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Biodegradation of Toxic Dyes using a Multi-Microbial System with *Pseudomonas putida* and *Lysinibacillus sphaericus* Consortium and Pathway Elucidation for Acid Yellow 42, Reactive Red 198 and Reactive Black 5

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ABSTRACT

Azo dyes, including Acid Yellow 42 (AY), Reactive Red 198 (RR), and Reactive Black 5 (RB), are persistent environmental contaminants known for their toxicity and resistance to conventional treatment methods. In this study, a co-culture of *Pseudomonas putida* and *Lysinibacillus sphaericus* was employed to investigate the biodegradation potential of these structurally diverse azo dyes. The microbial system was cultivated in minimal salt medium (MSM) under optimized conditions, demonstrating complete degradation of 50 ppm dye concentrations within 24 hours and 100 ppm within 48 hours at pH 7.2 and 34 ± 0.3 °C under continuous shaking at 180 rpm. Enzymatic profiling confirmed the active involvement of azoreductase, laccase, and NADH-DCIP reductase, suggesting a multi-enzyme facilitated degradation mechanism. To precisely map the transformation of dye molecules, electrospray ionization mass spectrometry (ESI-MS) was employed as the primary analytical tool. ESI-MS enabled the identification of intermediate metabolites formed during the degradation process, which were subsequently used to construct detailed biochemical degradation pathways for AY, RR, and RB. The strategic use of mass spectrometry-based metabolite tracking presents a powerful alternative to traditional analysis methods, offering direct insights into the molecular breakdown of complex pollutants. This work highlights a sustainable, enzyme-driven, and analytically streamlined route for azo dye degradation, with potential application in industrial wastewater bioremediation.

Keywords: *toxicity, co-culture, laccase, ESI-MS, wastewater bioremediation*

1. INTRODUCTION

The global expansion of textile industries has intensified the release of synthetic dyes into water bodies, introducing a class of industrial pollutants that are chemically diverse and biologically resistant. Azo dyes, which make up a significant portion of all synthetic dyes produced annually, have become particularly problematic due to their structural complexity and environmental persistence. Among these, Acid Yellow 42 (AY), Reactive Red 198 (RR), and Reactive Black 5 (RB) are widely utilized for their vivid color profiles and application efficiency across various fiber types. However, the same chemical features that make these dyes industrially valuable—namely their azo linkages and sulfonated aromatic backbones—also make them extremely recalcitrant in natural ecosystems[1].

Acid and reactive dyes represent two distinct yet equally challenging dye classes in wastewater treatment. Acid dyes are primarily applied to protein-based textiles and are characterized by their water solubility and affinity for ionic bonding. Reactive dyes, designed for cellulose-rich fabrics, exhibit strong covalent binding capabilities but suffer from low fixation efficiency, resulting in high levels of dye loss during the dyeing process. Effluents containing these dyes often carry high chemical oxygen demand (COD), intense coloration, and bioactive degradation products—factors that contribute to aquatic toxicity, ecosystem disruption, and long-term ecological risk[2].

Although traditional treatment approaches such as flocculation, oxidation, and adsorption are commonly used, they are frequently inefficient for complete dye mineralization and often lead to the generation of toxic secondary waste. In recent years, biological degradation has emerged as a compelling alternative. Bacterial consortia, in particular, offer the advantage of metabolic diversity, enabling the breakdown of structurally varied and chemically stable dyes through cooperative enzymatic action[3]. The global expansion of textile industries has intensified the release of synthetic dyes into water bodies,

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The use of multi-species microbial systems has shown promising results in tackling the limitations of single-species degradation. Enzymes such as azoreductases, oxidases, and dehydrogenases, when secreted in concert by different bacterial strains, can attack a wide range of dye bonds and intermediate compounds. Yet, despite numerous studies highlighting decolorization efficiency, the precise metabolic breakdown routes of many dyes remain underexplored—particularly when it comes to resolving the identity and fate of degradation intermediates[4].

In this study, we focus on a bacterial co-culture composed of *Pseudomonas putida* and *Lysinibacillus sphaericus*, chosen for their complementary enzymatic profiles and high tolerance to dye stress. Rather than relying on conventional monitoring tools, we apply electrospray ionization mass spectrometry (ESI-MS) as the central technique to map the degradation dynamics of AY, RR, and RB. Through mass spectrometric tracking of molecular intermediates, this work establishes definitive degradation pathways for each dye, offering new insights into how microbial systems process complex xenobiotics. The objective is not merely to achieve decolorization, but to clarify the biochemical transformations underpinning complete degradation—thus advancing the design of targeted, mechanism-driven bioremediation strategies[5].

