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Research Article

Biotransformation of the Textile Dye Reactive Black 5 with *Aspergillus niger* and Analysis of Ligninolytic Enzyme Activity

Diah Ayu Prawitasari[1*], Edwan Kardena[2]

[1]*Department of Environmental Engineering, Faculty of Civil Engineering and Planning, Universitas Islam Indonesia, Sleman 55584, Indonesia*
[2]*Department of Environmental Engineering, Faculty of Civil Engineering and Environmental Engineering, Bandung Institute of Technology, Bandung 40132, Indonesia*

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ABSTRACT

Removal of textile dye using biological processes is considered more cost-effective, generates no hazardous byproducts, and is ecologically friendly. This study analyzes the removal of the synthetic dye Reactive Black 5 (RB5) using the biological agent *Aspergillus niger*. The Treatment occurred in a 250 mL Erlenmeyer flask containing a mixture of dye solution. The preliminary research treatment involved varying dye concentrations, which were then analyzed by examining enzyme activity and fungal growth at different shaker rotations. The initial findings on concentration variation indicated that it was effectively removed up to approximately 90%. Further research determined that the highest concentration, 200 ppm, was used. The results obtained with rotation variations up to the sixth day were 95% for 100 rpm and 93% for 150 rpm. Dye removal utilizes laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) as degrading enzymes. The highest amount of enzyme activity was achieved at 100 rpm, corresponding to the most effective dye removal. The enzyme activity at the 100-rpm variation was observed for MnP (62.2 U/L), LiP (32.2 U/L), and laccase (16.6 U/L). The rapid growth of the *Aspergillus niger* fungus is associated with the biomass, which influences the biosorption mechanism. Biodegradation pathways indicate the cleavage of aromatic bonds, with toluene and propanoic acid identified as the final products.

1. Introduction

The global textile industry generates a substantial amount of waste, accounting for 8.2% of the plastic that ends up in Indonesia's oceans (Kasih 2021). Textile waste commonly contains dye, metals, and various other contaminants. The concentration of these contaminants typically ranges from 50 to 2,500 Pt-Co, with approximately 10 to 15% of the dye being discarded as waste during the dyeing process (Lellis *et al.* 2019). Synthetic dyes, which are complex organic compounds, are the primary component used in this process. These dyes are known for their resilience in environments that can be harmful to ecological systems. Reactive Black 5 (RB5), an azo dye, is widely used in the textile industry due to its stability and the range of dyes it produces. The presence of RB5 in industrial effluents is a cause for environmental concern, as it possesses toxic, carcinogenic properties and exhibits resistance to natural degradation, which may lead to contamination of water sources and pose risks to aquatic ecosystems.

Biological treatment methods, particularly bioremediation using microorganisms, have emerged as effective and environmentally friendly alternatives for addressing pollution caused by textile dyes. While physical and chemical methods may offer higher efficiency, they are more costly and may generate hazardous byproducts (Abbasi 2017). Recent studies have shown that white rot fungi are commonly employed to remove dyes; however, the brown rot

* Corresponding Author
E-mail Address: diahayuprawitasari@uii.ac.id

fungus *Aspergillus niger* also demonstrates the capacity to degrade pollutants. *Aspergillus niger*, a widely distributed brown mold, has demonstrated potential in degrading various complex organic compounds. This fungus is considered a promising candidate for waste treatment due to its cost-effectiveness, efficiency, and environmental sustainability (Cohen *et al.* 2021). Its ability to degrade azo dyes is primarily attributed to the production of ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, which facilitate the breakdown of the complex aromatic structures of azo dyes (Asses *et al.* 2018). Previous research has demonstrated that *Aspergillus niger* is capable of degrading various azo dyes through enzymatic mechanisms. Previous research has confirmed that *Aspergillus niger* efficiently degrades RB5 through the production of ligninolytic enzymes (Ekanayake & Manage 2022). However, the specific mechanisms and the role of each enzyme in the biodegradation process of RB5 by *Aspergillus niger* remain inadequately explored, warranting further investigation.

Therefore, this study aims to investigate the ligninolytic enzyme capabilities of *Aspergillus niger* and analyze the biotransformation processes involved in the biodegradation of Reactive Black 5. A more comprehensive understanding of this biodegradation mechanism is expected to contribute to the development of more efficient bioremediation strategies for mitigating textile dye pollution in the environment.

2. Materials and Methods

2.1. Inoculation of *Aspergillus niger*

Inoculation is conducted in PDA media at a concentration of 39 g/L, which requires sterilization via autoclave. *Aspergillus niger* ITBCC151 is incubated for one week. The mycelium was collected in a test tube containing distilled water and then transferred to a Potato Dextrose Broth solution (24 g/L) in a 250 mL Erlenmeyer flask. The flask was incubated at 37°C for 24 hours.

2.2. Textile Dyes Removal by *Aspergillus niger*

Aspergillus niger was cultivated from PDA (Potato Dextrose Agar) media to liquid media, Potato Dextrose Broth (PDB). Inoculum from PDA was dissolved in 5 mL of Tween (85%) solvent. The initial total cell count of spores is 10^6-10^7 cells/mL, then pour into a flask. The removal procedure utilized a 250 mL Erlenmeyer flask containing 200 mL of PDB media with optimum conditions of 30 °C and pH 5, and a synthetic dye solution at a concentration of 200 ppm. The flask was incubated for 6 days, and 5 mL samples of the filtrate were collected every 24 hours. The investigation involves isolating the initial concentration variation of the synthetic dye. Concentrations of 200, 100, 50, and 25 ppm were varied. The optimal circumstances include an initial pH of 5, a temperature of 30°C, and a rotational speed of 100 rpm. The optimal environmental parameters were established based on previous studies. The optimal parameters for use are a pH of 5, a temperature of 30°C, and shaker rotation speed changes of 250, 200, 150, 100, and 50 rpm. The removal process was conducted utilizing a 250-milliliter Erlenmeyer flask in a batch. A 5-milliliter sample was collected daily and incubated for six days in an incubator shaker. A 5-milliliter filtrate culture sample was subsequently centrifuged for 20 minutes at 6,000 rpm to evaluate dye removal. Dye removal is determined by the absorbance at the peak wavelength of Reactive Black 5, which is 600 nm.

2.3. Measurement of Enzyme Activity and Protein

The protein assay was performed with the Barford method. The standard curve utilizes Bovine Serum Albumin (BSA) at concentrations ranging from 10 to 100 µg/L for the standard and from 1 to 10 µg/L for the microassay, with intervals of 1 µg/L, resulting in a linear curve as the protein measurement standard. One hundred microliters of BSA samples at varying concentrations or one hundred microliters of supernatant samples were combined with five milliliters of Bradford reagent. The mixture was homogenized using a vortex and allowed to stand for 15 minutes, following which its absorbance was measured at a wavelength of 595 nm.

