**Immobilization of *Bacillus subtilis* and *Bacillus cereus* in PVA and Na-Alginate Matrix for Azo Dye Decolorization in Batik Waste**

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**Abstract.** The batik industry is one of the most developed industries in Indonesia. Batik making uses natural materials and dyes. However, over time, the batik industry began to use synthetic dyes. The use of synthetic dyes also affects the waste produced by the batik industry. The type of dye that is often used in the batik industry is azo dye, such as naftol Black. Most of the dyes become waste and have toxic properties and have the potential to damage the environment, so further processing is needed before the waste can be disposed of into the environment. Several methods have been applied, such as adsorption, coagulation, electro fenton process, and photocatalytic decolorization. However, these methods are less efficient. One alternative that can be used in color degradation is to utilize microbes by immobilizing them. Immobilization can prevent the release of bacterial cells from the matrix, can interact with harsh environments. Immobilization using consortium microorganisms produces better degradation, some bacteria that can decolorize azo dyes are *Bacillus subtilis* and Bacillus cereus. This research studies the preparation of microbial consortium immobilized to determine the effectiveness and influence of the use of immobilized microorganisms on the decolorization process of dyes in batik waste and to determine the effect of microbial composition and type of dye on the use of microorganisms on the decolorization process of dyes in batik waste.

# INTRODUCTION

The batik industry is one of the most developed industries in Indonesia. Batik is a traditional culture that can compete in the market. Batik initially used natural materials and dyes. However, over time, the batik industry began to use synthetic dyes because they can improve the quality of colors in batik and are easier to obtain. The use of synthetic dyes also has an impact on the waste generated by the batik industry. The types of dyes that are often used in the batik industry are azo dyes, such as Naftol Black and Naftol Green B [1]. Basically, azo dyes are xenobiotic compounds that have an azo bond which indicates that the dye lacks electrons. azo dyes contain sulfide groups (SO32-) or other electron donor groups that result in electron deficiency [2]

The amount of use of coloring materials is also proportional to the amount of coloring materials that eventually become waste products. Most of the dyes used have toxic properties and have the potential to damage the environment, so further treatment is needed before the waste can be discharged into the environment. Some physical and chemical methods that can be used are adsorption, coagulation, and Electro Fenton Process. However, these methods still have some shortcomings. The adsorption method requires a long time in its application [3]. Meanwhile, the coagulation method is not environmentally friendly and uses hazardous materials and causes corrosion [4].The electro Fenton process can only be applied under special conditions and requires a complex process [5]. One alternative that can be used in color removal is by utilizing microbes. The biodegradation process

in color removal does not require large costs, the sludge produced is less, and of course it is friendly to the environment [6].

The use of microorganisms in the form of bacterial consortia results in better degradation rates than single bacteria, some bacteria that can decolorize azo dyes include *c*and *Bacillus cereus* [7]. *Bacillus subtilis* is a species known to be able to degrade synthetic dye wastewater, one of which can degrade azo dyes. *Bacillus subtilis* can produce lignin peroxidase (LiP), and laccase enzymes that can degrade complex aromatic compounds into simpler compounds so that they can degrade dyes [8]. *Bacillus subtilis* bacteria can absorb azo dyes optimally for up to 72 hours. After more than 72 hours, there is no significant absorption [9]. *Bacillus cereus* bacteria can degrade azo dyes at optimal conditions with a temperature of 37oC. *Bacillus cereus* bacteria proved to be optimal in degrading azo dyes [10]. *Bacillus cereus* bacteria can work in the absorption of azo dyes for up to 48 hours. The absorption carried out by *Bacillus cereus* runs gradually with a constant change in the percentage of absorption [11]. Batik waste comes from various types of different dyes, so the use of *Bacillus subtilis* and *Bacillus cereus* bacteria together can be a solution in the absorption of azo dyes.

Cell immobilization is a technique to hold microorganisms in a matrix. Immobilization techniques are used for pollutant remediation and have several advantages in the reuse cycle, more resistant to the environment, and easy to separate from the treatment solution [12]. Immobilized cells are more stable, this is because immobilization can prevent the release of bacterial cells from the matrix, can interact with harsh environments [13]. In addition, it can be used repeatedly, reducing the cost of replacing inactive cells [14].