2. MATERIALS AND METHODS

2.1 Chemicals and Analytical Reagents

The azo dyes selected for this study included Acid Yellow 42 (CI No. 18950, chemical formula: $C_{16}H_9N_4Na_3O_{10}S_3$, molar mass: 635 g/mol), Reactive Red 198 (CI No. 18221, chemical formula: $C_{27}H_{18}ClN_7Na_4O_{15}S_5$, molar mass: 986 g/mol), and Reactive Black 5 (CI No. 20505, chemical formula: $C_{26}H_{21}N_5Na_4O_{19}S_6$, molar mass: 991 g/mol). These dyes were employed to assess the degradation capacity of the microbial co-culture. The chemical reagents used during the dye degradation process included glucose as the carbon source and nutrient broth for bacterial growth. For the ESI-MS analysis, the following chemicals were used: methanol (HPLC grade), acetonitrile (HPLC grade), and water (HPLC grade) were used as solvents for sample preparation. Formic acid (99%) was employed to aid in the ionization process during the mass spectrometry analysis. These solvents and reagents facilitated the ionization of metabolites and intermediates formed during the dye degradation process, allowing for detailed identification of molecular structures and degradation pathways[6].

2.2 Preparation of Dye Solutions for Laboratory Degradation Trials

To initiate controlled degradation experiments, three target azo dyes—Acid Yellow 42 (AY), Reactive Red 198 (RR), and Reactive Black 5 (RB)—were selected for their industrial relevance and structural diversity. For each dye, stock solutions were formulated at two defined concentrations: 50 mg/L and 100 mg/L. High-purity distilled water served as the solvent to maintain sterility and avoid interference from residual ions or impurities. Once prepared, the dye solutions were stored at 4 °C in amber containers to prevent photodegradation and maintain chemical stability prior to use in microbial assays[7].

2.3 Microbial Strains and Culture Preparation

The bacterial strains *Lysinibacillus sphaericus* (MTCC 9523) and *Pseudomonas putida* (MTCC 2445) were sourced from the Microbial Type Culture Collection (MTCC), Chandigarh. To enhance their capacity for dye decolorization, both strains were routinely cultured in nutrient broth according to the standard protocols provided by MTCC. For the cultivation, a small inoculum from the frozen stock was transferred into a 250 ml Erlenmeyer flask containing 100 ml of nutrient broth. The culture was incubated at 30 °C with continuous agitation at 150 rpm for 24 hours. After incubation, the resulting bacterial suspension was harvested and used as the inoculum for all subsequent dye decolorization experiments involving AY, RR, and RB[8].

2.4 Optimization for Dye Decolorization and Degradation using Response Surface Methodology (RSM)

A structured optimization approach was implemented to maximize the decolorization and degradation of AY, RR, and RB, employing Response Surface Methodology (RSM) with a Central Composite Design (CCD). The study focused on evaluating the influence of three key variables: temperature (26–34 °C), pH (5.0–7.0), and agitation speed (100–200 rpm). Glucose was chosen as the carbon source due to its affordability and simplicity, while nutrient broth was used to provide essential nitrogen for bacterial growth, in accordance with MTCC guidelines. Given that azo dye concentrations in textile effluents are typically found in the range of 50–90 ppm, a working concentration of 50 ppm was initially used for the experiments, representing a realistic concentration of pollutants in industrial wastewater. Additionally, to test the robustness of the microbial co-culture, a higher dye concentration of 100 ppm was also included, simulating more severe contamination conditions typically encountered in heavily polluted environments[9].

2.5 ESI-MS Analysis

After 120 hours of incubation, the dye-degraded samples were centrifuged at 5000 rpm for 10 minutes to separate any remaining biomass. The clarified supernatant was then analyzed using Electrospray Ionization Mass Spectrometry (ESI-MS) to identify the metabolic byproducts formed during the degradation process[10]. A 2 µL portion of each sample was directly injected into the mass spectrometer for analysis, with water as the mobile phase to enhance ionization efficiency. The ESI-MS analysis was conducted under both positive and negative ionization modes to ensure a comprehensive metabolite profile. The instrument was configured with a capillary voltage of 4000 V, an injection voltage of 4500 V, a capillary temperature of 300°C, and a drying temperature of 320°C. Additional parameters included a nebulizer pressure of 35 psi, a dryer flow rate of 9.5 L/min, and a scanning range of 50–1200 m/z, with each scan completed in 1 second. The multiplier voltage was set to 1100 V, and helium was introduced as the collision gas at a flow rate of 10 ml/min. The acquired mass spectra were analyzed to examine the molecular structures and fragmentation patterns of the degraded dye metabolites, providing insights into their transformation pathways[11].

