# Crude Enzyme of Aspergillus sp. 3 Immobilized in Chitosan-Beads to Decolorize Batik Effluent

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# Crude Enzyme of *Aspergillus* sp. 3 Immobilized in Chitosan-Beads to Decolorize Batik Effluent

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**Abstract.** Batik is one of Indonesian's cultures which has a unique symbolic meaning and has high aesthetic value for the Indonesian. The number of industries engaged in this business will bring new problems to the surrounding environment because batik effluent can pollute the river. This untreated dye effluent is very dangerous and can damage the environment because it is toxic, carcinogenic, and even mutagenic. One of the effluent treatment methods is by a biological method. The indigenous *Aspergillus* sp. 3 fungi are isolated from batik effluent, taken from the batik industry in Banyumas regency. The utilization of fungi for effluent treatment can be done by adsorption and enzymatic method. Degradation using enzymes is known to be more effective. Aspergillus fungi contain ligninolytic enzymes. Ligninolytic enzymes play an important role in degrading lignin on lignocellulosic substrates. This research is aimed to apply fungal enzyme immobilization for decolorization of batik effluent. Chitosan-based beads components are made with a combination of chitosan, STPP 2%, and phosphate buffer. Enzyme immobilization is done by immersing the chitosan solution in the Ligninolytic enzyme solution. Ligninolytic enzymes that are immobilized into chitosan will form beads that will be dissolved into batik effluent. The development of enzyme immobilization techniques is applied to batik effluent with a percentage of effluent decolorization until 96,8%. The best treatment results can reduce the value of Total Dissolved Solids (TDS) from 16,500 mg/L to 4005 mg/l and can also reduce the pH value of the effluent.

## 1 Introduction

Batik is one of Indonesian's cultures which has a unique symbolic meaning and has high aesthetic value for the Indonesian. The official recognition from UNESCO for batik, positively correlated with the number of requests. The government has advised civil servants to wear batik on certain days, especially on the commemoration of National Batik Day. The general public is also increasingly proud to use batik, both for the old and the youngster [1]. There are various types of batik in Indonesia, including written, stamped, and printed batik [2]. Batik making is inseparable from natural and synthetic dyes. Natural dyes come from animals (lac dyes) or plants such as roots, stems, leaves, skin and flowers. Meanwhile, synthetic dyes come from chemicals such as indigosol, naphtol, rapid, base, indanthreen, procion, and others [3].

The high demand for batik has made more and more batik businesses emerge. The number of companies engaged in this industry will bring new problems to the surrounding environment because batik effluent can pollute the river. Based on the results of data collection, it is known that the value of batik production in Indonesia reaches 407.5 billion rupiah per month or the equivalent of 4.89 trillion rupiah per year. Most of the batik industry in Java uses artificial dyes [4].

Efforts that can be made in overcoming the problem of batik effluent are establishing a wastewater treatment plan (WWTP). The process of building WWTP needs funding, either from self-help in the batik industry, utilization of local government budget or Corporate Social Responsibility (CSR) funds. This process is important because factual conditions prove that the batik industry's awareness of managing effluent is still low. In fact, Pekalongan Regency, one of the batik centers in Indonesia, the WWTP has actually been built but is currently not used by the existing batik industries [4].

Chitosan [poly((b-1-4)2-amino-2-deoxy-Dglucose)] is a deacetylation form of chitin that contains more than 5000 glucosamine units and is the second most important biopolymer in the world after cellulose. The amine and hydroxyl groups in chitosan have the ability to covalently bond with metal ions and act as chelating agents. Chitosan can absorb pollutant heavy metals such as lead (Pb) due to the presence of amine and hydroxyl groups which are Lewis basic (electron pair donors). Chitosan will exchange protons owned by polluting metals with electrons that are owned by nitrogen (N) [5]. Chitosan has a $-NH_2$ group which has a pair of free electrons that are reactive to other compounds so that it makes chitosan easy to modify. One form of modification of chitosan is to change chitosan into beads thus the structure of chitosan is more organized. Beads are one of the adsorbents that is frequently used in the adsorption process. This is due to the ease of which silica is produced and the surface properties (pore geometric structure and chemical properties on the

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surface) which can be easily modified. Therefore, in this study, beads are made from chitosan [6].