In general, cell immobilization uses polyvinyl alcohol (PVA). PVA is a synthetic polymer that is also widely used in the immobilization process due to its elasticity, durability, high chemical stability, low material cost, and non-toxicity to microorganisms. PVA has sticky properties, so the beads will stick and clump together. The addition of sodium alginate can prevent clumping of the beads. Sodium alginate has a high adsorption [5]. Trapping with cross-linked sodium alginate is the easiest way of immobilization [15]. Sodium alginate (Na-alginate) is odorless, white to brownish yellow powder, tasteless, can form colloids, with 1% alginate concentration having a pH of 5-7.5. The solubility properties of sodium alginate are insoluble in acidic conditions but soluble in alkaline conditions. Seaweed cell wall components consist of alginate and fucoidan which are rich in anion polysaccharides, namely negatively charged ions, and are mostly mixed with salts (sodium, potassium, calcium, magnesium), non-toxic, biocompatible, and more hydrophilic [13].

In this research, we studied the preparation of microbial consortium immobilized to determine the effectiveness and influence of the use of immobilized microorganisms on the decolorization process of dyes in batik waste and to determine the effect of microbial composition and type of dye on the use of microorganisms on the decolorization process of dyes in batik waste.

# MATERIALS AND METHODS

## Materials

In this study, the materials used include batik waste, *Bacillus subtilis*, Bacillus Cereus, Nutrient Agar, Nutrient Broth, Potassium Permanganate, Oxalic Acid, Sulfuric Acid, Na-alginate, PVA (Polyvinyl Alcohol), CaCl2 solution, Thiosulfate, and distilled water. While the tools used consist of filter paper, beaker, Ose, gauze, syringe, dropper, incubator shaker, thermometer, funnel, watch glass, Erlenmeyer, measuring cup, test tube, fat cotton and spatula.

## Methods

### Bacterial culture

A total of 20 grams of nutrient agar was dissolved into 1 Liter of distilled water. The media is poured into a test tube as much as 5 mL and positioned obliquely. Wait for the media to turn solid. Plant bacteria on the media using an Ose. Store the media that has been planted for 24 hours.

### Growth curve generation

Liquid media consists of yeast extract 5 g/L, (NH4)2SO4 0.7 g/L, KH2PO4 1 g/L, CaCl2.2H2O 0.2 g/L, MgSO4.7H2O 0.15 g/L, ZnSO4.7H2O 2.5 g/L, MnSO4.H2O 0.8 g/L, and FeSO4.7H2O 0.7 g/L and batik waste in 1

Liter of distilled water. Then put the liquid media into a 100 ml Erlenmeyer glass as much as 40 ml. Enter the microbial culture into the Erlenmeyer using an Ose. Close the Erlenmeyer using cotton and gauze so as not to be contaminated. Put into an incubator with 140 rpm and 35oC temperature. Curve making is done by measuring the absorbance value of the solution with a UV-Vis Spectrophotometer at a wavelength of 600 nm every 4 hours within 48 hours.

### Immobilization on PVA Na-Alginate

The liquid medium was centrifuged at 10,000 rpm for 15 minutes to collect the bacteria in the sediment. PVA (12% w/v) and Na-alginate (3% w/v) were dissolved at 60°C. The PVA and Na-alginate solution was cooled and then mixed with bacteria (10% w/v), then added dropwise into a solution of boric acid solution (3% w/v) and CaCl2 (5% w/v) using a 50 mL syringe. After the microbial consortium was mixed with the matrix, the solution was stirred for 30-50 minutes to solidify, washed thoroughly with water and stored at 4°C for 24 hours.

### Decolorization of Batik Waste

The results of microbial immobilization as much as 2 grams were put in an Erlenmeyer containing 25 mL of 600 mg/L Reactive Black 5, 12,500 mg/L Orange II, and mixture of 600 mg/L reactive black 5 and 12,500 mg/L orange II solution. The flask was incubated at 30oC on a shaker incubator at 140 rpm for 72 hours. After the specified time, the solution was filtered, centrifuged and the dye concentration was monitored at the respective wavelengths using a spectrophotometer. The wavelengths of Orange II and Reactive Black 5 were 484 nm and 597 nm, respectively. The colour removal efficiency was calculated using equation (1). Where Ai and at represent absorbance at zero and time [16].