3. RESULTS AND DISCUSSIONS

3.1 Decolorization and Degradation Condition Optimization

The microbial strains *Lysinibacillus sphaericus* and *Pseudomonas putida* were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, and optimized for the decolorization of AY, RR, and RB. To ensure their metabolic activity, the bacterial strains were regularly enriched in nutrient broth. The cultures were grown in nutrient broth at 30°C for 24 hours with continuous shaking at 180 rpm to prepare inocula for decolorization experiments. Optimization of the degradation conditions was performed using Response Surface Methodology (RSM) with a Central Composite Design (CCD), focusing on the influence of temperature, pH, and agitation speed. A total of 20 experimental trials were conducted, and the results were analyzed using a second-order polynomial model. The CCD model predicted that optimal decolorization efficiency for all six experimental sets (AY50CO, AY100CO, RR50CO, RR100CO, RB50CO, RB100CO) would be achieved at 32°C, pH 5.5, and 120 rpm. These optimized conditions were validated through confirmatory experiments, which demonstrated over 90% decolorization efficiency within 120 hours. This result confirmed the robustness of the RSM-CCD model and the reliability of the predicted conditions. All subsequent decolorization experiments were carried out under these optimized parameters to maximize degradation efficiency[12].

3.2 Analysis of AY, RR, and RB Degradation Pathway by *Pseudomonas putida* and *Lysinibacillus sphaericus* Consortium

3.2.1 ESI-MS Analysis

The degradation products of AY, RR, and RB were extracted and examined through Electrospray Ionization Mass Spectrometry (ESI-MS) to determine the metabolic intermediates formed during the biodegradation process. The breakdown of these dyes commenced with the cleavage of the azo bond, which is a crucial step in their degradation.

For AY, an eluted product at RT 3.45 min exhibited a molecular ion peak at m/z 195.25, corresponding to 4-Methyl-4'-aminobiphenyl (Product 1), suggesting the transformation of the dye into aromatic amines. Another detected compound, 4-Amino-3-methylbenzenesulfonic acid (m/z 187.22, Product 2), indicated sulfonated intermediate formation. Further oxidation yielded products such as 4-Methyl-4'-aminobiphenylsulfonic acid (m/z 249.30, Product 3), 4-Hydroxybenzenesulfonic acid (m/z 174.18, Product 4), and 4-Hydroxy-3-methylbenzenesulfonic acid (m/z 188.20, Product 5).

In the case of RR, azo bond cleavage resulted in the formation of 4-Aminobenzenesulfonic acid (m/z 173.19, Product 6) and 3-Amino-5-chloro-1,2,4-triazine (m/z 147.55, Product 7). Hydroxylation led to the emergence of 4-Amino-3-hydroxybenzenesulfonic acid (m/z 189.18, Product 8). Another metabolite, 2-Naphthalenesulfonic acid, 4-hydroxy-7-[(4-methoxyphenyl) amino] (m/z 317.36, Product 9), pointed to oxidative modifications. The detection of 3,5-Dihydroxy-1,2,4-triazine (m/z 129.09, Product 10) and 4-Hydroxy-3-hydroxybenzenesulfonic acid (m/z 190.17, Product 11) confirmed progressive fragmentation into smaller compounds.

For RB, initial cleavage of the azo bond produced intermediates such as 4-Amino-5-hydroxy-2,7-naphthalenedisulfonic acid (m/z 316.32, Product 12) and 4-Amino-5-hydroxy-1,3-benzenedisulfonic acid (m/z 269.20, Product 13). Further oxidation led to 5-Hydroxy-2,7-naphthalenedisulfonic acid (m/z 302.26, Product 14) and 5-Hydroxy-1,3-benzenedisulfonic acid (m/z 255.19, Product 15). The final metabolic products, including Maleic acid (m/z 116.07, Product 16), Succinic acid (m/z 118.09, Product 17), and Fumaric acid (m/z 116.07, Product 18), suggested extensive breakdown of the dye molecules.

