Screening of microfungi from spent mushroom for decolorizing and removing heavy metals from batik effluent and its toxicity

by Ratna Dewi

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Abstract. The concentrated colors and heavy metals contained in batik waste. Therefore, decolorization and removal of heavy metals is required. The purpose of this study was to screening microfungi for decolorizing and removing heavy metals from batik effluent, and obtaining information about the toxicity of the treatment results. Obtained 3 genera isolated from spent mushrooms, namely *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp. The superior microfungi for decolorizing and removing heavy metals from batik effluent is *Penicillium* sp., and its isolate can reduce the toxicity after the treatment results.

1. Introduction

The batik industry has progressed quite rapidly since the entry of Indonesian batik in the Objects Masterpieces of the Oral and Intangible Heritage of Humanity by The United Nations Educational, Scientific and Cultural Organization (UNESCO). Along with these advancements the batik industry has a negative impact. Its effluent has a negative effect on the environment and public health. The batik industry can produce contaminants that can damage the ecosystem that comes from the dyes used. The batik industry can produce contaminants which can damage the ecosystem that comes from the dyes used.

The heavy metals contained in batik effluent. The highest metal content in the batik effluent was Zn, which is 8.279 mg/L [1]. Heavy metals are high molecular weight metal elements. Heavy metals, in low levels are generally toxic to plants and animals, including humans. Based on the Decree of the Minister of Environment No. KEP-51 / MENLH / 10/1995 of 1995 concerning quality standards for effluent for industrial activities, the quality standard of Zn metal in industrial effluent is 5 mg/L [2].

Pollutants in batik effluent can be either organic or inorganic compounds, namely carbohydrates, proteins, fats, surfactants and aromatic organic substances (dyes and heavy metals, dyeing substances, alkalis, acids and salts) whose levels exceed the quality standards set by the government. The color content caused by 185 mg / L exceeds the government's quality standard of 50 mg / L [3].

Very small amounts of color in water (10-50 mg / l) have a negative effect on aesthetic values, water clarity and solubility of gases in water bodies [4]. Batik effluent always affects the receptors of adjacent water bodies so that it has a negative impact on health if it consumes polluted water. If absorbed in the human body through drinking water which is affected by effluent from water bodies and accumulates in the human body through the food chain, it causes toxicity and carcinogenicity. Exposure to chemicals, especially dyes, to the skin can cause irritation and allergies with symptoms of

itching, dry and reddish skin, and cracks. This skin damage will facilitate the entry of chemicals, especially those that are toxic, into the body. In addition, organic compounds resulting from the batik industrial process are toxic so that they endanger health [6]. Therefore it is necessary to have processing to eliminate its toxicity.

The processing of batik effluent can use physical methods, for example ultrasonification and chemistry, for example coagulation. This method is effective, but requires high costs and can produce secondary waste that is harmful to the environment. The biological method is a more profitable method because it is simpler, cheaper, environmentally friendly, and does not produce secondary waste in the form of large amounts of sludge sedimentation. Biological processing is by utilizing microbial activity to degrade the content contained in the waste into non-toxic materials or give a low pollution effect [7,8]. One of the microbes that can be used for effluent treatment is fungi.

Fungi were chosen as decolorizing agents that are able to degrade toxic color components because they have the ability to transform, changing from hazardous chemicals in waste to less or harmless forms [4,9]. The advantages of using a fungus as a decolorizing agent are that it is more economical, easy to obtain and safer to use as a decolorizing agent. Therefore, it is necessary to look for superior fungi as agents for removing the color of batik effluent in practical forms, namely the product of a formula that can be applied in industry as well as its toxicity effect on living things in the environment.

Fungi can interact with heavy metals in the biosorption process in two ways, namely depending on cell metabolism and not depending on cell metabolism. A process that depends on cell metabolism, the absorbed metals enter across the cell membrane. Transport of heavy metals across fungal cell membranes has the same mechanism as the transport of essential metal ions such as Ca, Mg and K. Processes independent of cellular metabolism can occur through physical adsorption and ion exchange. The cell walls of fungi, which consist of polysaccharides, proteins and functional groups such as carboxylates, hydroxyl, sulfates, phosphates and amino acids, can bind to heavy metals [10].

Fungi can degrade dyes and heavy metals [11]. *Aspergillus* sp. has the ability to decolorize Reactive Blue dye by 95% [12]. *Aspergillus flavus* was able to decolorize and detoxify Malachite Green dye by 97.43% [13]. *P. ostreatus* was able to decolorize the Cibacron Black W-NN dye by 100% [14]. The percentage of batik effluent decolorization by *Penicillium* sp. amounted to 24.304% [15].

