans* at the three studied media.

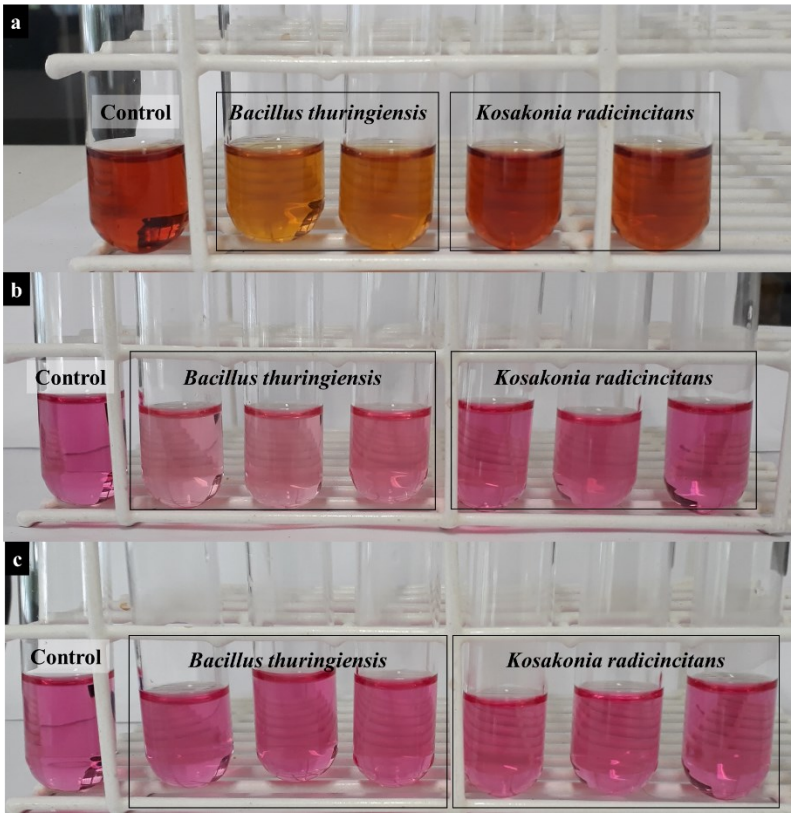

Figure 6: Samples of the decolourization result at BHI (a), MSG (b), and MS (c) media for both isolated cultures.

Since single isolated culture presented exciting perspectives to be explored, the experimentation of bacterial consortium was realized to verify the viability of higher yields and better processes.

4.3.6 Dye-degrading kinetic by *B. thuringiensis* and *K. radicincitans* as a consortium

Degradation of azo dyes is usually performed by a consortium of species. It is expected that the co-metabolism of a microbial community promotes better conditions and results for biodegradation and mineralization of azo dyes. Some microbial consortia had achieved yields and objectives that no individual strain could reach successfully in biodegradation studies (Neifar et al. 2019; Shindhal et al. 2021; Samuchiwal et al. 2021). Besides, mixed culture evaluations are more similar to practical situations since aseptically conditions can add more cost in the process, higher stability over environmental stress such as composition, pH, or temperature variations. The synergistic action can occur by different pathways: i) a microorganism causes dye biotransformation, rendering it more reachable to another organism that would not be able to act on this dye at its original state; ii) an only microorganism promotes some solution decolourization by the modification of the chromophore; however, the complete degradation is not achieved, and the metabolic products may have a toxic nature, as at an anaerobic reduction of azo dyes. Once this unwished metabolite is taken as a nutrient source by another microorganism, the complete degradation, leading to carbon dioxide, ammonia, and water, can be achieved (only by mixed populations).

This mineralization reaction assures no potentially harmful degradation products are released into the environment (Kandelbauer and Guebitz 2005).

In this study, the consortium evaluation revealed a well-development at both media, with MSG promoting better conditions for higher decolourization yield (Figure 7). Compared to the isolated kinetic study, the decolourization process achieved better results in a minor period, with approximately 36% of decolourization yield within 12 h, associated with a synergic effect.