The ESI-MS spectra of the dye-degraded samples (AY50CO, AY100CO, RR50CO, RR100CO, RB50CO, RB100CO) were obtained to identify the molecular masses of the metabolic byproducts formed during the degradation process. The spectra (Figures 1) provide insights into the molecular mass and fragmentation patterns of the metabolites, helping to elucidate the transformation pathways of the azo dyes[13].

The degradation process was driven by enzymatic activity, beginning with azoreductases that catalyze azo bond cleavage to generate aromatic amines. The presence of hydroxylated and desulphonated intermediates implied the role of peroxidases in oxidative degradation. Additionally, hydrolases contributed to the breakdown of larger intermediates into smaller organic acids. The involvement of catechol dioxygenases further supported the aerobic cleavage of aromatic rings, facilitating the eventual mineralization of the dye structures.[12] The identification of low-toxicity intermediates such as succinic acid and maleic acid suggests that the degradation process ultimately leads to the formation of compounds that can be further metabolized into CO_2 and H_2O, ensuring complete mineralization of the dyes.

3.3 Proposed Mechanism underlying the Biodegradation of AY, RR, and RB by *Pseudomonas putida* and *Lysinibacillus sphaericus* Consortium

According to the identified intermediates and degradation pathways, the detailed molecular degradation mechanisms for AY (Figure 2), RR (Figure 3), and RB (Figure 4) are proposed based on the enzymatic transformations observed in *Pseudomonas putida* and *Lysinibacillus sphaericus*. The degradation process of all three dyes begins with the reductive splitting of azo bonds by azoreductase enzymes, resulting in the production of aromatic amine compounds.

For AY, azoreductase-mediated cleavage results in the formation of 4-Methyl-4'-aminobiphenyl and 4-Amino-3-methylbenzenesulfonic Acid, which undergo hydroxylation catalyzed by peroxidases to form 4-Hydroxybenzenesulfonic Acid and 4-Hydroxy-3-methylbenzenesulfonic Acid. The oxidation of these intermediates leads to the breakdown of the biphenyl core, forming 4-Methyl-4'-hydroxybiphenylsulfonic Acid, which is further degraded by hydrolases into smaller organic acids, ultimately yielding Maleic Acid, Succinic Acid, and Fumaric Acid as final mineralization products.