The baglog spent mushroom that has been studied by [16][17] is able to decolorize indigosol yellow dye effluent. Contaminant fungi found in spent mushrooms, namely the genus Aspergillus, Fusarium, Paecillomyces, Penicillium, Rhizopus, Syncephalastrum, and Trichoderma [18]. These fungi have the potential to reduce metal levels and decolorize batik effluent. The advantages of using fungal mycelium found in spent mushroom for the decolorization process and metal absorption in batik effluent are because it is more economical, easy to obtain and safer to use as a decolorizing agent.

This research is an initial description that spent muhroom overgrown with contaminant fungi can be used to decolorize batik effluent, but further research is needed on the effectiveness of isolated fungal cells, given that the growing media for fungi contains lignin, it is expected that the growing fungi have lignin-degrading enzymes. It is known that fungi that can degrade lignin are able to degrade other xenobiotic compounds. The purpose of this study was to screening microfungi for decolorizing and removing heavy metals from batik effluent, and obtaining information about the toxicity of the treatment results.

2. Method

2.1. Materials and apparatus

The tools and instrument used in this research are glassware, pH meters, UV-Vis spectrophotometers, UV lamps, filter paper, petri disk, Erlenmeyer flask AAS (Atomic Absorption Spectrophotometry) and

the materials that used were Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), wikolazka medium. Batik effluent is collected in the Batik home industry at Sokaraja Banyumas.

2.2. Making Potato Dextrose Agar (PDA) medium [19]

A total of 200 grams of peeled potatoes are cut into small pieces then boiled in 500 ml of distilled water and filtered. So that as much as 20 grams is mixed with 250 distilled water then heated. The agar that has dissolved in distilled water is then mixed with boiled potato water and added 20 grams of dextrose. The medium mixture is added with distilled water until it reaches a volume of 1000 ml, the pH is made neutral (pH 7). The medium is then put into Erlenmeyer and corked with cotton, then sterilized using autoclave at a temperature of 121^{o} C with a pressure of 2 atm for 20 minutes.

2.3. Isolation and identification of microfungi from spent mushroom P. ostreatus

Isolation was carried out by direct planting using PDA medium. Spent mushroom *P. ostreatus* selected the middle part which is not exposed to the environment, then cut into cubes with a size of 1 cm x 1 cm. The samples were placed in a petri dish containing 10 ml of PDA medium which had been previously given chloramphenicol, then incubated at room temperature for 7 days. The isolated isolates were then purified in a petri dish containing new PDA medium until a pure culture was obtained (single isolate) [20].

Identification of isolates using isolate morphological characters. Observation of morphological characters includes macroscopic observations (colony morphology) and microscopic observations (cell morphology). Macroscopic observations include colony color, reverse color, colony surface texture, colony type. The fungal isolates were then characterized using the identification book [21].

2.4. Screening of microfungi using solid medium to decolorize dye

The selection of fungi on a solid medium that has the potential to decolorize the batik effluent was carried out after the isolates were obtained from the isolates. Selection by test on wikolazka medium (10 g Glucose; 0.25 g Yeast extract; 2 g KH_2PO_4; 0.5 g $MgSO_4x7H_2O$; 0.8 Mm $MnCl_2x7H_2O$; 0.1 g $CaCl_2$; 0.5 g Ammonium Phosphate; 20 ml Sodium acetate, 20 g agar) which is exposed to the dye. Each fungal isolate measured the diameter of the clear zone and the diameter of the colonies formed every day up to 10 days of incubation, then measured the dry weight. The best results are used as superior fungi in the selection process.

2.5. Decolorization of different batik effluent

At this stage a different batik effluent was used, namely Naphtol and Indigosol. 5 plugs (circle of fungal isolates made with a cork drill with a diameter of 5 mm) the best selected fungal isolates were inoculated into Erlenmeyer flask (250 ml) containing 100 ml of medium aseptically. The medium was covered with cotton and coated with a wrapper, then incubated using a shaker at a speed of 55 rpm at room temperature for 5 days. After forming the fungal isolate pellets, then inserting batik effluent into the cultivation medium and incubating it using a shaker at a speed of 55 rpm at room temperature for 8 days. The incubated effluent was analyzed for color change by comparing it with a spectrophotometer. The medium with the greatest color change intensity was defined as the best cultivation medium [22][23][24].

Color analysis expressed as percentage of decolorization. Decolorization measurements were carried out in each treatment using the spectrophotometric method (using the U-1900 UV-VIS spectrophotometer). The absorbance of the effluent samples before and after treatment was measured using a spectrophotometer at a wavelength of 645 nm. The percentage of decolorization is measured according to these following formula [25]:

$$\text{Decolorization \%} = \frac{(\text{Initial absorbance} - \text{Absorbance at after treatment})}{\text{Initial absorbance}} x\ 100$$

2.6. Analysis of heavy metals

2.6.1. Distruction of heavy metals (SNI 2004; 06-6992.8-2004). A total of 50 ml of batik effluent, namely samples without mycelium treatment (before) and samples that had been treated with mycelium (after), were heated until their volume was less than 20 ml, then added 10 ml of HNO3 and 2 ml of H_2O_2. After that it is heated again until the volume is 10 ml. The batik effluent is filtered using Whatman no. 42 then added distilled water until the volume reaches 50 ml.