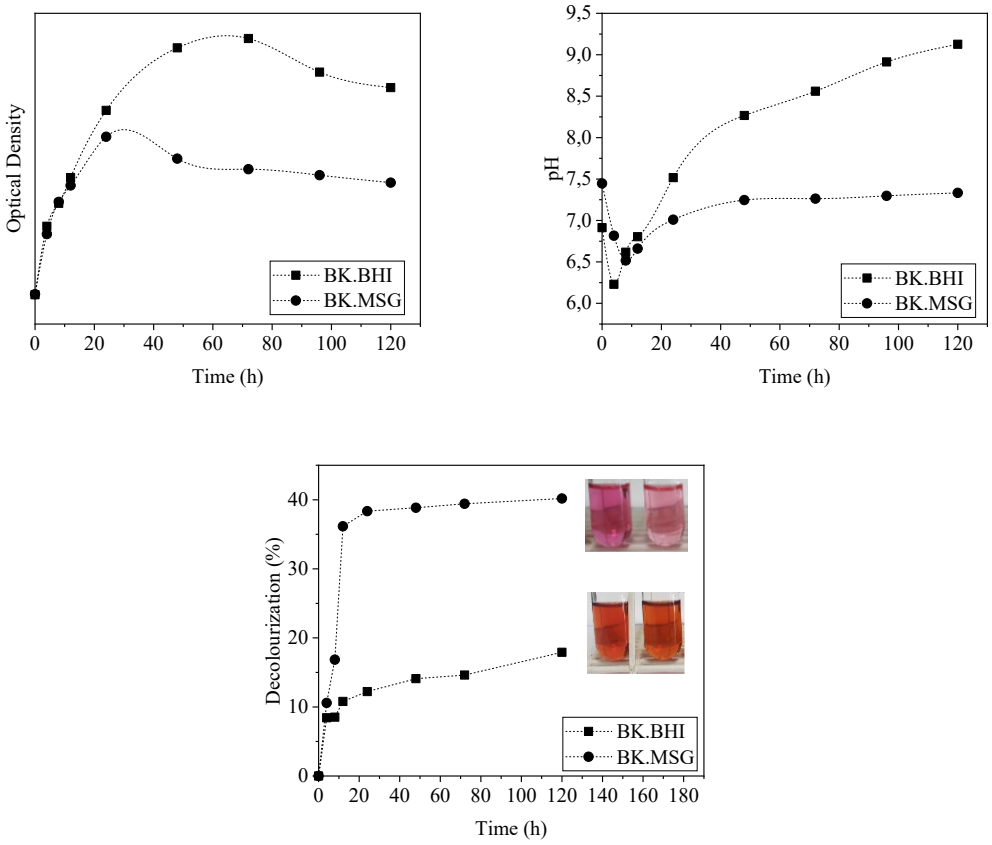

Figure 7: Microbial growth, pH, decolourization rates, and picture of the consortia system at BHI and MSG media.

It is not easy to reproduce and effectively interpret the results when using mixed cultures because it only provides a wide view of what is happening in the system, difficult the identification and quantification of individual culture growth, hampers the elucidation of the degradation mechanism (Didier de Vasconcelos et al. 2021). For these reasons, the use and deep comprehension of colour removal by single bacterial cultures are essential since it promotes a more straightforward interpretation of experimental observations and reproducibility (Thakur et al. 2014).

4.3.7 Phytotoxicity assay

Phytotoxicity assays are expected to present a reduction in toxic levels with the coloured wastewater. Some negative results in toxicity assays are related to the formation of some toxic substances, such as aromatic amines, that can act as an inhibitory factor (Tabasum et al. 2019; Carvalho et al. 2020; Didier de

Vasconcelos et al. 2021). Nevertheless, some of these experiments reveal that an excellent decolourization index is not necessarily associated with completing degradation processes.