𝐷𝑒𝑐𝑜𝑙𝑜𝑟𝑖𝑧𝑎𝑡𝑖𝑜𝑛 (%) = 𝐴𝑖−𝐴𝑡 × 100% (1)

𝐴𝑖

### Screening electron microscope (SEM) analysis

SEM analysis was conducted to determine the morphology which is the shape or surface state of the microorganism consortium that has been immobilized on Na-Alginate.

### Fourier transform infra-red (FTIR) analysis

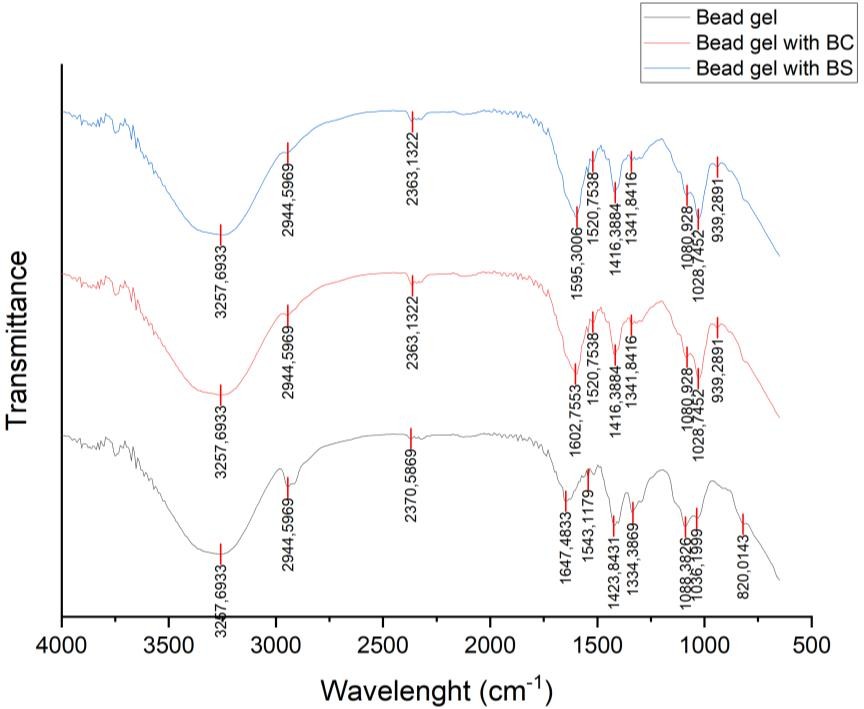
FTIR (Fourier Transform Infra-Red) analysis with transmittance mode was used to characterize the presence of certain chemical groups in the sample.

### Chemical Oxygen Demand Analysis (COD)

The COD test was conducted by taking a 100 ml sample into a 250 ml Erlenmeyer. Add KMnO4 0.01 N a few drops into the test sample until pink in color. Add 5 ml of 8 N H2SO4. The mixture is heated to 105°C. Pipette 10 ml of 0.01 N KMnO4 standard solution and continue heating to boiling. Boiling is done carefully for 10 minutes. After 10 minutes of boiling, 10 ml of 0.01 N Oxalic Acid was added. Excess Oxalic Acid is titrated with 0.01 N KMnO4 standard solution until a pink color appears.