2.6.2. Analysis of heavy metal content [26]. Analysis of the metal content of Zn, Cu, Cd using a set of AAS (Atomic Absorption Spectrophotometry) with a sensitivity level of 0.003.

2.7. Preliminary toxicity test

The preliminary toxicity test basically aims to reduce the risk of harm posed to humans, so that the toxicity test in this study was carried out on test animals because of ethical constraints, not allowing direct toxicity tests on humans. Toxicity test is carried out by a test to determine the potential of a compound as toxic, to recognize the biological / environmental conditions for the emergence of toxic effects. This test is performed on the survival rate of the fish.

3. Result and discussion

3.1. Fungi isolation of spent mushrooms

Fungal isolates were obtained from the Spent mushroom of *P. ostreatus* (Figure 1). The results of the research that had been carried out regarding the isolation of fungi were obtained 5 isolates of contaminant fungi from the spent mushroom of *P. ostreatus* fungi in addition to the *P. ostreatus* fungi itself (Table 1). Besides containing mycelium *P. ostreatus* also contains other fungal mycelium, namely contaminant fungi. Isolation was carried out by planting directly on the part of the contaminated spent mushroom using PDA medium. The isolated isolates were then purified in a petri dish containing the new PDA medium until a pure culture (single isolate) was obtained. These functions are assumed to have a role in the decolorization process.

Figure 1. Spent mushroom *P. ostreatus*

3.2. Identification of fungi

Identification is done by observing the morphological characteristics of the fungus both macroscopically and microscopically. The results obtained three kinds of fungi contained in spent mushroom consisting of 1 isolate of *Penicillium* sp., 2 isolates of *Trichoderma* sp., and 2 isolates of *Aspergillus* sp. According to Naiola (1993) the fungi that can grow on the spent mushroom include *Coprinus* sp., *Penicillium* sp., and *Aspergillus* sp. The contaminant fungi found in the mushroom growing medium in 7 genera, namely Aspergillus, Penicillium, Paecillomyces, Trichoderma, Rhizopus, Fusarium and Syncephalastrum [27]. Microbes isolated that from tropical Peatland that could have potential for applications [28].

Identification is done by observing the morphological characteristics of the fungus both macroscopically and microscopically. The morphological characteristics of the four fungi can be seen in Table 2. Morphological characteristics that can be observed from the three fungi are as follows, the colony is black, the colony is reverse color white, the surface texture of the colony is like flour with radial colony type, hyphae with septic, round conidium shape black, thought to be *Aspergillus* sp. The colony is gray, the colony reverse color is brown, the surface texture of the colony is velvety with a concentric colony type, the hyphae is not dissatisfied, the conidium is round and has a hyaline color, which is thought to be *Penicillium* sp. The colony is green, the colony is reversed in color, the colony is white, the surface texture of the colony is grained with concentric colony type, the hyphae is not dissatisfied, the conidium shape is oval and green is thought to be *Trichoderma* sp.

Table 1. The morphological data of the isolated and identified fungi.

Isolate	Characteristics of isolates								Code
	a	b	c	d	e	f	g	Identification of isolates	
1	Yellow turns green	Brownish yellow	Velvety	Spread	have septa	Round	Green	*Penicillium* sp.	Pn
2	White becomes black	White	Like flour	Spreads fast	have septa	Small, round	Black	*Aspergillus* sp.	As 1
3	White becomes gray brown, then black	White	Like flour	It spreads fast	have septa	Small, Round	Black	*Aspergillus* sp.	As 2
4	Dark green	White	Grain	Radial	have septa	Oval	Green	*Trichoderma* sp.	Tc 1
5	Light green becomes dark	White	Grain	Radial	have septa	Oval	Green	*Trichoderma* sp.	Tc 2

a. Colony color
b. Reverse colony color
c. Colony surface texture
d. Colony type
e. Hyphae
f. Conidia form
g. Conidia color

The content of spent mushroom *P. ostreatus* apart from mycelium from the white oyster mushroom itself also still has mycelium from other fungi that play a role in decolorizing batik effluent. Based on the isolation results from the spent mushroom *P. ostreatus*, three fungi were obtained that could grow on PDA medium. The results of fungal isolation were then identified by observing the distribution pattern, colony surface color, color pigmentation on the reverse of the fungal colony, margins or the edge of the colony and microscopic observation.