The enzyme activity was assessed using a spectrophotometer over 5 minutes, with measurements taken at 1-minute intervals. The laccase enzyme was evaluated using the ABTS substrate reagent at an absorption wavelength of 420 nm (Rahman *et al.* 2013). The enzymatic activity of Lignin peroxidase (LiP) was evaluated at a wavelength of 650 nm (Bholay *et al.* 2021). The enzymatic activity of manganese peroxidase (MnP) was evaluated at a wavelength of 610 nm (Hariharan & Nambisan 2013). One unit of enzyme activity is defined as the quantity of enzyme in moles of substrate per minute (U/L), with a conversion of 1 micromole (1 µmol = 10^{-6}). The formula for measuring enzyme activity is:

$$U/L = \frac{(A_t - A_0).\ Vtot\ (mL)\ .\ 10^6}{\epsilon\ .\ d.\ Venzim\ (mL)\ .\ t}$$

A : Absorbance (nanometers)
Volume : (milliliters) total volume (milliliters)
Venzim : Volume of enzyme supernatant (mL)
ϵ : Molar biosorptivity factors: Lacasse (36000 M^{-1} cm^{-1}); LiP (49900 M^{-1} cm^{-1}); MnP (31620 M^{-1} cm^{-1})
d : Path length (centimeters)
t : duration (minutes)

2.4. Biotransformation of Dyes Using FT-IR and Toxicological Assessment

The results of the removal of dye and *Aspergillus niger* biomass were characterized by biotransformation using FT-IR to analyze the profile of azo compound bonds. Analysis with FT-IR (4000 –400 cm^{-1}) provides results on the profile of chemical bond reduction that occurs. For more specific types of compounds, the GCS/MS test is preceded by a hexane-ethyl acetate solvent mixture (1:1).

2.5. Scanning Electron Microscopy Analysis of *Aspergillus niger* Biomass

Aspergillus niger hyphae identification was taken using a Scanning Electron Microscope (SEM) at magnifications of 1000x and 4000x. This morphology illustrates the attributes of *Aspergillus niger* before and after removal. This demonstrates the biosorption activity of *Aspergillus niger* cells in the dye removal process.

2.6. Statistical Analysis using Non-parametric Kruskal-Wallis

Evaluation of the effect of rotational speed (50, 100, 150, 200, 250 rpm) on Reactive Black 5 decolorization was conducted using statistical analysis. After a 6-day incubation on an orbital shaker, the decolorization percentage was measured spectrophotometrically. Due to non-normal data distribution, the results were analyzed using the non-parametric Kruskal-Wallis test ($\alpha = 0.05$) with three replicates per speed. A significant Kruskal-Wallis result was followed by Dunn's post-hoc test for pairwise comparisons.

3. Results

3.1. Characteristic of the Textile Dye Reactive Black 5

This is shown in the FT-IR results of the RB5 compound powder. From the results, the wave peak at 3448.72 cm^{-1}, followed by peaks at 1342.46 cm^{-1}, 1132.21 cm^{-1}, and 665.44 cm^{-1}, indicates the presence of hydroxyl groups (-OH) and bonds (-NH), which are difficult to distinguish. The appearance of the wave peaks at 1629.85 cm^{-1} and 1492.90 cm^{-1} also indicates the presence of the aromatic ring group with double C bonds (-C=C-) and the presence of the open azo chain (-N=N-), followed by the appearance of peaks at 842.89 cm^{-1} and 740.67 cm^{-1}, which indicate the ortho and para aromatic rings. The peaks at 1415.75 cm^{-1} and 1342.46 cm^{-1} also indicate the presence of organic sulfate and sulfonate (-S(=O)2-O-) as the auxochrome of the dye (Figure 1).

3.2. Removal Dye Treatment using *Aspergillus niger*

Several external factors, including temperature, pH, and shaker rotation speed, influence the removal of dye by the fungus *Aspergillus niger*. The optimum temperature and pH used are based on the literature review of previous studies, with a temperature of 30°C and a pH of 5. Variations in the initial dye concentration for the RB5 dye show a reduction in dye, achieving removal efficiencies of 96%, 97%, 99%, and 99% for initial dye concentrations of 200 ppm, 100 ppm, 50 ppm, and 25 ppm, respectively, up to day 6. The result shows that the 25 ppm concentration variation already exhibited a significant dye reduction on the second day with an efficiency of 44%, the 50 ppm concentration variation on the third day with an efficiency of 50%, while the 100 ppm and 200 ppm concentration variations were on the fifth day (Figure 2).

The application of dye concentration must take into account the fungus's maximum capacity to prevent growth interference and ensure the dye is removed to its optimal limit. The subsequent treatment involves varying rotation speeds at 250, 200, 150, 100, and 50 rpm, with an initial concentration of 200 ppm (Figure 3A). The removal results show the optimum conditions for the highest removal process, which are the rotation speeds of 100 rpm and 150 rpm, also observed visually (Figure 3B). The efficiency achieved by the sixth day for each rotation speed of 100 rpm and 150 rpm was 95% and 93%, respectively. Meanwhile, the lowest rotation speed of 50 rpm is 78%. The second removal of synthetic dyes with the lowest efficiency occurs at rotation speeds of 250 rpm and 200 rpm, with the efficiency value of RB 5 dye being 63% at 250 rpm and 71% at 200 rpm.

The results of the Kruskal-Wallis test revealed a statistically significant difference in dye concentrations