# RESULT AND DISCUSSION

## Characterization of Gel Beads

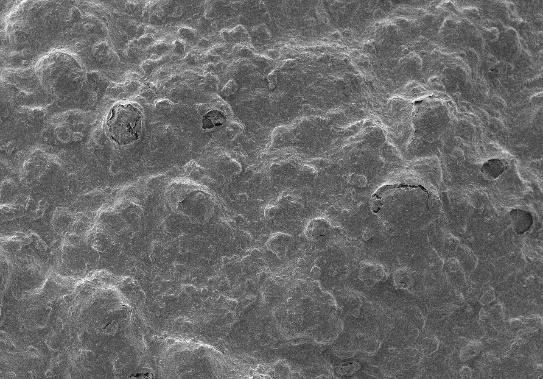


Wavelength

**Figure 1.** Fourier Transform Infrared (FTIR) Results

Comparison of Fourier Transform Infrared spectra on PVA-Alginate matrix, matrix containing Bacillus Cereus, and matrix containing *Bacillus subtilis* can be seen in **Figure 1**. Each spectra gives identical results. This shows that no reaction occurs in the matrix when bacteria are added. Na-alginate compounds contain hydroxyl, ether, and carboxyl groups [17]. The hydroxyl group (O-H) is visible at a wavelength of 3257 cm-1. At wavelengths of 1647 cm-1 and 1423 cm-1 indicate the presence of carboxylate compounds. There is a C-H hydrocarbon compound at a wavelength of 2944 cm-1. The C-O group is shown at a wavelength of 1334 cm-1. The wavelength of 1080 cm-1 indicates the presence of ether compounds (C-O-C). In the matrix containing bacteria, there are curves at wavelengths of 1602 cm-1 and 1595 cm-1 which indicate the presence of azo compounds (-N=N-) [18]. This is caused by the addition of dyes to the matrix containing bacteria so that the bacteria can adapt to the new environment.

To determine the characteristics of the bead gel, SEM analysis was also conducted. SEM (Scanning Electron Microscopy) analysis was used to evaluate the shape and surface structure of the beads produced. SEM is used as an important tool in the characterization of gel beads to determine the shape, size, composition, and quality of the beads produced.



**Figure 2.** SEM micrograph (150x) of beadgel surface morphology

**Figure 2** shows the scan results at 150x magnification. The pores on the bead gel surface are clearly visible, with a round shape indicating the presence of cells trapped inside the bead gel. The gel bead formed has a rough and dense surface, this is in accordance with the literature [19]. The pores formed show how the cross-linked polymer layer is formed inside the beadgel. This process of forming a cross-linked polymer layer allows for a denser structure, which is caused by the interaction between PVA-Na alginate [20].

## Effect of Bacteria and Dyes on Decolorization

Decolorization is a color removal process used to reduce the color density of effluent. Color decolorization is needed so that the discharged waste does not interfere with water quality and the surrounding environment. Based on the experiments that have been carried out, the decolorization percentage data is obtained in the following table:

**Table 1.** Percentage of Decolorization in Reactive Black 5 Dye

|  |  |
| --- | --- |
| **Variable (BC:BS)** | **% Decolorization** |
| 0:1 | 23,9882 |
| 1:0 | 19,18212 |
| 1:1 | 19,89882 |
| 1:2 | 22,47049 |
| 2:1 | 13,23777 |
| In the synthetic dye experiment, reactive black | 5 was examined using UV-Vis spectrophotometry with a |

wavelength of 597 nm. In **Table 1.** the highest decolorization percentage is found in the ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) 0: 1 with a decolorization value of 23.99% while the lowest decolorization percentage is found in the ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) 2: 1 with a decolorization value of 13.24%. *Bacillus subtilis* proved to be able to decolorize Methylene Blue dye which has similar properties to Reactive Black 5, which is carcinogenic, difficult to decompose, and belongs to the class of azo [8]. Based on these data, it is evident that *Bacillus subtilis* is able to decolorize textile dyes, including Reactive Black 5.

The effectiveness of decolorization at a ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) 0: 1 is high compared to other ratios due to the optimal ability of *Bacillus subtilis* to decolorize the dye for 72 hours [9]. compared to *Bacillus cereus* which is only able to optimally decolorize the dye for 48 hours [11]. The ability of *Bacillus subtilis* to decolorize is longer, resulting in a higher percentage of decolorization compared to other ratios.

**Table 2.** Percentage of Decolorization in Orange II Dye

|  |  |
| --- | --- |
| **Variable (BC:BS)** | **% Decolorization** |
| 0:1 | -67,321 |
| 1:0 | -17,589 |
| 1:1 | -24,018 |
| 1:2 | -67,411 |
| 2:1 | -1,163 |
| In the Orange II synthesized dye experiment, | it was examined using UV-Vis spectrophotometry with a |

wavelength of 484 nm. In **Table 2** the percentage value of decolorization is negative, a negative decolorization value indicates that there is no decrease in absorbance during measurement. In general, the negative value that occurs in the positive control and negative control indicates that there is no decrease in absorbance in the control variable [21]. In the Orange II synthesized dye experiment, it was examined using UV-Vis spectrophotometry with a wavelength of 484 nm. In **Table 2** the percentage value of decolorization is negative, a negative decolorization value indicates that there is no decrease in absorbance during measurement. In general, the negative value that occurs in the positive control and negative control indicates that there is no decrease in absorbance in the control variable [21]. In the Orange II dye experiment, NaOH was added so that the pH of the dye increased. This is caused by solution conditions that do not support bacterial growth. In addition, under alkaline conditions, the PVA-Alginate matrix can be damaged and dissolved so that the percentage value of decolorization becomes negative.