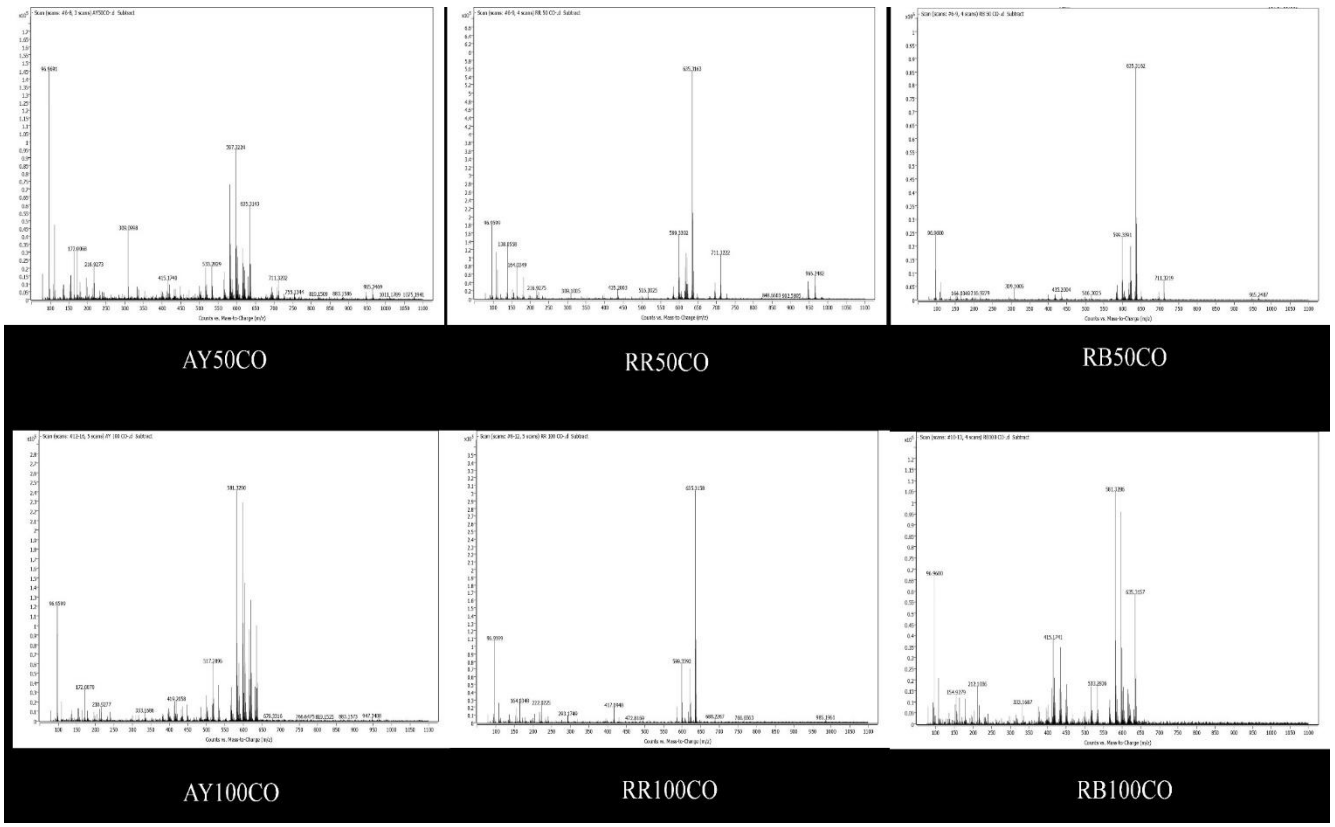

Figure 1. ESI-MS spectra of AY50CO, AY100CO, RR50CO, RR100CO, RB50CO, and RB100CO showing the molecular masses of degradation byproducts.

A similar mechanism is observed for RR, where azoreductase action generates 4-Aminobenzenesulfonic Acid and 3-Amino-5-chloro-1,2,4-triazine, which are hydroxylated by peroxidases to form 4-Amino-3-hydroxybenzenesulfonic Acid and 2-Naphthalenesulfonic acid, 4-hydroxy-7-[(4-methoxyphenyl) amino]. Further oxidation produces 4-Hydroxybenzenesulfonic Acid and 3,5-Dihydroxy-1,2,4-triazine, and subsequent ring-cleavage reactions lead to the formation of Maleic Acid, Succinic Acid, and Fumaric Acid, ensuring near-complete degradation.

In the case of RB, azoreductase cleavage results in the formation of 4-Amino-5-hydroxy-2,7-naphthalenedisulfonic acid and 4-Amino-5-hydroxy-1,3-benzenedisulfonic acid, which are further oxidized by peroxidases to 5-Hydroxy-2,7-naphthalenedisulfonic acid and 5-Hydroxy-1,3-benzenedisulfonic acid. These intermediates undergo desulfonation and hydroxylation before being degraded into smaller organic acids such as Muconic Acid, Succinic Acid, and Fumaric Acid, facilitating complete mineralization.

Previous studies have shown that NADH: quinone oxidoreductases, commonly present in biodegrading bacteria, play a crucial role in electron transfer during azo dye reduction, linking the degradation process to cellular respiration. The reducing equivalents generated in these pathways are transported to the electron transport chain, facilitating dye mineralization. Additionally, it has been reported that hydrolase-mediated desulfonation steps contribute to the conversion of sulfonated intermediates into more biodegradable forms. Given that *Pseudomonas putida* and *Lysinibacillus sphaericus* possess the genetic and enzymatic potential for azo dye degradation, future research should explore the regulation of these key enzymes to enhance the efficiency of dye bioremediation[14].

In this study, the degradation of Acid Yellow 42 (AY), Reactive Red 198 (RR), and Reactive Black 5 (RB) by the co-culture of *Pseudomonas putida* and *Lysinibacillus sphaericus* was investigated through detailed molecular profiling using Electrospray Ionization Mass Spectrometry (ESI-MS). The ESI-MS spectra of the dye-degraded samples revealed key insights into the metabolic byproducts formed during the degradation process. The mass spectrometric analysis identified several intermediates, corresponding to the breakdown of the azo dyes into smaller aromatic amines and other metabolites.

The observed molecular masses and fragmentation patterns suggest that the azo bond cleavage, a crucial phase in the breakdown process of these dyes, occurred predominantly through reductive processes facilitated by azoreductase enzymes. This cleavage resulted in the formation of aromatic amine intermediates, which were then oxidized and hydroxylated, as evidenced by the presence of oxygenated metabolites. The formation of such metabolites indicated the successful breakdown of the aromatic structures of the dyes.