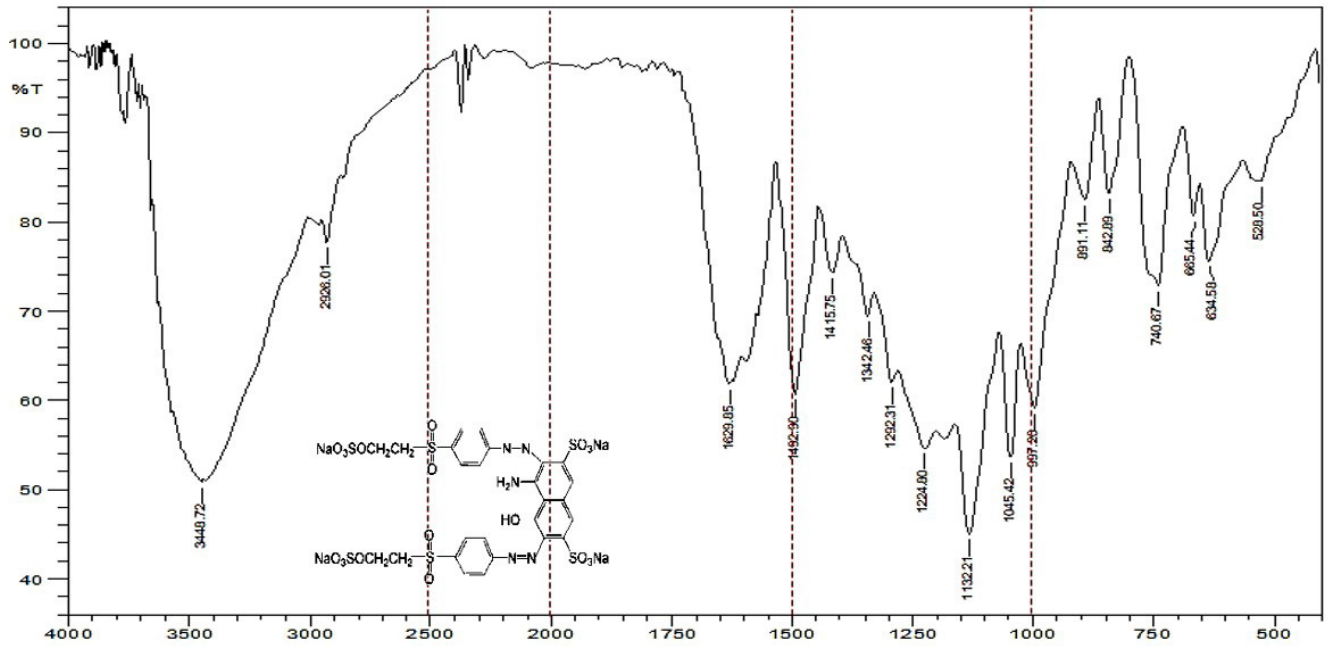


Figure 1. Chemical characteristic of reactive Black 5 by FTIR

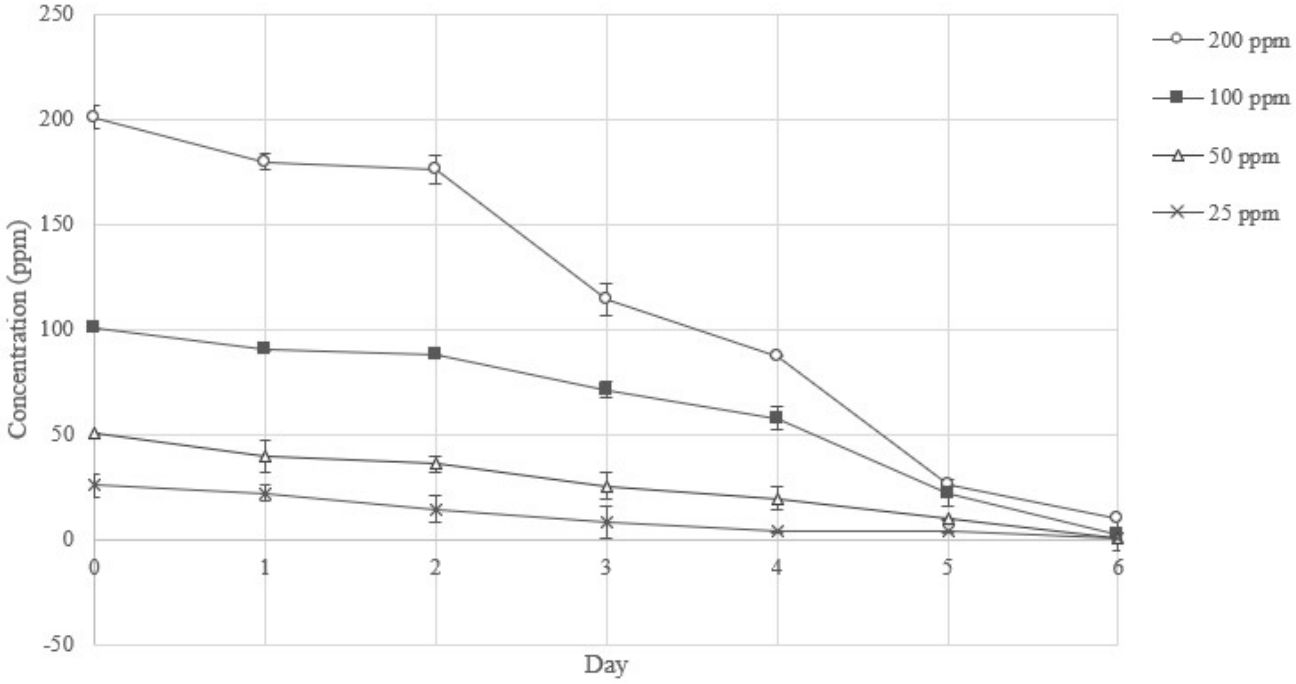


Figure 2. Decreasing of reactive Black 5 in various concentrations

across the different rotation speeds ($p = 0.0091$). This suggests that varying the rotation speed has a significant effect on the dye concentration levels. These findings suggest that rotation speed plays a crucial role in the efficiency of the dye removal process using *Aspergillus niger*, highlighting the importance of optimizing operational parameters for improved performance.

3.3. Analysis of Growth and Morphology of *Aspergillus niger* Fungal Isolates

The growth curve graph shows the exponential phase for dye variations of 250 and 200 rpm reaching day 3, while 150 and 100 rpm reach day 4, and 50 rpm reach day 2 (Figure 4). The exponential phase is the phase where cell division occurs at the maximum rate.