The first fungal isolates obtained had the following characteristics: the distribution pattern was concentric with the color of the colony's surface green and smooth, the pigmentation formed on the reverse of the colony was yellow, the margins or the edges of the colony were flat. Microscopic fungi have a round conidium shape, long conidiophores, single and branched at the ends and have septa hyphae. These characteristics belong to the genus Penicillium [21], so based on this it can be seen that

the first fungal isolate was *Penicillium* sp. The results of macroscopic and microscopic observations can be seen in Figure 2.

The second isolate obtained was characterized by a concentric distribution pattern with a black colony surface, rough surface and uneven edges and no pigmentation on the other side of the colony. Microscopic observation showed that *Aspergillus* sp. has a conidium with a round shape and is black. Single conidiophores, unbranched, long, perpendicular and the tip forms a vesicle. These characteristics are the same as those of the Aspergillus genus[21][29], so it can be seen that the second fungal isolate is *Aspergillus* sp. Picture of the morphology of the *Aspergillus* sp. shown in Figures 5 and 6.

The third isolate obtained has a concentric distribution pattern, the colony surface looks rough with green color, and does not form pigmentation on the other side of the colony. Conidiophores of *Trichoderma* sp. branching, the branching towards the end looks shorter and shorter. The conidium is semicircular or oval and has a bulkhead on the hyphae. Based on the results and observations, these characteristics are owned by *Trichoderma* sp[21]. The complete observation image can be presented in Figures 5 and 6.

The results of the identification are also in accordance with the identification that has been done by [30]. Fast-growing vegetative hyphae, with septate, branched, upright conidiophores which are always unbranched and beseptate, conidia are formed like chains with a very distinctive brush-like shape are characteristics of *Penicillium* sp. Isolates, and *Aspergillus niger* is characterized by a fast growing colony, with white mycelium becoming brown or gray, then black and purplish conidia; conidia are small, globose / round, coarse, 4-5 µm in diameter. While the characteristics of *Trichoderma* sp. as revealed by [31][32]. Colony of *Trichoderma* spp. white, yellow, light green, and dark green. *Trichoderma* spp. multicellular lined up to form fine threads (hyphae) in the form of flattened, insulated, branching to form webbing (mycelium) which can grow rapidly and produce spores of millions of spores [31]. *Trichoderma* spp. have hyphae with septa, branched and have smooth walls, colorless, 1.5-12 μm diameter. Branches of hyphae form right angles on the main branch. The tip of the conidiophores is a round conidia, flat walls with a gloomy green, whitish green, bright green or slightly greenish [32].

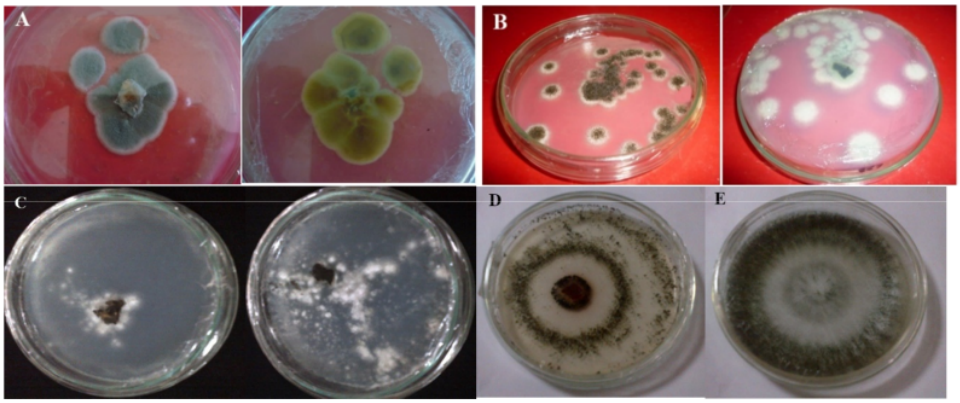

Figure 2. Picture of isolate colony: (A) surface & reverse of Pn colony, (B) surface & opposite As1 colony, (C) As2 colony surface which was initially white to brownish gray, then black, (D) Tc1 colony surface , (E) the surface of the Tc2 colony

Trichoderma sp. can contaminate the cultivation of shiitake (*Lentinus edodes*) [33]. According to Contaminant fungi that can grow in the spent mushroom include *Penicillium* sp. and *Aspergillus* sp. [34]. *Trichoderma aureoviridae*, *Penicillium citrinum*, and *Aspergillus flavus* and *A. fumigatus* were

found to be contaminant fungi in spent mushroom [35]. These fungi are suspected of being involved in the decolorization process of batik effluent.