In addition to the initial reductive cleavage, the spectra also revealed the involvement of peroxidases, which catalyzed the further oxidation of the aromatic amines. These products underwent subsequent degradation through hydrolytic and desulfonation processes, leading to the generation of low-molecular-weight compounds such as succinic acid, maleic acid, and fumaric acid. These smaller metabolites are indicative of near-complete mineralization of the dyes, as they represent common intermediates in the microbial degradation of aromatic compounds.

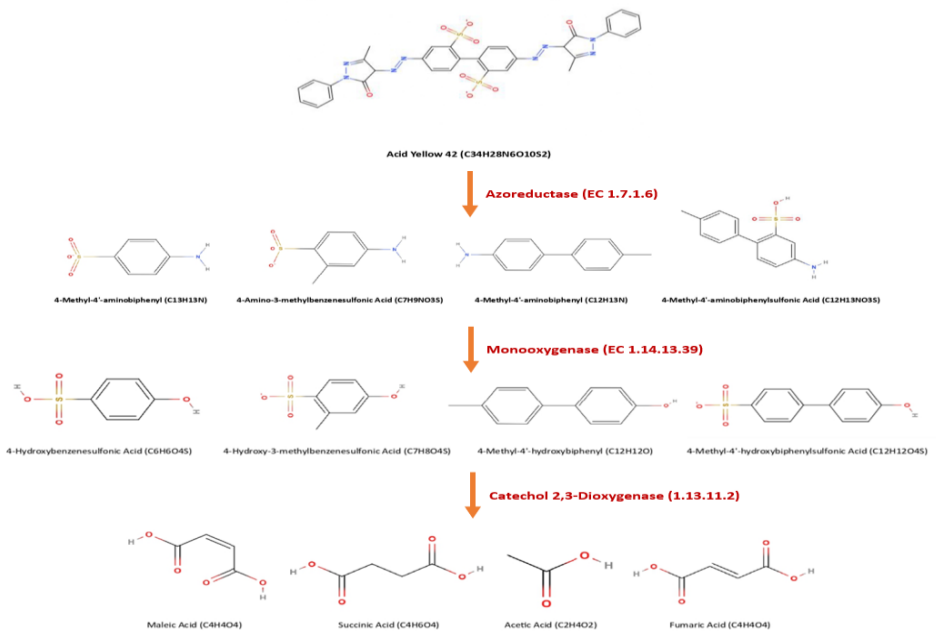

Figure 2. Proposed metabolic pathways for the biodegradation of dye AY by Pseudomonas putida and Lysinibacillus sphaericus consortium.

The ESI-MS data also demonstrated the synergistic role of the two bacterial species in enhancing the degradation process. The complementary enzymatic activities in the co-culture system facilitated the decomposition of intricate dye structures into simpler compounds, preventing the accumulation of toxic intermediates. This analysis provides a in-depth insight into the degradation mechanism, highlighting the critical roles of azoreductases, peroxidases, and hydrolases in the efficient mineralization of azo dyes.