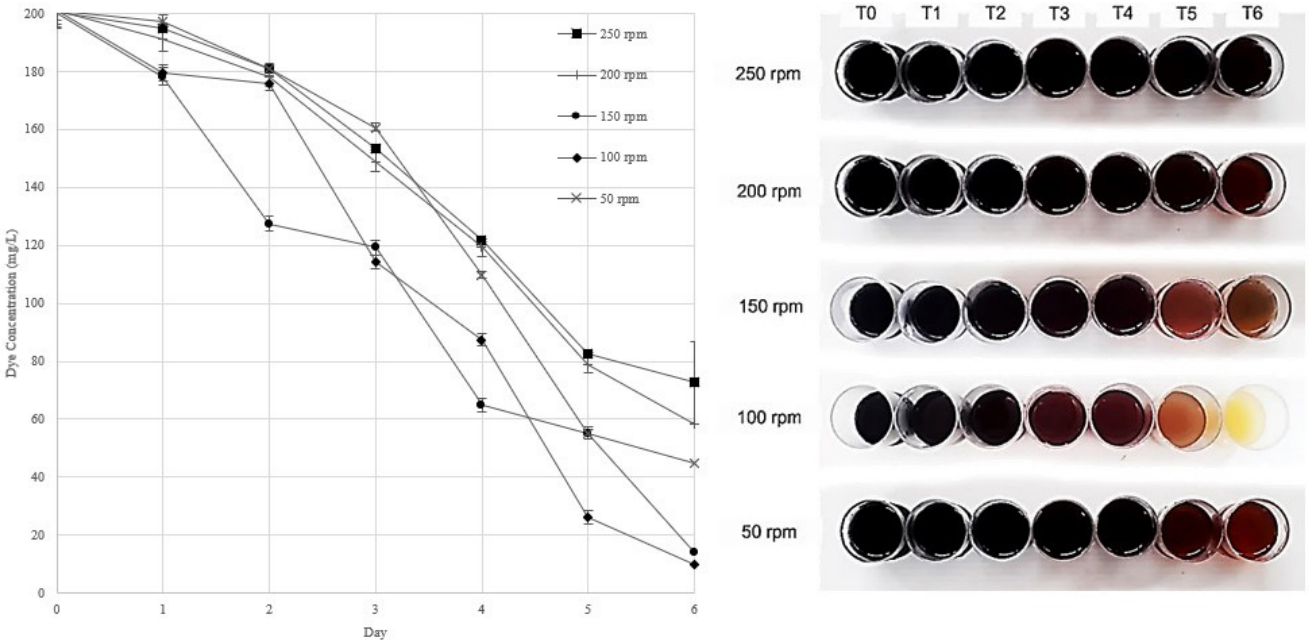


Figure 3. Decreasing of removal in rotation variation (A) visualisation of decreasing removal, (B) of reactive Black 5

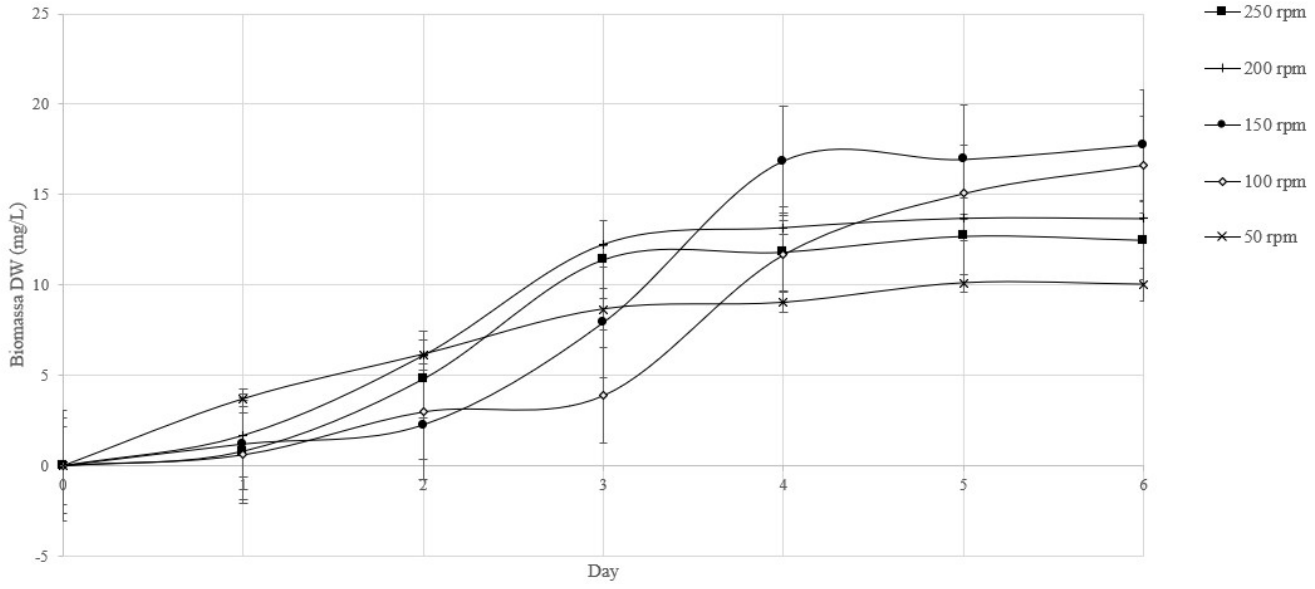
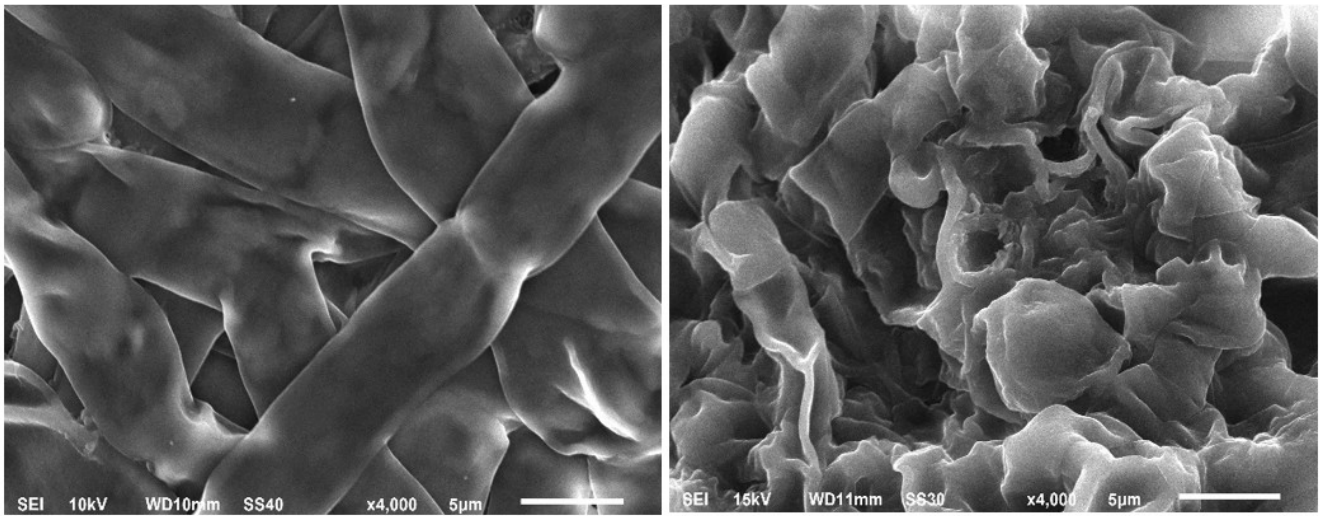


Figure 4. Growth curve of *Aspergillus niger* biomass on reactive black 5

The hyphae in the control mycelium biomass without dye have more structured filaments without any damage (Figure 5). Meanwhile, pellets with synthetic dyes exhibit changes in hyphal filaments that tend to be more shriveled and less structured in their formation. The biosorption capacity can also be evaluated by examining the % removal at the beginning of the process. In this study, days 0 and 2 can be assumed as the determinants of the % dye removal in the biosorption mechanism before reaching equilibrium conditions.

3.4. Analysis of Enzyme Activity in the Process of Removing the Synthetic Dye Reactive Black 5

The enzymes tested in this screening process are oxidase enzymes from brown-rot fungi, specifically laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP). The three enzymes are induced to accelerate the oxidation reaction against the targeted substrate.