The analysis of phytotoxicity (Figure 8) revealed the toxic nature of Reactive Red 141 to the *Lactuca sativa* seeds. Germination rate was lower with Reactive Red 141 (60%, i.e., 6 of 10 seeds germinated, on average) when compared to tap water (80%, i.e., 8 of 10 seeds germinated, on average). The Reactive Red 141 degradation products were significantly inhibitory for the germination of the plants, which indicates the formation of phytotoxic compounds. Azo dyes and some of their metabolites present carcinogenic, toxic, and mutagenic properties to the environment and humankind (Kalme et al. 2007). Bacterial azo dye degradation comprises the cleavage of azo bonds and the intermediates breakdown. However, three mechanism routes promote the carcinogenic activation of azo dyes: i) direct oxidation of azo linkage to diazonium salts with highly reactive electrophilic behaviour; ii) oxidation of azo dyes in the presence of structures formed by free aromatic amine groups; and iii) reduction and cleavage of the azo bond with consequent formation of aromatic amines (Brown and De Vito 1993; Chung 2000).

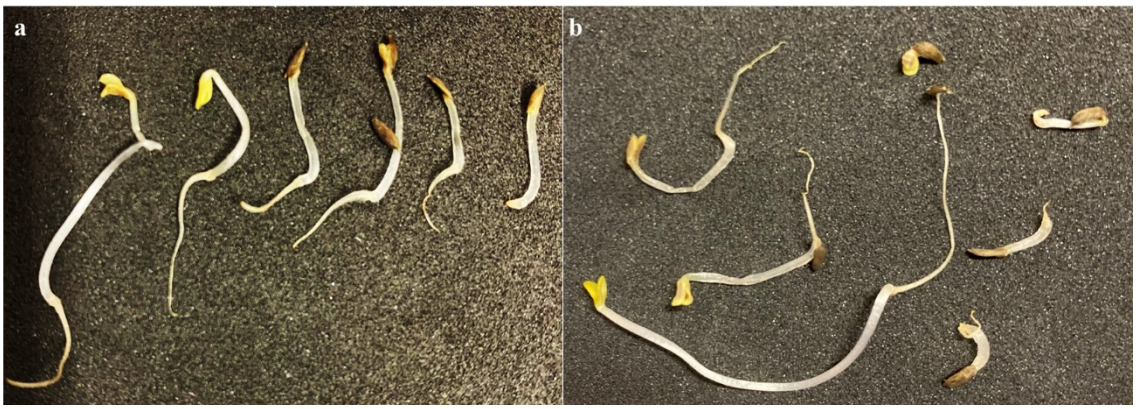

Figure 8: Phytotoxicity experiments: (a) in RR-141 solution, (b) tap water.

4.3.8 Biodegradation analysis

The HPLC-MS analysis was performed to confirm the dye quantity reduction. The chromatograms of dye solutions at the initial and after each studied media treatment were obtained (Figure 9). The HPLC profile of the RR-141 solution in both media exhibited a single peak at retention times of 4.53 and 4.63 min to BHI and MSG, respectively (matrix effect - chromatography). The assay results of the solution after treatment exposed peaks corresponding to RR 141 with reduced intensity, which indicates that the compound was not completely degraded. The absence of different peaks in the chromatograms of treated solutions contributes to a possible adsorption strand by the biomass generated, which can be observed in Figure 10 that shows the concentrated biomass after a centrifugation process for some studied strains (Pearce et al. 2003; Rodrigues et al. 2014; Siddiqui et al. 2018). Once adsorption seems to have a more significant influence on the decolourization phenomena, another speculation can be done relating the effect of culture media and the biomass composition and adsorption, consequently.