The treatment of effluent containing dyes, including those from the batik industry, is usually processed conventionally among others, by means of chlorination, deposition, absorption with activated carbon or using biological agents. The method consists of chlorination and deposition, the sediment formed is usually burned which will result in the formation of chloroxide and carbon dioxide compounds, whereas the use of activated carbon can only absorb organic pollutants that have low molecular weight, while compounds with high molecular weight are not eliminated. Microbiological processing can only decompose compounds that are biodegradable while non-biodegradable compounds will remain in sediment or sludge which will return to the environment. Therefore it is necessary to develop a more effective technology to reduce the concentration of dye in wastewater, one of which is the immobilization method of enzymes with chitosan [7].

*Aspergillus* sp. 3 produces extracellular enzymes such as ligninolytic enzymes consisting of cellulase, xylase, and ligninase, which are the main enzymes capable of breaking down lignin and cellulose waste. Ligninolytic enzymes play a role in degrading lignin on lignocellulosic substrates. This enzyme is also able to degrade various recalcitrant compounds and complex pollutants such as dyes [8]. Ligninolytic enzymes can remodel dyes through redox reactions, where ligninolytic enzymes will completely oxidize carbon compounds to $CO_2$ and $H_2O$. [8]. Ligninolytic enzymes can remodel dyes through redox reactions, where ligninolytic enzymes will completely oxidize carbon compounds to $CO_2$ and $H_2O$ [8].

In this experiment, batik effluent was decolorized using the enzyme immobilization method on chitosan-based beads. In the batik-making process, the types of blue dyes commonly used are indigosol blue and naphthol red synthetic dyes, which are disazo-aromatic chemical compounds that are carcinogenic. This research was conducted with the aim of applying the fungal enzyme immobilization technique in the batik effluent decolorization process using the chitosan-based beads method.

## 2 Methods

The materials used in this study included isolates of *Aspergillus* sp. 3, 2% chitosan, 1% acetic acid, 2% Sodium Tripolyphosphate (STPP), a solution of NA-Buffer Phosphate 0,5 M, Potato Dextrose Broth (PDB), as well as indigosol blue and naphthol red batik effluent. Meanwhile, the tools used include incubator shakes, spectrophotometer, pH meter, syringe, and glassware.

### 2.1 Samples and Chemicals Preparation

The samples of Indigosol Blue 04B (IB) batik effluent used for the bioremediation in this investigation were collected from several domestic batik effluent discharge drains and kept in a refrigerator at 4°C. The indigenous fungal isolates used were *Aspergillus* sp. 3, isolated from dye effluent soil and batik effluent.

### 2.2 Preparation of culture condition

The isolates were maintained in a PDB medium at room temperature, then isolates of *Aspergillus* sp. 3 were incubated using an incubator shaker. The lignolytic enzyme from the isolate was taken to be immobilized with chitosan-based beads.

### 2.3 Preparation of chitosan based beads

The preparation of chitosan beads was carried out at a ratio of enzyme: chitosan (1: 3) with a fixed volume of 10 mL, so that the amount of enzyme added was 2.5 ml and chitosan was 7.5 mL. The concentration of chitosan used is 2%, meaning 2 grams in 100 mL of solution, where the solvent used is 1% acetic acid. Making beads is done by taking a solution (enzyme-chitosan mixture) using a syringe and then slowly dropping it into a 20 mL solution of 2% STPP solution, meaning 2 grams in 100 mL of distilled water. Then the bead droplets were left to stand for 120 minutes of contact time. The formed beads are then filtered and stored in a solution of 0.5 phosphate buffer then stored in a refrigerator and ready to be used for the decolorization process.