The PVA-Alginate matrix can be damaged and can dissolve because alginate levels that are too low can cause unstable gel beads. Alginate functions as a binder. If the alginate concentration is too low, the structure of the PVA- Alginate Matrix cannot be formed properly, so the Matrix is easily dissolved in water. Whether or not it dissolves also depends on the type of salt that binds it. Sodium salts, other alkali metals, and ammonia are examples of salts that can dissolve in water [22]. In the Orange II dye experiment, NaOH was added so that the pH of the dye dropped

below 7 [23]. In bacilus subtilis and bacilus cereus bacteria pH 7-8. this can also affect the percentage of decolorization, because the bacteria are not at the optimal pH for the bacteria to work

**Table 3.** Percentage of Decolorization Mixture Dyes

|  |  |  |
| --- | --- | --- |
| **Variable (BC:BS)** | **Wavelength (cm-1)** | **% Decolorization** |
| 0;1 | 484 | -10,667 |
|  | 597 | 7,280 |
| 1;0 | 484 | -12,5 |
|  | 597 | 35,302 |
| 1;1 | 484 | -10,667 |
|  | 597 | 23,901 |
| 1;2 | 484 | 7,5 |
|  | 597 | 20,467 |
| 2;1 | 484 | -21,833 |
|  | 597 | 3,434 |

Based on the data in **Table 3** the results of the percentage value with a wavelength of 484 nm with a ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) bacteria 1: 0 with a decolorization value of 4.3%. For the results of the percentage value with a wavelength of 597 nm with a ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) bacteria 1: 0 with a decolorization value of 35%. In Figure 4.4 the percentage of decolorization has a negative value. A negative decolorization value indicates that there is no decrease in absorbance during measurement [21]. This is caused by solution conditions that do not support bacterial growth as well as damage and dissolution of the PVA- Alginate matrix in alkaline conditions which causes the percentage value of decolorization to be negative. In addition, the dye experiment was given NaOH so that the dye was homogeneous, but this caused the pH of the dye to decrease. In *Bacillus subtilis* and *Bacillus cereus* bacteria pH 7-8. this can also affect the percentage of decolorization, because the bacteria are not at the optimal pH for the bacteria to work [23].

The effectiveness of decolorization at a ratio of *Bacillus cereus* (BC) and *Bacillus subtilis* (BS) 1:0 was highest compared to the other ratios at a wavelength of 484 nm and a wavelength of 597 nm. This could be due to the optimal ability of *Bacillus subtilis* in decolorizing the dye for 72 hours [9].

## Effect of Bacteria and Dyes on COD Levels

Chemical oxygen demand is one of the parameters used to determine water quality. According to the Minister of Environment and Forestry Regulation, COD levels that meet wastewater quality standards are 150 mg/L. Based on the experiments that have been carried out, data on the results of COD reduction are obtained in **Table 4**, **Table 5**, and **Table 6**.

**Table 4.** Percentage of COD Reduction in Reactive Black 5 Dye

|  |  |  |
| --- | --- | --- |
| **Variable (BC:BS)** | **COD (mg/L)** | **% Reduction** |
| 0:1 | 2308,174 | 86,469 |
| 1:0 | 2349,391 | 86,22737 |
| 1:1 | 2308,174 | 86,469 |
| 1:2 | 2473,043 | 85,50249 |
| 2:1 | 2947,444 | 82,72146 |

The COD level of Reactive Black 5 before the application of decolorization was 17058.408 mg/L. After the decolorization process there was a significant decrease in COD levels. The results of COD reduction on reactive black 5 are shown in **Table 4**. In the 0:1 and 1:1 variable there was a decrease in the best COD levels of 86.47%. Meanwhile, the 1:0, 1:2, and 2:1 variable resulted in a decrease in COD levels of 86.23%, 85.5%, and 82.72%, respectively.