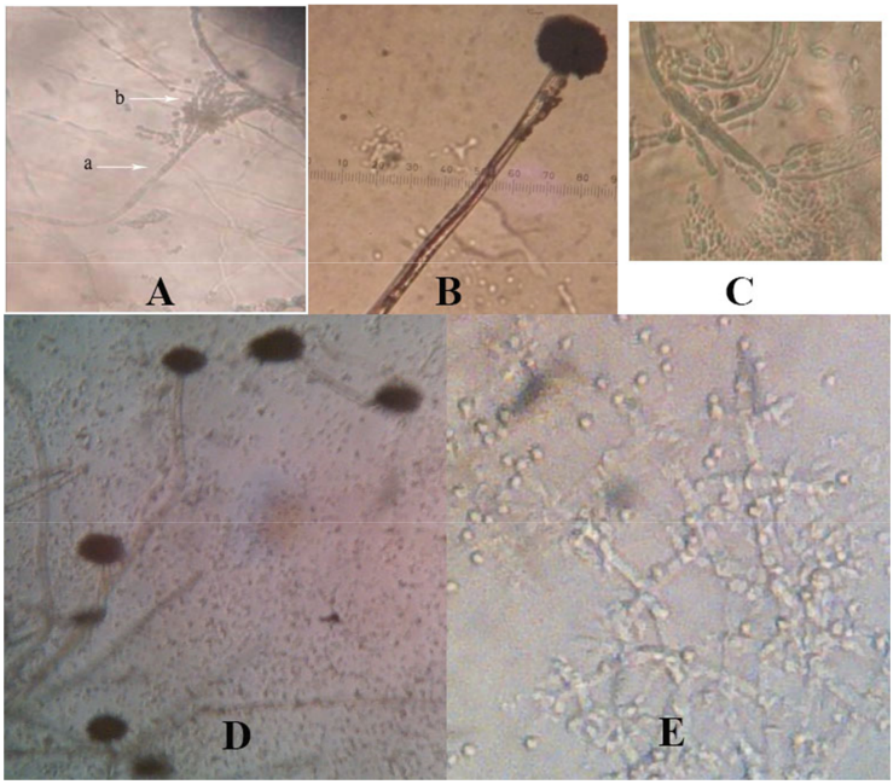

Figure 3. Microscopic observation of selected fungal isolates from spent mushroom. Microscopic image of isolates: (A) Pn : a. conidiophores, b. conidium, (B) As1, (C) Tc1, (D) As2, (E) Tc2.

3.3. Selection of fungi on solid medium

The selection of fungi on a solid medium that has the potential to decolorize the batik effluent was carried out after the isolates were obtained from the isolates. The selection was tested on the medium that was exposed to the dye effluent. Each fungal isolate measured the diameter of the clear zone and the diameter of the colonies formed every day up to 10 days of incubation, then measured the dry weight. The best results are used as superior fungi in the selection process.

The results of the study showed that the five isolated fungal isolates did not show a clear zone around the colony, but when compared with the control (left side plate), these isolates were able to reduce indigosol blue batik dye effluent (Figure 4-8).

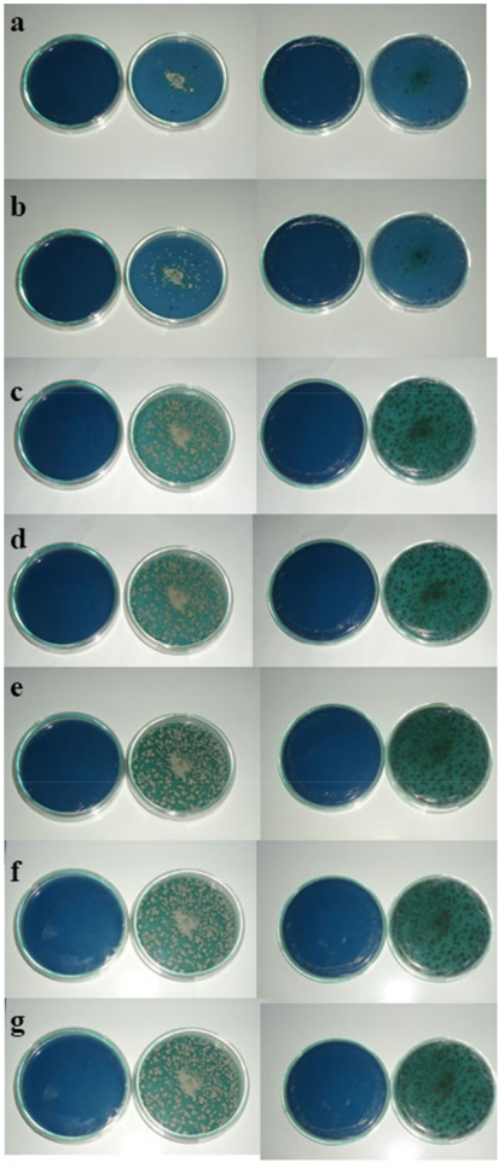

Figure 4. Growth and color reduction in solid medium by Pn isolates on day 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g) and their comparison with control.