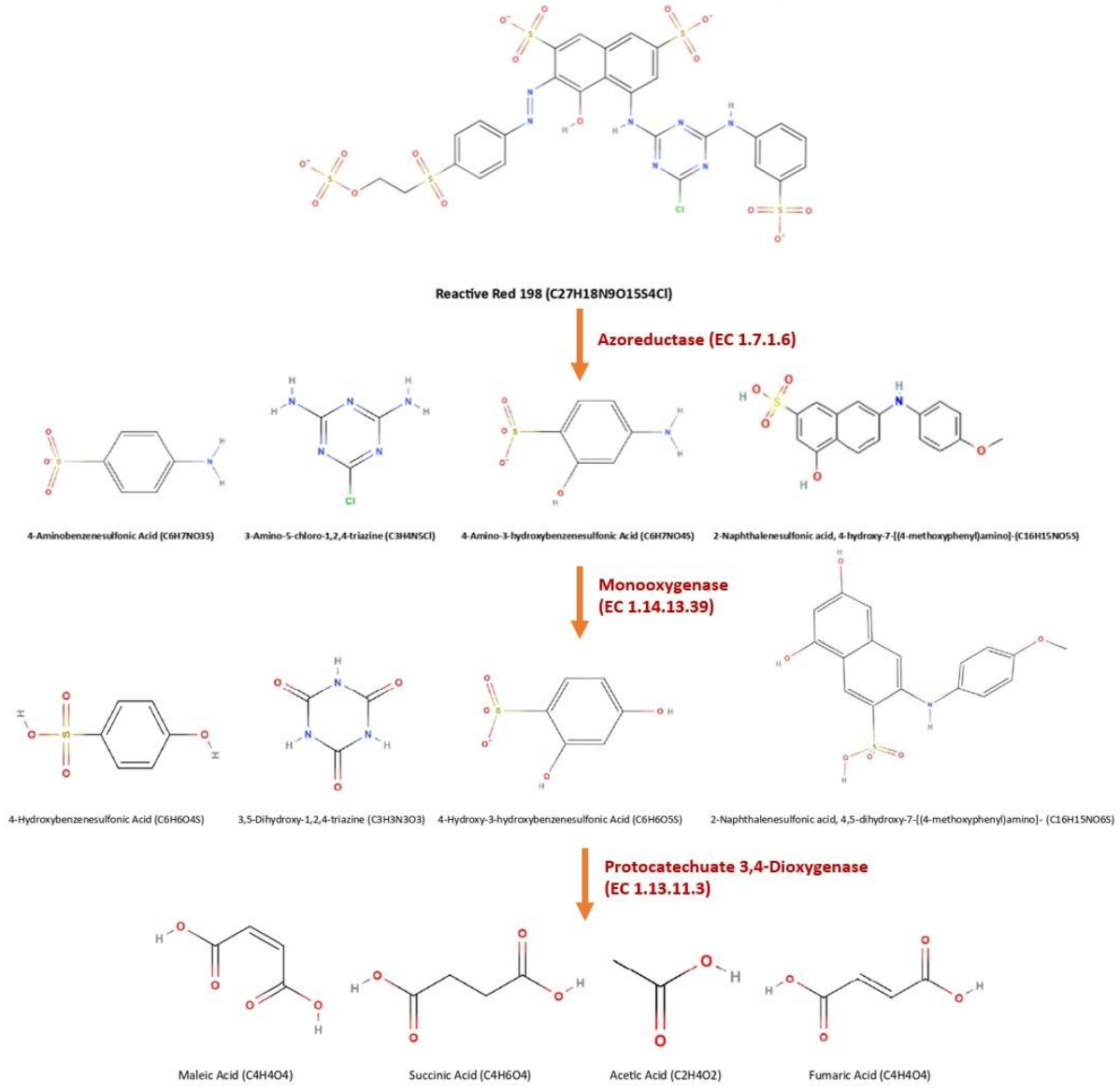

Figure 3. Proposed metabolic pathways for the biodegradation of dye RR by *Pseudomonas putida* and *Lysinibacillus sphaericus* consortium.

Overall, the ESI-MS results underscore the effectiveness of the co-culture system in facilitating the complete breakdown of these recalcitrant dyes. The identification of key intermediates and the elucidation of the degradation pathway offer valuable insights into the metabolic processes involved, paving the way for the development of more efficient microbial degradation strategies for textile wastewater treatment.