The waste decomposition process requires the help of cellulose enzymes to break the glycosidic bonds. *Bacillus subtilis* and *Bacillus cereus* are types of bacteria that can produce cellulose enzymes so that they can help reduce COD levels. The Bacillus genus of bacteria can be used to decompose proteins and is effective for cleaning carbon in water [24]. In the decolorization process, protease and azoreductase enzymes play more roles. These enzymes will break the azo bond in reactive black 5. Reactive black 5 will be degraded into Butyl cyclohexane sulfonate, 4-

(hydroxymethyl) phenol, and 4-hydroxy-3,4-dihydronaphthalene-2,7-disulfonic acid [11]. However, the COD levels obtained after the decolorization process still do not meet the quality standards.

**Table 5.** Percentage of COD Reduction in Orange II Dye

|  |  |  |
| --- | --- | --- |
| **Variable (BC:BS)** | **COD (mg/L)** | **% Reduction** |
| 0:1 | 6943,498 | -3,457 |
| 1:0 | 7226,906 | -7,679 |
| 1:1 | 6943,498 | -3,457 |
| 1:2 | 7410,619 | -10,417 |
| 2:1 | 7130,973 | -6,25 |

. The results of COD reduction on reactive black 5 are shown in **Table 5**. Decolorization of Orange II dye type does not have a significant effect on COD levels. There is no decrease in COD levels in each variable used. In the 0:1 variable, there was an increase in COD levels of 3.46%, the 1:0 variable increased by 7.68%, the 1:1 variable increased by 3.46%, the 1:2 variable increased by 10.42%, and the 2:1 variable increased by 6.25%.

The decolorization process in Orange II dyes also occurs with the help of protease and azoreductase enzymes that break the azo bond so that it can be degraded. Orange II degradation will produce 1-amino-2-naphthol and sodium 4-amino-benzene sulfonate [9]. The absence of a decrease in COD levels is due to waste conditions that do not support the development of bacteria. Similar results were also obtained in the dye mixture variable. *Bacillus subtilis* bacteria can grow at an optimal pH of 7 [25], while *Bacillus cereus* bacteria can grow at an optimal pH of

4.5 to 9.5 [26]. In making orange II synthetic dye, it is necessary to add NaOH so that the naftol dye can dissolve in water. NaOH is added as much as 50% by weight of naftol or 0.16 M. The addition of NaOH with a concentration of

0.16 M will produce an alkaline solution pH and is not in accordance with the optimal conditions for bacterial growth.

**Table 6.** COD Reduction in Mixture Dyes

|  |  |  |
| --- | --- | --- |
| **Variable (BC:BS)** | **COD (mg/L)** | **% Reduction** |
| 0:1 | 18809,524 | -5,932 |
| 1:0 | 20163,81 | -13,559 |
| 1:1 | 21217,143 | -19,492 |
| 1:2 | 17455,238 | 1,695 |
| 2:1 | 18659,048 | -5,085 |

Based on **Table 6**, the same thing also happened to the decolorization results of the Reactive Black 5 and Orange II dye mixture. In the variables 0:1, 1:0, 1:1, and 2:1 there was an increase in COD levels by 5.93%, 13.56%, 19.49%, and 5.09%, respectively. While in the 1:2 variable there was a slight decrease in COD levels by 1.7%.

The decrease in COD levels that are not optimal is also caused by solution conditions that do not support bacterial growth. In addition, in alkaline conditions, the PVA-Alginate matrix can be damaged and dissolved, thus increasing COD levels in the solution [27].

# CONCLUSION

From the research that has been done, it can be concluded that *Bacillus subtilis* and *Bacillus cereus* bacteria have the potential to remove reactive black 5. Decolorization is done by mixing the matrix containing bacteria into the dye solution. Decolorization was carried out at a temperature of 30oC and a rotation speed of 140 rpm for 72 hours. The best percent decolorization result was obtained with the variable *Bacillus cereus* and *Bacillus subtilis* 1:0 in the color mixture variable with a wavelength of 597 cm-1, which was 35.3%. However, in this variable there was no decrease in COD levels. The second best variable is the 0:1 variable on reactive black 5 dye with a percent decolorization of 23.99%. It is necessary to adjust the operating conditions so that the bacteria can grow and degrade the dye more effectively. In this variable there was a decrease in COD levels by 86.47%. From the results that have been obtained, it can be used as a reference for further development to produce methods with better percent decolorization.

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