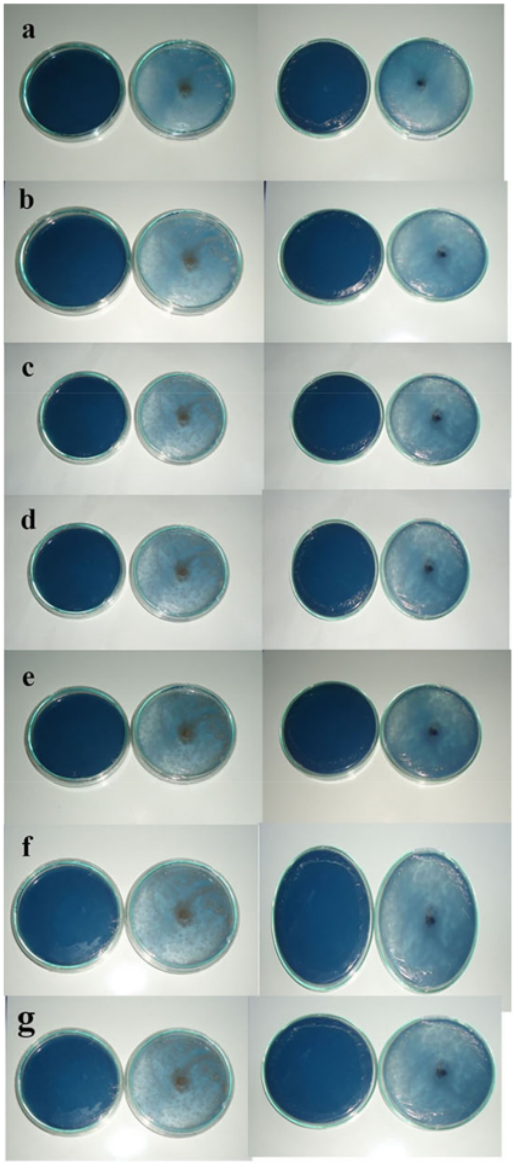

Figure 5. Growth and color reduction in solid medium by As1 isolates on day 1 (a), 2 (b), 3 (c), 4 (d),5 (e), 6 (f), 7 (g) and their comparison with control.

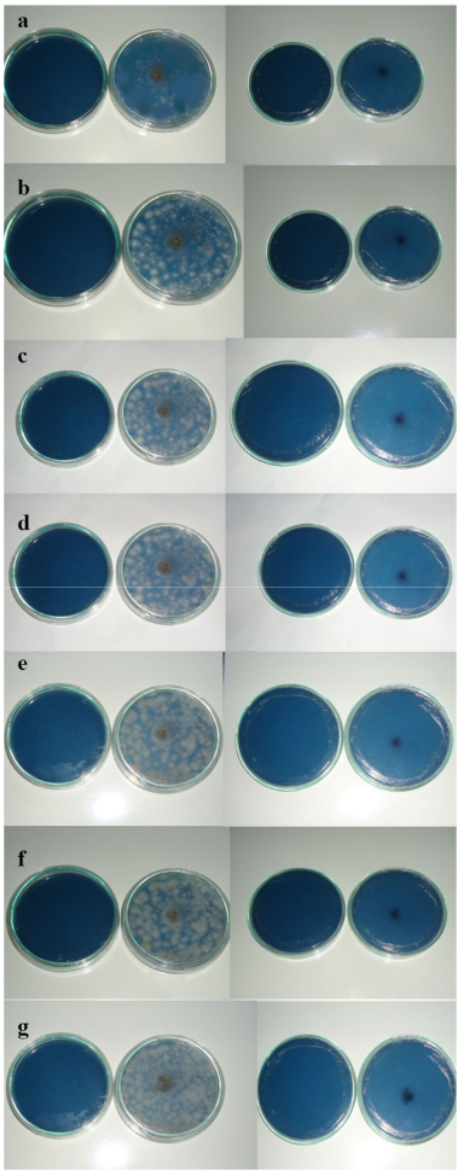

Figure 6. Growth and color reduction in solid medium by As2 isolates on day 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g) and their comparison with control.