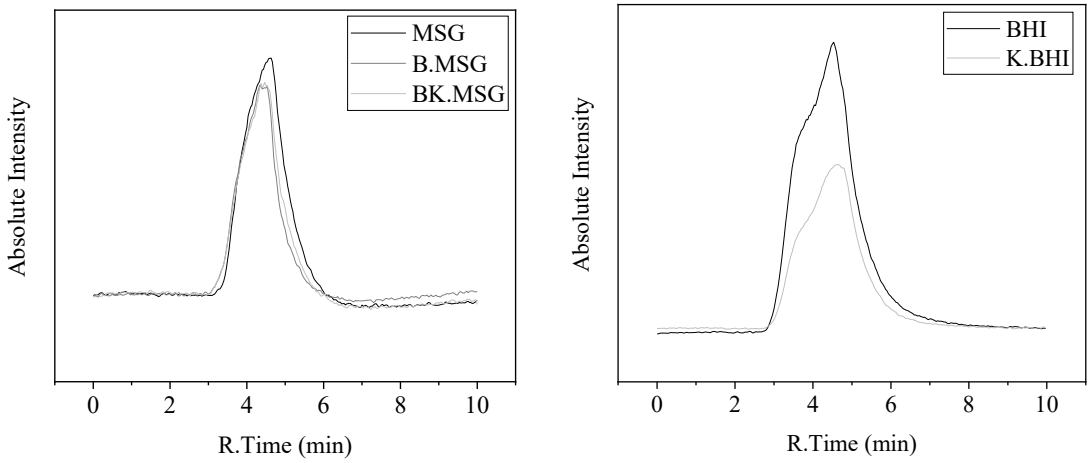

Figure 9: Chromatograms of RR-141 solutions in both media (MSG and BHI) studied and their degraded systems samples (B.MSG, BK.MSG and K.BHI).

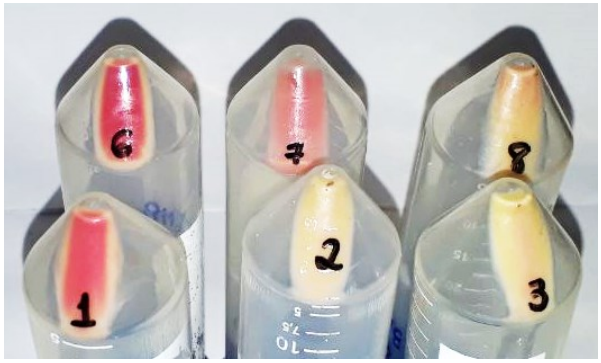

Figure 10: Biomass of resultant treatment solutions and the adsorbent behaviour of some of them.

4.3.9 Enzyme identification

All tested strains showed, after 24 h, a halo formation represented by a more translucid area surrounding the CFU (Figure 11) that could be associated with extracellular enzyme production (Barros et al. 2013). Considering the halos measurement (Table 6), *K. radicincitans* presented the best results, which suppose good development (Figure 5, OD graphic) and consequent enzymatic activity. In addition, the cell growth on dye supplemented BHI agar medium with white colonies aspect can be associated with dye decolouring potential. Since cell mat colouring results from dye biosorption, the maintenance of the original mat colour indicates biodegradation by an enzymatic process (Karim et al. 2018).

Oxidative and reductive enzymes are considered vital parts in azo dye biodegradation (Mahmood et al. 2016). Azo dye remediation intermediated by enzymes can be intra or extracellular. However, the high complexity of azo dyes hamper their diffusion through cell membranes, so the preferable route is by the enzyme release in the extracellular environment (Sari and Simarani 2019). Microbial strains used in the decolourization process must have efficient enzymes and a transport system to permit the absorption of dyes in cells (Mahmood et al. 2016). Enzymes improve the reductive cleavage of azo bonds, producing intermediate metabolites posteriorly degraded by aerobic or anaerobic mechanisms (Pandey et al. 2007).