A B

Figure 5. SEM results of biomass pellets with synthetic dye at 4000x magnification for (A) control and (B) dye treatment

The research results show that a rotation speed of 100 rpm yields the most optimal oxidase enzyme activity, with laccase enzyme activity reaching 16.6 U/L (Table 1), LiP enzyme activity at 32.7 U/L (Table 2), and MnP enzyme activity reaching 62.2 U/L (Table 3). The sufficiency of dissolved oxygen influences this at 100 rpm aeration, which also increases the nutrients utilized for the induction of extracellular enzymes. Meanwhile, at 250 and 200 rpm, excessive aeration hurts biomass growth. As previously mentioned, the cell structure will easily break and quickly die due to excessive molecular friction, causing the induced enzymes to become less effective, even though fungal growth rapidly increases at the beginning of the growth period.

The activity of the laccase enzyme at each rotation speed variation tends to show maximum activity on the third and fourth days (Figure 6A). Laccase enzyme was also detected starting from day 1. The activities of LiP and MnP were already detectable on the second day and showed a tendency to increase to a maximum on the third and fourth days. Additionally, enzyme activity correlates with protein concentration. The increase in enzyme activity also indicates a rise in protein concentration every 24 hours (Figure 6B and C). Under optimal conditions of 100 rpm agitation the daily decrease in oxygen concentration and pH levels results in a reduction in dye concentration.

3.5. Analysis of the Biotransformation of the Removal of the Synthetic Dye Reactive Black 5

The results of the dye removal were analysed using FT-IR to observe the bond profiles that have been removed by comparing the results before and after Treatment. The results of the removal of Reactive Black 5 dye show that several wave peaks have disappeared from the FT-IR of the initial dye. This disappearance is observed at several wave peaks between 1500–500 cm^{-1}, indicating the transformation of aromatic compounds into simpler compounds.

Additionally, the open azo bond at the wave number 1630–1575 cm^{-1} is not detected, indicating the cleavage of the double azo bond (Figure 7). The presence of functional groups in the degradation products of the RB5 dye is also evident from the FT-IR results. The wave peak at 1381.03 cm^{-1} indicates tertiary phenol, 1244.09 cm^{-1} indicates the presence of ester, 881.47 cm^{-1} indicates C-O-O for the presence of acetic acid, 1082.07 cm^{-1} indicates C-O stretching, 3427.51 cm^{-1} indicates the presence of hydroxyl (-OH) bonds, and 2856.58 cm^{-1} indicates the presence of (-C-H).

The results of RB5 dye removal were also subjected to GC/MS testing to identify specific compounds resulting from enzymatic degradation. The GC/MS results reveal multiple wave peaks, indicating the presence of several compounds (Figure 8). The

Table 1. Laccase enzyme activity

Rotation (rpm)	Day					
	1	2	3	4	5	6
250	0.9±0.1	8.0±0.2	10.5±0.3	9.1±0.4	7.9±0.4	4.1±0.1
200	0.7±0.6	7.3±0.1	10.5±0.1	8.8±0.5	7.6±0.5	4.1±0.1
150	1.8±0.2	9.8±0.6	12.9±0.1	14.5±0.1	13.4±0.6	8.7±0.3
100	0.3±0.5	3.9±0.7	11.2±0.4	16.6±0.1	14.8±0.1	6.2±0.1
50	0.5±0.4	1.5±0.3	4.2±0.5	5.7±0.4	6.2±0.2	4.9±0.6

Table 2. Lignin peroxidase (LiP) enzyme activity

Rotation (rpm)	Day					
	1	2	3	4	5	6
250	0	5.3±0.2	26.9±0.3	27.3±0.1	24.3±0.1	14.8±0.5
200	0	4.5±0.2	27.5±0.1	29.2±0.1	26.0±0.3	17.5±0.4
150	0	8.3±0.1	30.0±0.5	32.6±0.5	29.9±0.4	20.4±0.1
100	0	7.7±0.4	31.9±0.4	32.7±0.2	30.6±0.3	22.2±0.1
50	0	1.1±0.1	26.7±0.5	25.2±0.4	23.6±0.1	6.2±0.3

Table 3. Mangan peroxidase (MnP) enzyme activity

Rotation (rpm)	Day					
	1	2	3	4	5	6
250	0	7.3±0.5	43.9±0.6	45.4±0.1	39.2±0.7	29.2±0.1
200	0	12.3±0.4	46.7±0.1	45.4±0.2	43.2±0.5	33.3±0.3
150	0	13.6±0.3	56.7±0.3	61.9±0.2	58.2±0.5	48.8±0.5
100	0	15.9±0.3	63.4±0.3	62.2±0.3	60.3±0.1	52.3±0.3
50	0	5.8±0.1	25.1±0.1	41.1±0.1	40.7±0.1	21.8±0.3