### 2.4 Decolorization of chitosan based beads.

This test was carried out by adding beads to Indigosol Blue batik effluent (pH 8) with a concentration of 10% (10% Batik effluent and 90% additional water), 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% (100% Batik effluent without additional water). Those samples are incubated. Beads were added as much as 3 g per mL of effluent, meaning 30 beads for 10 mL of effluent solution. Incubation was carried out using a shaker at 150 rpm for 3x24 hours. The decolorization results were measured using a spectrophotometer at a wavelength of 604 nm (Dewi et al., 2019) [7]. The percentage of decolorization is measured by the formula (1).

$$D(\%) = (i\text{-}f/i \quad (1)$$

D = Decolorization
i = Initial absorbance
f = Final absorbance

The best decolorization results are used to measure pH and TDS value.

### 2.5 The effect of beads on decolorization in different types of batik effluent

The effect of beads on decolorization in different types of waste was carried out. Beads were tested on 2 types of batik effluent (Indigosol Blue and Naphthol Red). Determination of the percentage of decolorization is as much as 5 ml of batik effluent samples that have undergone decolorization and have been separated from the mycelium solids are taken and the absorbance is

measured using a UV-VIS spectrophotometer. At this stage the mycelium in the liquid medium is added with the effluent. Decolorization was carried out at 25% and 50% effluent concentration.

## 3 Results and discussion

Chitosan can be modified into various forms, namely flakes, granules, gels, fibers and membranes. One of the modifications of chitosan is into the form of granules (beads). This modification is through a swelling process, namely by dissolving chitosan in acetic acid until a gel is formed. As a result of this study, chitosan was used as an immobilization material for enzymes secreted by *Aspergillus* sp.3. These unpurified enzymes (crude enzymes) were successfully immobilized by chitosan, characterized by their white color and 2-5 mm in diameter (Fig.1).

**Fig. 1.** Crude Enzyme of *Aspergillus* sp. 3 Immobilized in Beads.

Chitosan is used as a supporting matrix in this study because it is better than other supporting matrices. Lipase which was crosslinked to chitosan was able to produce biodiesel while lipase immobilized in silica [10]. These beads can serve as an enzyme immobilization matrix. Enzyme immobilization is a process of entrapment of enzymes into a polymer matrix or binding of enzymes to a carrier material. Enzyme immobilization serves to maintain the stability of the enzyme without reducing the catalytic activity of the enzyme [11].

Observations on color parameters began by observing the color content of batik effluent. Color can be observed visually by comparing the color of the water sample with the untreated Indigosol Blue effluent. Enzymes immobilized in chitosan beads were able to decolorize batik effluent in various effluent concentrations (Fig 2. a,b). The concentrated effluent turned clear. One of these color changes is thought to be done due to the binding of the effluent dye to the chitosan beads. This is proven by the surface of the beads that look blue from the previous white color (Fig 2. c,d)

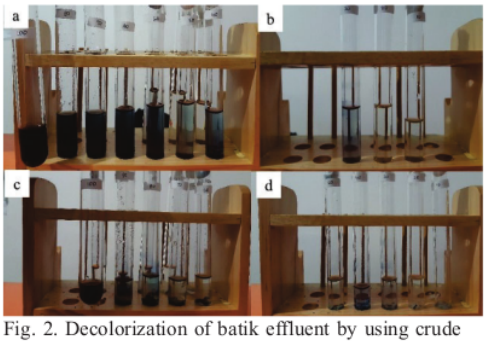


Fig. 2. Decolorization of batik effluent by using crude chitosan immobilized enzyme in the form of beads. Color reduction in each treatment (a, b), dye adsorption on beads in each treatment (c, d).

**Table 1.** Spectrophotometry Result of Batik Effluent Decolorization.

| Concentration of Effluent (%) | Initial Absorbance | Final Absorbance | % Decolorization |
|---|---|---|---|
| **10** | 0.239 | 0.200 | 16.3 |
| **20** | 0.267 | 0.188 | 29.6 |
| **30** | 0.258 | 0.196 | 24.3 |
| **40** | 0.320 | 0.266 | 16.9 |
| **50** | 0.587 | 0.182 | 68.9 |
| **60** | 0.808 | 0.184 | 77.2 |
| **70** | 1.318 | 0.177 | 86.6 |
| **80** | 1.877 | 0.187 | 90.03 |
| **90** | 5.324 | 0.184 | 96.5 |
| 100 | 5.324 | 0.169 | 96.9 |