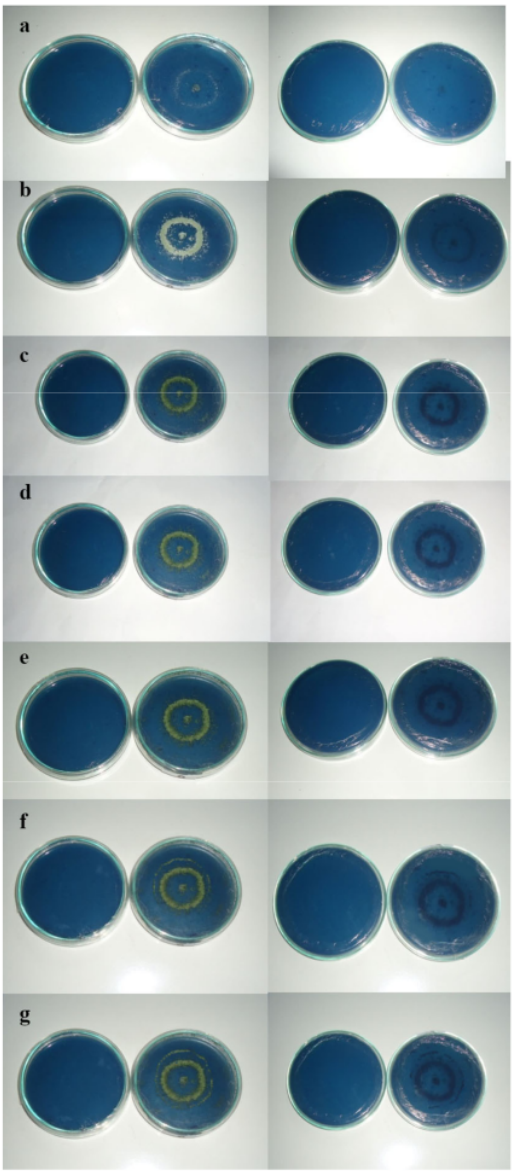

Figure 7. Growth and color reduction on solid medium by Tc1 isolates on day 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g) and their comparison with control

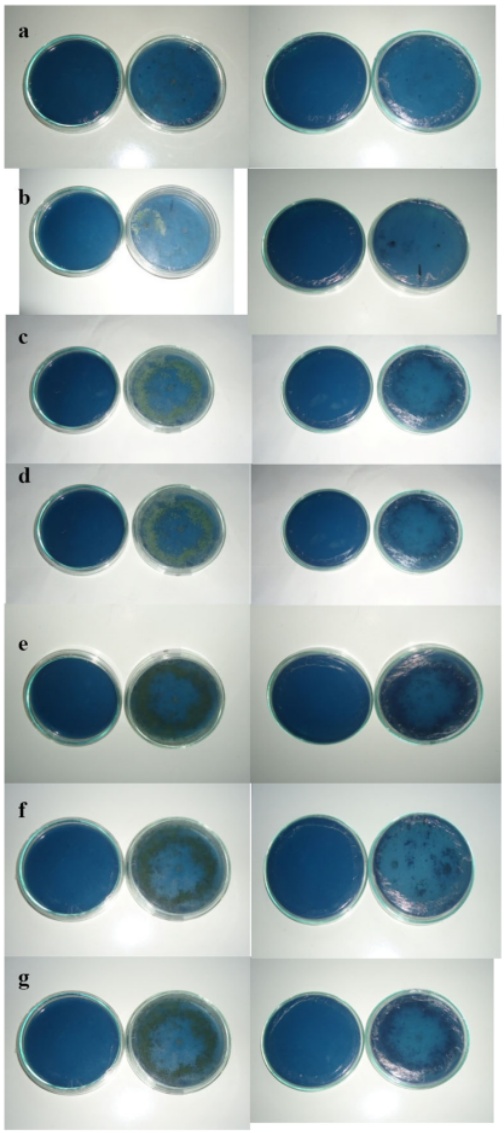

Figure 8. Growth and color reduction on solid medium by Tc2 isolates on day 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g) and their comparison with control.

Likewise, the naphtol dye effluent, the brown color in the effluent can look faded, but the growth is very thin. Colony diameter growth data and mycelium dry weight are presented in Figure 9 and 10. This proves that the five isolates were able to grow on the medium containing batik dye effluent. *Penicillium* sp. LA2 can absorb orange II dye [36]. *Aspergillus niger* SA1 was able to decolorize the colors of acid red 151, orange II, sulfur black, and *drimareneblue* K2RL [37]. *Aspergillus niger*, *Trichoderma viridae*, and *Penicillium verrucosum* which were able to reduce textile effluent methyl orange and methyl red [38].

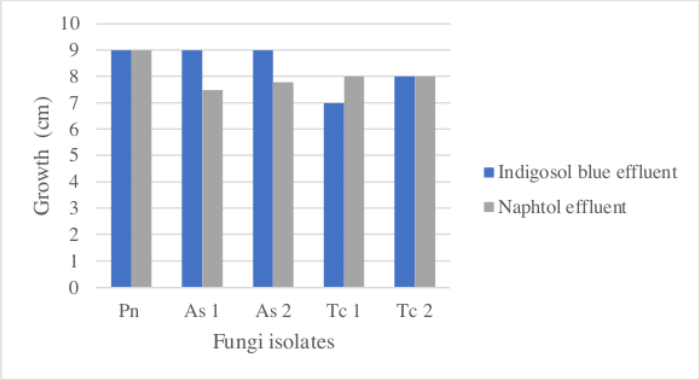

Figure 9. The diameter of the isolate on the solid medium contains effluent of batik dye