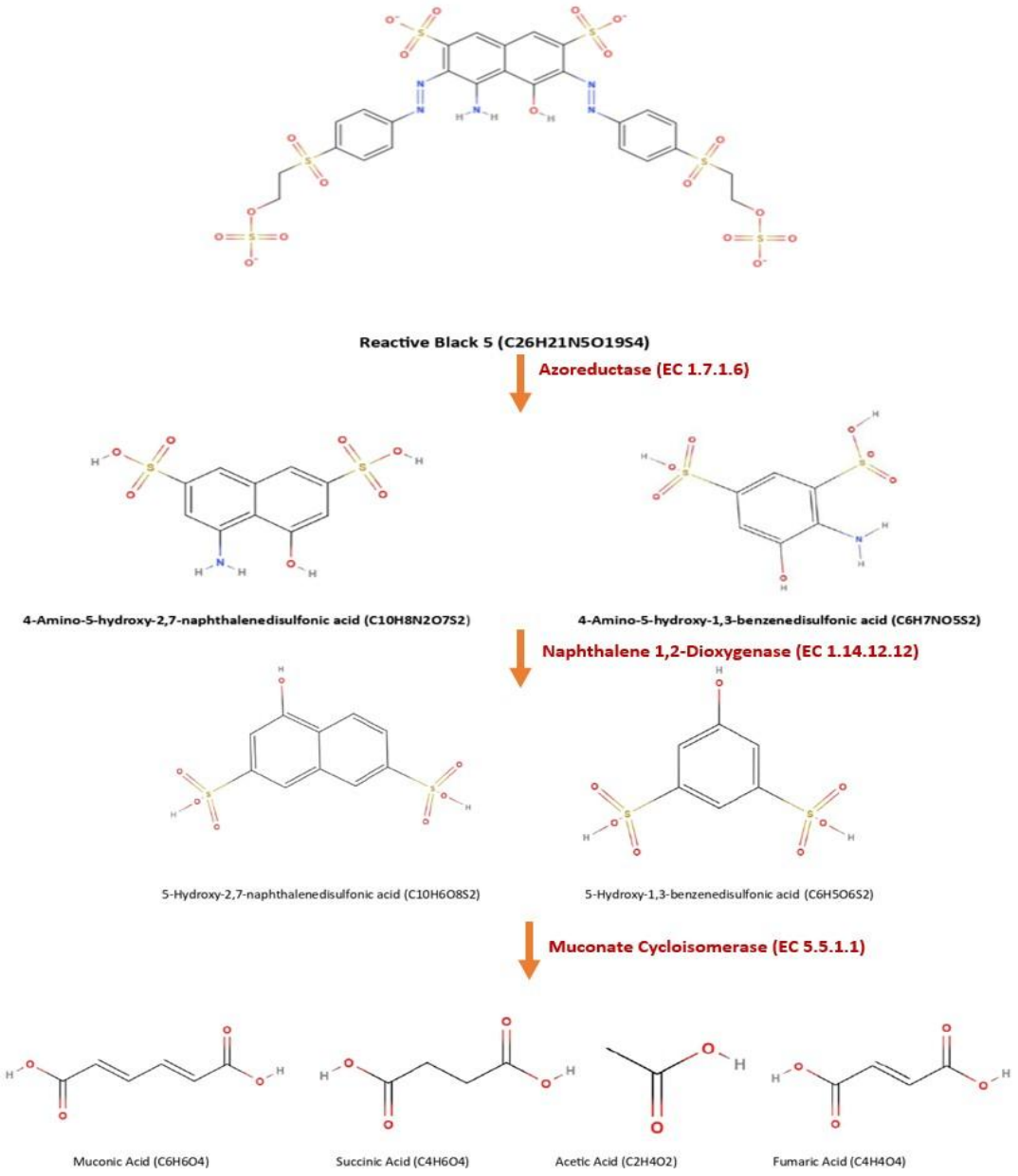

Figure 4. Proposed metabolic pathways for the biodegradation of dye RB by *Pseudomonas putida* and *Lysinibacillus sphaericus* consortium.

4. CONCLUSION

This study highlights the successful biodegradation of Acid Yellow 42 (AY), Reactive Red 198 (RR), and Reactive Black 5 (RB) using a co-culture system of *Pseudomonas putida* and *Lysinibacillus sphaericus*. ESI-MS analysis revealed the degradation of these azo dyes into low-toxicity organic acids, signifying near-complete mineralization. Unlike traditional single-strain approaches, the co-culture system demonstrated enhanced decolorization efficiency and accelerated degradation, emphasizing the potential benefits of multi-microbial systems for wastewater treatment. The increasing environmental burden of textile effluents underscores the need for sustainable bioremediation methods. Azo dyes, with their resistant structures, pose significant challenges to degradation, but the combined enzymatic activities of azoreductases, peroxidases, and hydrolases effectively broke down the dyes into safer metabolites. These findings reinforce the promise of co-culture microbial degradation as an eco-friendly solution for dye-contaminated industrial wastewater. Future research should focus on scaling this approach for practical applications in real-world settings, as well as optimizing microbial interactions for improved degradation rates. Overall, this study provides important insights into the potential of microbial consortia for environmental pollution management and offers a pathway toward more effective, biological treatment strategies for industrial wastewater.

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6. AUTHOR CONTRIBUTION

Conceptualization, M.N. and L.K.S.; methodology, M.N. and L.K.S.; formal analysis, M.N.; investigation, M.N.; data curation, M.N.; writing—original draft preparation, M.N.; writing—review and editing, M.N. and L.K.S.; supervision, L.K.S.; All authors have reviewed and approved the final version of the manuscript for publication.

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