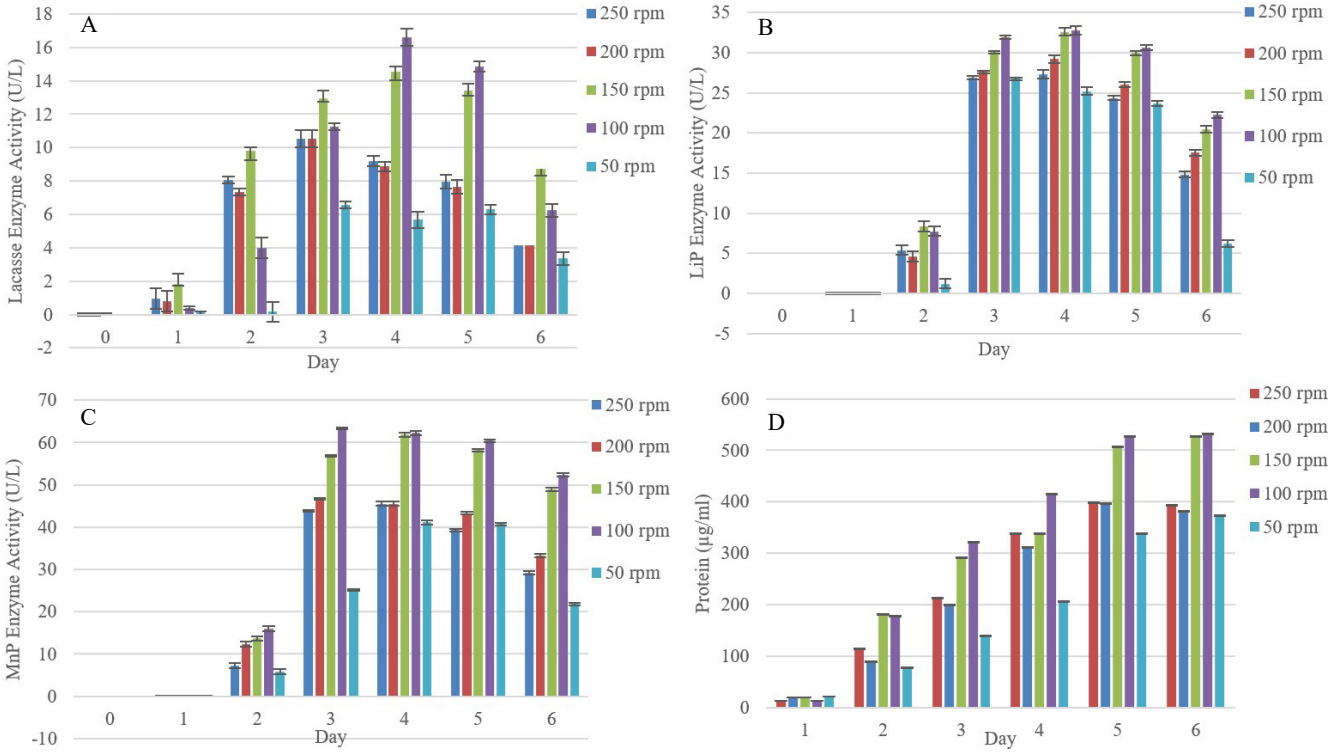


Figure 6. Activity enzyme of lakase (A), LiP (B), MnP (C), and protein (D) for 6 days

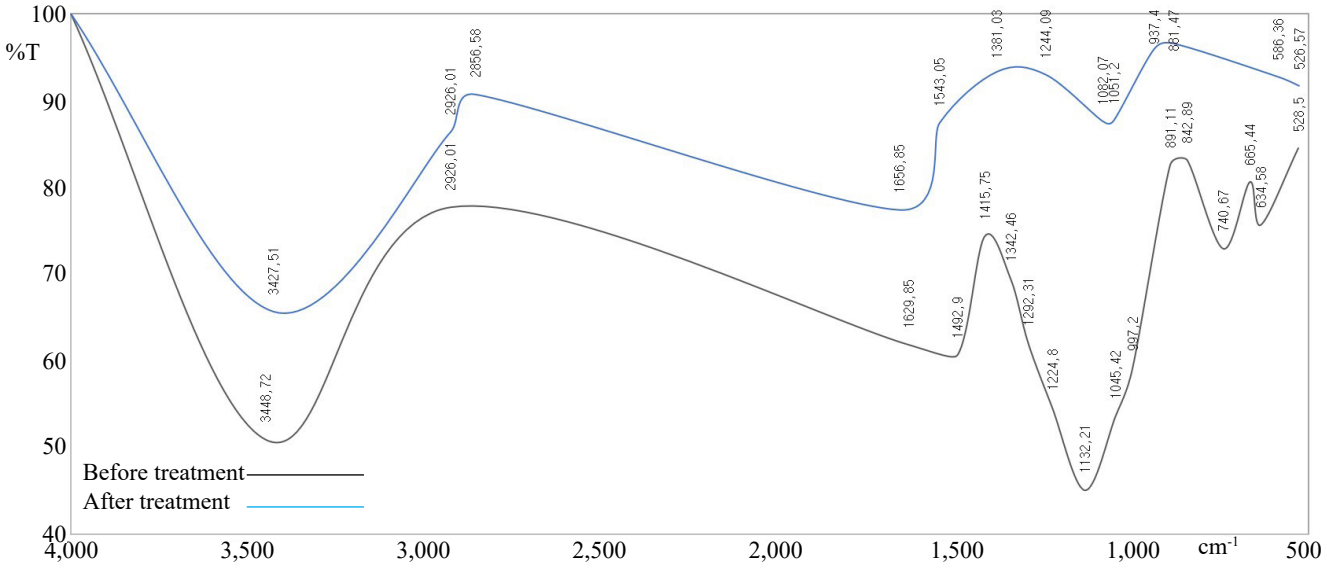
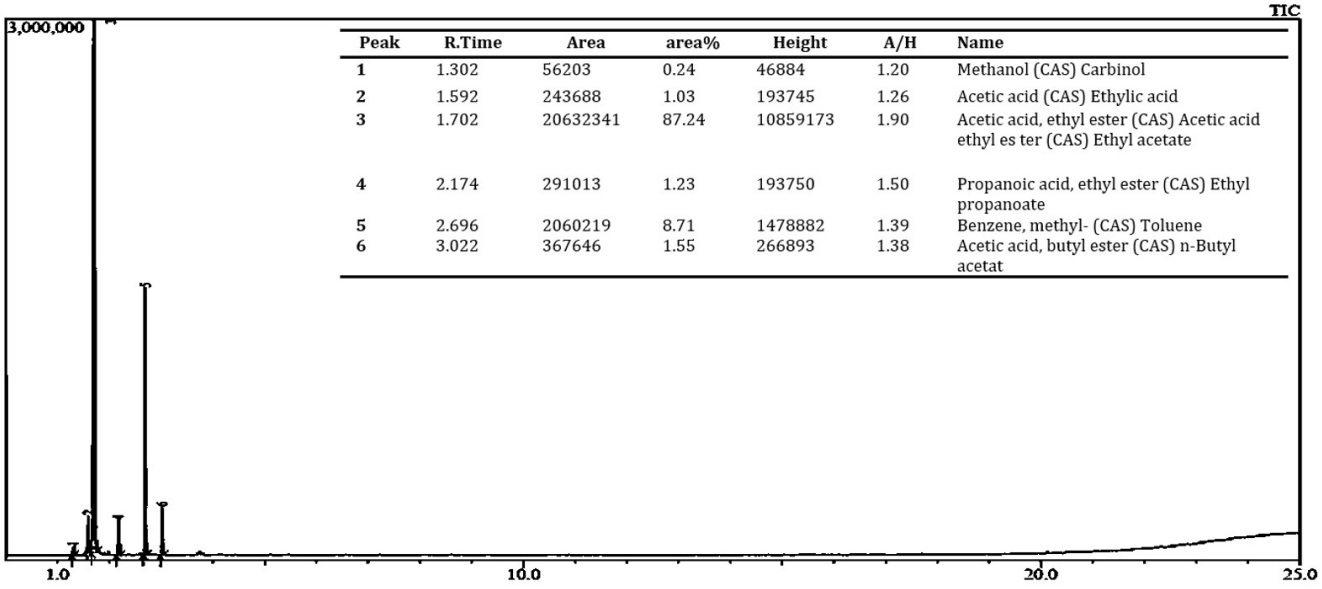


Figure 7. FT-IR before and after the reactive Black 5 removal mechanism



Peak	R.Time	Area	area%	Height	A/H	Name
1	1.302	56203	0.24	46884	1.20	Methanol (CAS) Carbinol
2	1.592	243688	1.03	193745	1.26	Acetic acid (CAS) Ethylic acid
3	1.702	20632341	87.24	10859173	1.90	Acetic acid, ethyl ester (CAS) Acetic acid ethyl es ter (CAS) Ethyl acetate
4	2.174	291013	1.23	193750	1.50	Propanoic acid, ethyl ester (CAS) Ethyl propanoate
5	2.696	2060219	8.71	1478882	1.39	Benzene, methyl- (CAS) Toluene
6	3.022	367646	1.55	266893	1.38	Acetic acid, butyl ester (CAS) n-Butyl acetat

Figure 8. GC/MS result peak of propanoic acid

compounds showed peaks, including toluene and propanoic acid.