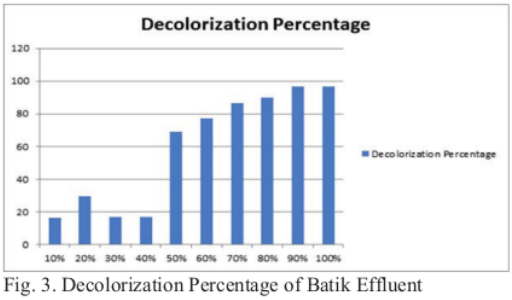


Fig. 3. Decolorization Percentage of Batik Effluent

**Table 2.** pH and reduce TDS of batik effluent before and after the optimum treatment.

| No. | Parameter | before | after |
|---|---|---|---|
| 1. | pH | alkali | neutral |
| 2. | TDS | 16.500 mg/L | 4.050 mg/L |

From this research, the results obtained that the highest percentage of decolorization is 96.8% at 100% effluent concentration (Table 1, Fig.3). The treatment resulted in a change in the color of Indigosol Blue effluent from dark blue to visually clearer. Apart from the adsorption process on chitosan, this is due to the role of the enzyme produced by *Aspergillus* sp.3. Enzymatic decolorization mechanism by fungi involved extracellular enzymes complex secreted by the fungus into the cultivation medium. The enzyme is lignin peroxidase (LiP), manganese dependent peroxidase (MnP), glucose oxidase 1, glucose oxidase 2, phenol oxidase, and laccase. The presence of these enzymes which are nonspecific to the substrate allows the fungus to decolorize batik effluent. This enzyme is responsible for breakdown of aromatic bonds in color compounds complex [12].

The decolorization results are supported by other parameters. Enzymes in the beads matrix can lower the pH and TDS value. The best treatment results can reduce the value of TDS from 16,500 mg/L to 4005 mg/l and can also reduce the pH value of the effluent (Tabel 2). This happens because the decolorization process reduces the value of turbidity and lowers the pH which was originally alkaline to neutral. The content of solids in the waters can be measured based on the total dissolved solids. TDS is defined as the content of various solutes (both organic, inorganic, other materials) with a diameter of < 10-3 m contained in a solution that is dissolved in water. The most common ions are calcium, phosphate, nitrate, sodium, potassium, magnesium, bicarbonate, carbonate and chloride. in this case there is a decrease in the value of TDS due to a decrease in solutes.

In this study, the effect of beads for decolorization on different types of waste was also carried out. Observation of color parameters was carried out on samples of other types of effluent, using a preliminary test of the color content of batik effluent. Color can be observed visually, by comparing the color of the sample water with the standard color. Water that has a low turbidity value usually has the same visible color value and actual color as the standard. The decolorization process of the two isolates was observed visually. The treatment resulted in the color changes of the effluent from dark blue to clear (Indigosol Blue) and marron to reddish clear (Naphthol Red). The color of the media in isolate 1 experienced a significant change in each treatment. At a lower concentration the color is clearer. The results of visual observations showed that the lower the concentration the higher the decolorization (Fig. 4).

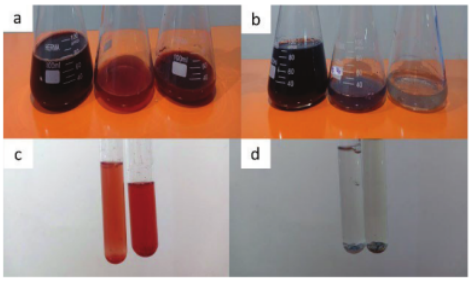


**Fig 4.** The Results Comparison of Visual Decolorization of Naphthol Red (a,c) and Blue (b,d) effluent using *Aspergillus* sp. 3

**Table 3.** Decolorization of *Aspergillus* sp. 3.

| Effluent Concentration (%) | Decolorization Percentage of Indigosol Blue | Decolorization Percentage of Naphthol Red |
|---|---|---|
| 25% | 94% | 63% |
| 50% | 91% | 61% |