Azoreductases, laccases and peroxidases are the enzymes often related to azo dye discolouration. The action of azoreductase is through reduction mechanisms mediated by a flavoprotein in the microbial electron transport chain (Misal and Gawai 2018), which converts azo dyes into colourless products (aromatic amines) (Pandey et al. 2007). The laccases act either by direct or indirect oxidation (Khlifi et al. 2010), through an unspecific free radical mechanism that results in phenolic products and prevents the formation of aromatic amines (Wong and Yu 1999). The peroxidase mechanism comprises the oxidation of the phenolic group and the production of a radical close to the azo bonds (Chivukula and Renganathan 1995).

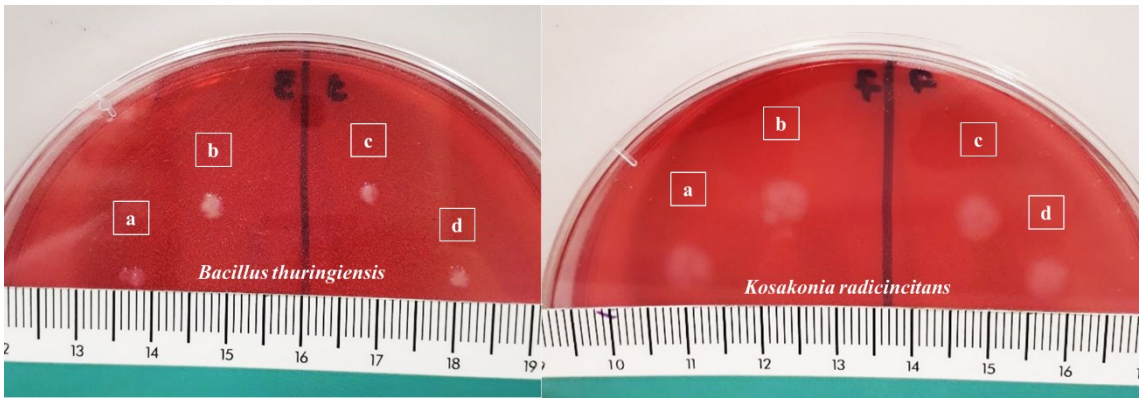

Figure 11: Enzyme activity detection assay by the halo formation.

Table 6: Measurements of the colony-forming unit and its halo for each bacteria species in the enzyme activity assay.

	Bacillus thuringiensis					***Kosakonia radicincitans***				
	a	b	c	d	Average	a	b	c	d	Average
Øcolony (cm)	0.310	0.275	0.180	0.235	0.250	0.570	0.545	0.580	0.500	0.549
Øcolony+halo (cm)	0.395	0.370	0.285	0.305	0.339	0.750	0.645	0.690	0.615	0.675
Øhalo (cm)	0.085	0.105	0.105	0.070	0.091	0.180	0.100	0.110	0.115	0.126

4.4 CONCLUSIONS AND FUTURE PERSPECTIVES

Coloured textile wastewater can negatively impact the environment when disposed incorrectly. Bacterial species isolated from the textile industry (activated sludge) were identified and tested for decolourization, considering dye solutions with BHI, mineral salt medium with and without glucose as culture medium. As a result, the bacterial isolates did not utilize the azo dye as their sole energy source and achieved exciting yields. *Kosakonia radicincitans* and *Bacillus thuringiensis* presented the bests decolourization results. The kinetic study revealed 21% in mineral medium with glucose by *K. radicincitans*, and 43% of decolourization in BHI by *B. thuringiensis.* The use of both cultures in the same reaction medium (MSG) promoted approximately 40% decolourization, indicating the potential use of mixed bacterial cultures as a bioremediation agent for cost-effective dye removal from textile effluent. Phytotoxicity assay and HPLC-

MS analyses suggested that the degradation was not complete. In addition, a potential enzyme action was inferred by the translucid halo formation observed, which can be associated with extracellular enzyme production. This work is believed to be an important contribution to the biodegradation of azo dyes by bacterial cultures isolated from activated sludge from the textile industry theme. Besides, the innovation in the utilization of *Kosakonia* sp. as a potential degradation agent.

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