4. Discussion

Reactive Black 5 (RB5) has the chemical formula $C_{26}H_{21}N_5Na_4O_{19}S_6$, a molecular weight of 991.8 g/mol, and the main functional groups are two reactive azo class chromophore groups and derivatives of anthraquinone, anthranilic, in the form of a tautomeric hydrazine. The chemical structure assigned to the RB5 compound includes auxochromes such as amino, sulfonate, and sulfone groups (García *et al.* 2017). The number of wave peaks indicates that this synthetic dye has a complex structure (Nandiyanto *et al.* 2019).

Optimum temperature conditions can enhance biosorption, which remains active with increasing temperature due to the interaction between cell biomass and the dye (Bankole *et al.* 2019). The effect of temperatures exceeding 30°C shows a decrease in degradation capacity. This affects cell viability, which decreases with increasing temperature, leading to the inactivation of degradation enzymes (Asses *et al.* 2018). The acidic pH range between 2 and 6 can also enhance the adsorption process, affecting the cell surface to become active in forming adsorbates and the presence of van der Waals forces between the negatively charged dye anions and the positively charged biomass cell cations (Sowjanya *et al.* 2020). Previous research has also shown that the optimum activity of manganese peroxidase and lignin peroxidase is in the pH range of 5-6 (Asses *et al.* 2018). Enzyme activity correlates with pH conditions, where pH is the main factor in catalyzing the enzyme reaction with the substrate. Therefore, the optimum conditions for the dye removal process were chosen at an initial pH of 5 and maintained at a temperature of 30°C. The enzymes produced later also play a role in the degradation process of organic compounds in the dye.

The initial concentration of the dye also plays a crucial role in determining the overall driving force of the adsorption system, which helps overcome the mass transfer resistance between the liquid phase and the solid phase. Therefore, this study demonstrates that *Aspergillus niger* can remove textile dyes at a tolerance concentration of 200 ppm; therefore, this maximum concentration is used for further research. This suggests that an increasing concentration of the dye can lead to saturation of the fungal cell surface, resulting in a slight reduction in the biosorption performance of the cells (Aderonke *et al.* 2020). Excessive concentrations can inhibit biomass growth due to the high concentration of sulfonic acid components in the dye, which inactivates enzymes by blocking their active sites and forming toxic byproducts, thereby affecting ongoing biological processes (Seyedi *et al.* 2020; Ameen *et al.* 2021; Srivastava *et al.* 2022).

Several factors, including temperature, pH, humidity, and nutrition, influence the growth of *Aspergillus niger* fungi. One of the factors that is quite important for biomass growth is the nutritional requirements. *Aspergillus niger* consumes the substrate contained in the Erlenmeyer reactor in the form of PDB medium and synthetic dye. The utilization of this substrate will affect the production of fungal biomass, as well as the production of several types of extracellular enzymes (Berger *et al.* 2022). It also affects the demyelination process of the dye.

Additionally, this pellet biomass can serve as a bioadsorbent in the biosorption process (Khan & Fulekar 2017). The biosorption process exhibits a greater affinity between the dye and the biomass cell wall, which comprises chitin, polysaccharides, proteins, lipids, and other functional groups, resulting in physical interactions between the active groups of the cell wall or covalent ion exchange (Jayaram *et al.* 2022). Biosorption of azo dyes by the surface of fungal cell biomass involves carboxyl, sulfonate, amide, and amine groups (Bouras *et al.* 2017).

The cell growth rate becomes rapid due to the cell division or reproduction process reaching its optimum point (Mirsha *et al.* 2021). The difference in exponential growth rates between these rotational speed variations is caused by agitation that affects oxygen transfer and substrate distribution, making both aspects more homogeneous. This can enhance the bioconversion process of the cell system and accelerate fungal growth (Ibrahim *et al.* 2015). However, excessively high rotation speeds also result in excessive shear stress on the biomass, which affects the size of the pellets, causing them to be smaller. Additionally, the increasingly smaller and denser pellet size is also influenced by shear stress, which affects the morphology of the cell structure, indicating that it is prone to breaking, as previously discussed (Ibrahim *et al.* 2015). The impact of the biosorption process of synthetic dyes by fungal biomass pellets is evident from the SEM results. This indicates the presence of a dye sorption mechanism in the surface area of the fungal filaments. This condition is influenced by synthetic dye compounds that inhibit fungal biomass growth because the dye adsorbed by the biomass hyphal aggregates generates electrostatic interactions on the biomass membrane with cationic dye bonds (Ruscasso *et al.* 2021; Kumaravel *et al.* 2022; Ogawa *et al.* 2024) This interaction, encompassing physico-chemical phenomena such as adsorption, deposition, and ion exchange, is a crucial mechanism

for dye removal, particularly by dead fungal cells (Lee *et al.* 2022). Conversely, living cells employ bioaccumulation and biodegradation via enzymatic action for dye remediation (Henning *et al.* 2022).

In this study, the targeted substrate is a synthetic dye that contains complex compounds. The chemical structure of the dye RB 5 is dominated by complex organic hydrocarbon compounds, such as phenolic and polycyclic compounds, which are the target substrates for fungal metabolites that are oxidized by enzymes like laccase, LiP, and MnP. The sufficiency of dissolved oxygen influences this at 100 rpm aeration, which also increases the nutrients utilized for the induction of extracellular enzymes (Ibrahim *et al.* 2015). The activity of this oxidase enzyme correlates with the reduction of dye at each variation of rotation speed, which has begun to show significant decreases on the 3rd, 4th, and 5th days. This suggests that the mechanism of dye biodegradation involves the oxidation of aromatic dye compounds. The presence of MnP and LiP enzymes, which appeared on the second day, indicates that these enzymes are inducible, meaning they can only be induced when a substrate is provided for the enzyme to target (Hou *et al.* 2020; Sosa-Martínez *et al.* 2020).