Based on Table 3, the solution with 25% concentration of Indigosol Blue resulted 94% of decolorization which is the highest. Meanwhile 50% concentration of Indigosol Blue resulted 91% of decolorization. The solution with 25% concentration of Naphthol Red resulted 63% of decolorization which is the highest. Meanwhile 50% concentration of Naphthol Red resulted 61% of decolorization. The decolorization process happened due to the metabolic activity with the enzymatic system. *Aspergillus* sp. 3 can decolorize Indigosol Blue batik effluent with an enzymatic system [13]. Ligninolytic enzymes immobilized using alginate succeeded in decolorizing batik effluent within 24 hours by 94.867% [14]. Apart from the secretion of enzymes in their metabolism, the decrease in color intensity due to the activity of fungal isolates was caused by two things, namely due to adsorption. Fig. 4c,d have shown the presence of adsorption on the surface of the beads. *Aspergillus* sp. biomass immobilization was able to decolorize indigosol blue batik effluent with the addition of tannic acid at 24, 48, 72 hours in agitation treatment 78.8%, 84%, 80.1%, respectively [15].

In addition, by comparing these two dye batik effluent, it can be seen that the indigo dye that was degraded using this fungal isolate had a higher degradation value than Naphtol red. This proves that Indigosol Blue effluent is easier to decompose than Naphtol Red effluent, or it can be said that Indigosol Blue effluent is more easily degraded than Naphtol Red effluent. The growth (colony diameter and dry weight of mycelium) of *Aspergillus* sp. and *Penicillium* sp. from spent mushroom was lower in Brown Naphthol than Indigosol Blue effluent batik [16].

The results show that these isolates can decolorize batik wastewater in a relatively wide pH range. This indicates that the fungus is suitable for practical treatment of dye wastewater. One mechanism for

decolorization is the presence of an enzyme, while pH was one of the factors that influence enzyme production. Enzyme system was one of the mechanisms of fungi involved in dye decolorization. Factors that need to be considered in the enzymes production by microorganisms include pH. Based on this research, information was obtained that optimal pH required for maximum decolorization [16]. The indigosol blue has pH 6, meanwhile Naphthol Red has pH 8. The factors affecting enzyme activity are enzyme concentration, substrate concentration, pH, temperature, and presence of inhibitors. Judging from the work of the enzyme ligninolytic, this enzyme works independently optimum at pH 3-4 and continues to decrease the pH value of the buffer increases. The optimum laccase enzyme is at pH 3-4 and a temperature of 35°C. Changes in pH have an effect on ionization change of the acid side chain amino on the active side. It plays a role in keeping the active side of the enzyme in conformation binds to the substrate and changing the substrate into a product. Acidity (pH) effect on amino acids building blocks of enzyme proteins. Carboxyl group amino acids tend to bind ions $H^+$ in an acidic atmosphere [17,18].

Differences in the results of effluent decomposition in the treatment of effluent types and different concentrations also occurred in the results of previous studies and other studies. Mycelium growth of *Aspergillus* sp. 3 which were grown in purple Indigosol, Green Indigosol, and Naphthol dyed batik effluent at 10x dilution was better than 5x dilution. Mycelium growth of *Aspergillus* sp. 3 is not very optimal at more concentrated effluents or at higher concentrations [19]. Immobilization of 3 types of fungi biomass almost complete to decolorize of different dyes classes (azo and triphenylmethane dyes) after 24 h [20]. Microbes are more tolerant of natural and synthetic dye residues of the indigo textile effluent type [21]. This is presumably due to differences in the structure of the dye. Based on their chemical structure, textile dyes are divided into several types, namely in terms of color formation: chromophore groups [22,23], long chain organic compounds [24]. The aromatic complex structure of dyestuffs makes it is recalcitrant and stable so it is difficult to degrade [25, 26]

## 4 Conclusion

The conclusion of this research is that the fungal enzyme immobilization technique using the chitosan-based beads method was successfully applied for batik effluent decolorization. Chitosan bead enzymes can decolorize batik effluent with the highest value being 96.8% at 100% effluent concentration. In different types of effluent, Indigosol Blue effluent is more easily decomposed than Naphtol Red waste by using chitosan enzyme beads.

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