The appearance of oxidizing enzymes on the second day suggests that the biosorption mechanism primarily drove dye removal. The mechanism of dye removal after day 2 is primarily driven by the biodegradation of aromatic compounds in reactive dyes by oxidase enzymes. These oxidizing enzymes are extracellular enzymes that are only induced when the substrate is available in the cells (Ekanayake & Manage 2022). Laccase, LiP, and MnP enzymes are extracellular oxidizing enzymes that can only be induced in the presence of substrates and mediators (Hou *et al.* 2020). Therefore, there is a possibility that enzymes can be detected as proteins (Polak *et al.* 2022). Enzymes are proteins composed of amino acids linked by polypeptide chains and forming a primary structure (Lewis & Stone 2022). This allows for the possibility of other enzyme activities besides laccase, LiP, and MnP, which can also degrade dyes enzymatically. Other enzymes involved are suspected to be tyrosinase, deaminase, oxyreductase, and azoreductase (Kamal *et al.* 2022).

The involvement of dissolved oxygen and a decrease in pH are caused by metabolic reactions and enzyme activity as biocatalysts for the degradation of synthetic dyes (Das *et al.* 2023). Oxygen also acts as a redox mediator due to the presence of free electron pairs, allowing it to bind to fungi and function as a catalyst easily, the oxidase enzymes, which then attack the aromatic groups in textile dyes (Pundir *et al.* 2024). The optimum dye removal was achieved at rotation speeds of 150 rpm and 100 rpm. Rotational speeds of 150 rpm and 100 rpm provide sufficient oxygen to degrade non-toxic metabolite compounds, allowing oxidase enzymes to easily target aromatic hydrocarbon substrates (Khan *et al.* 2021). This indicates that sufficient oxygen can enhance the enzymatic dye removal mechanism.

Biotransformation of Reactive Black 5 decolorization using *Aspergillus niger* is conducted to elucidate the possible enzymatic biodegradation pathway of Reactive Black 5. It begins with the cleavage of the asymmetric azo double bond and the oxidative cleavage of the azo double bond (Figure 9). Then, the deamination and hydroxylation reactions mediated by oxidase enzymes produce naphthalene-1,2,8-triol and sulphuric acid mono-[2-(toluene-4-sulfonyl)-ethyl] ester (Adnan *et al.* 2015). Oxidation of the azo bond forms ortho-hydroxyl compounds with hydroxyl groups while simultaneously releasing N2 in molecular form. This is caused by the abstraction of electrons from the azo bond by laccase, resulting in the formation of a cation stabilized by resonance, which is attacked by water nucleophiles, thereby producing a hydroxyl group. The compound naphthalene-1,2,8-triol undergoes several ethyl carboxyl oxidation reactions (Khandare & Govindwar 2016). Then, the ethyl carboxylate compound undergoes a decarboxylation reaction, resulting in the products phenyl acetate and ethylic acid. Next, the intermediate compound phenyl acetate undergoes C-chain cleavage oxidation. After that, a carboxylation and methylation reaction occurs, producing propanoic acid. Then, for the toluene product, a dihydroxylation and methylation reaction occurs. On the other hand, the compound sulphuric acid mono-[2-(toluene-4-sulfonyl)-ethyl] ester will undergo desulfonation and hydroxylation reactions, resulting in the formation of toluene (Figure 9).

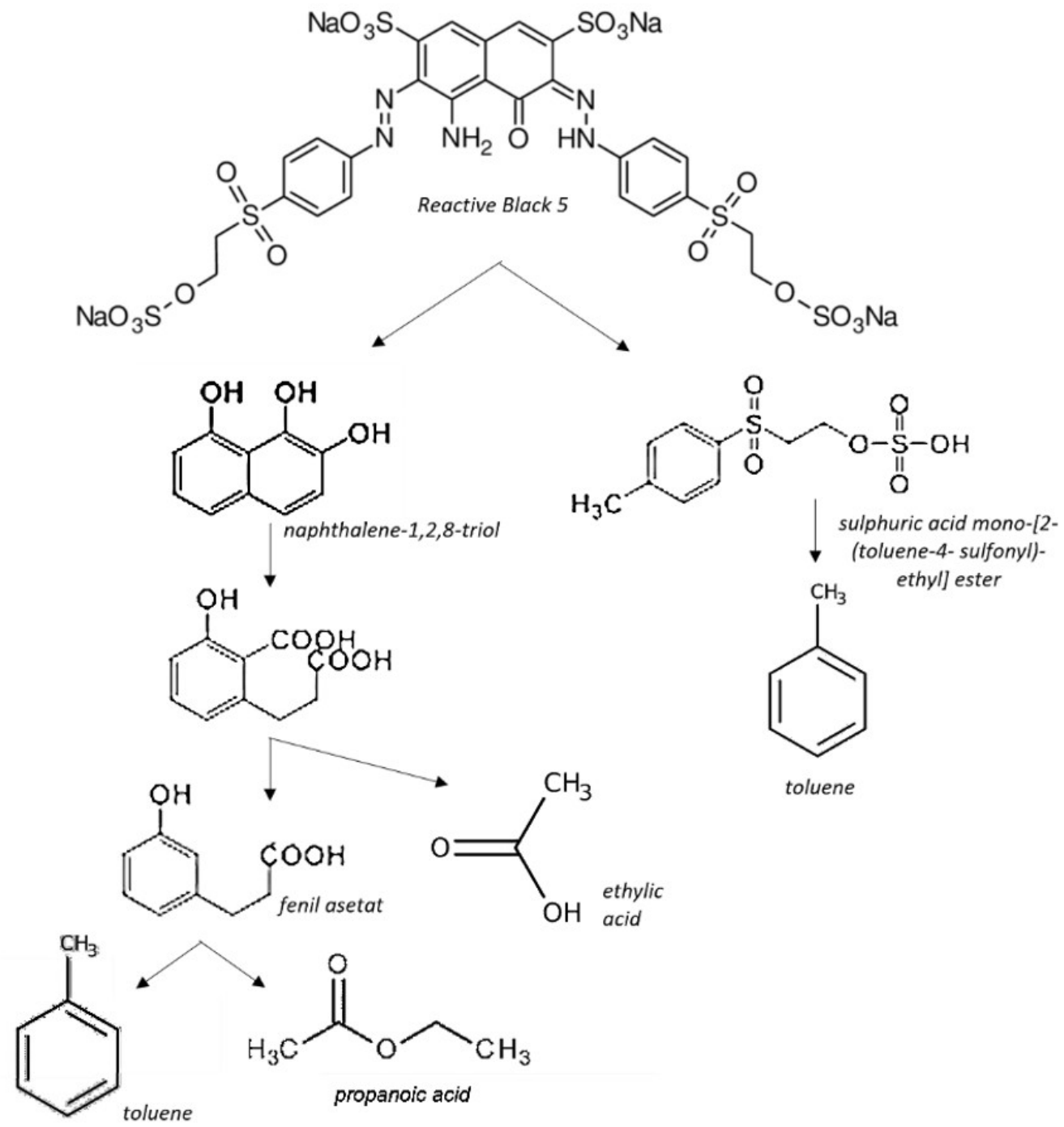


Figure 9. Biotransformation pathway of removal of reactive Black 5 by *Aspergillus niger* using ligninolytic enzymes

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