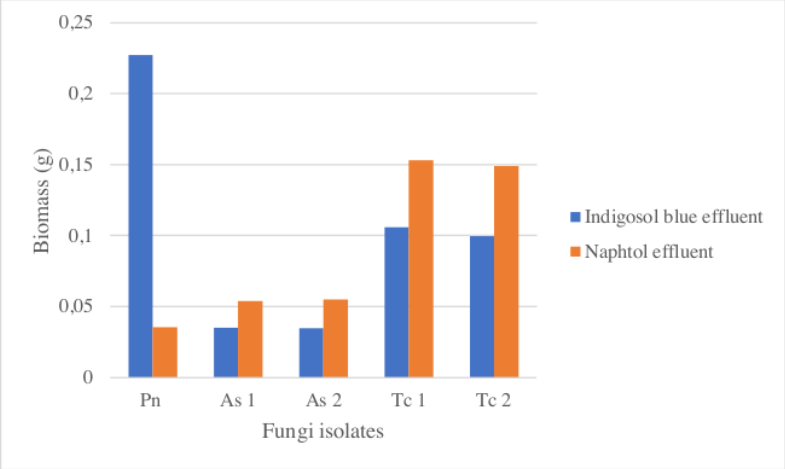

Figure 10. The dry weight of mycelium in solid medium contains dye effluent

Observation results and measurements of the clear zone diameter, the diameter of the colonies formed and the measured dry weight indicate that the data cannot be used to determine the best results used as superior fungi in the selection process (Figures 9 and 10). Therefore, quantitative measurements are made for decolorization (Figure 11). However, from the measurement results of the diameter the colonies formed and the measured dry weight showed that Isolates Pn and As 1 were the two isolates with the highest yields, so in quantitative measurements these two isolates were selected.

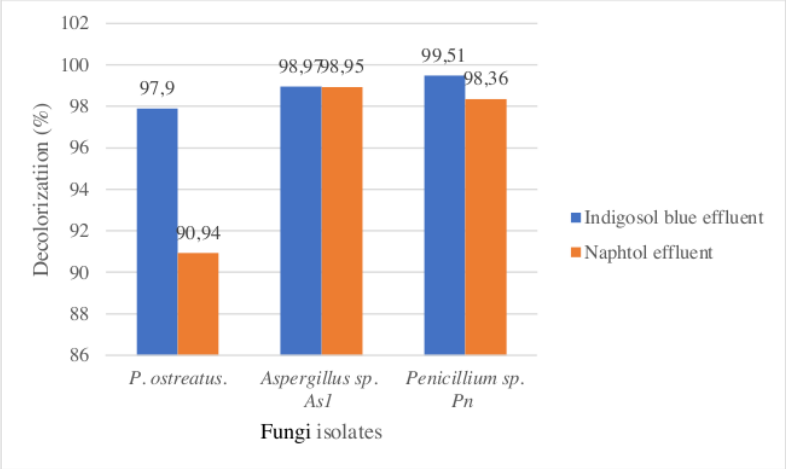

Figure 11. Percentage of decolorization data for different batik effluent

Figure 11 shows the percentage of decolorization between the two selected isolates (*Penicillium* sp. (Pn) and *Aspergillus* sp. 1 (As1)) compared to *P. ostreotus* (Po) as a control isolate which has the ability to decolorize dye. Pn 1 isolates had a higher decolorization ability than As1 and Po, by 99.51%, 98.97%, 97,9%, respectively for Indigosol blue effluent; and in Naphtol effluent respectively by 98.36%, 98.95%, 90.94%.

At this stage using naphtol and indigosol dye effluent, as well as contaminant fungi isolated from effluent (*Aspergillus* sp.- Isolates As1, and *Penicillium* sp.-Isolate Pn) because it is thought to have more ability among the isolates that were isolated and the preliminary test. Isolate *Aspergillus* sp. have the ability to eliminating the color of batik wastewater and reducing physicochemical parameters [39][40]. Likewise with the components of Cr, sulfide, ammonia, phenols, and oil-fats from batik effluent, isolates *Aspergillus* sp. reported to be able to reduce his concentration [41][42]. At this stage, *P. ostreatus* isolates were also used because they were known to have the ability to decolorize. The results of the measurement of % of batik liquid waste decolorization showed that the three isolates had the ability to reduce color with different values. The highest average percentage of decolorization was *Penicillium* sp. on indigosol effluent treatment (Figure 11). Based on the research, the fungus that has the best decolorization ability is *Penicillium* sp. in indigosol effluent by 99.5%. This is by *Penicillium* sp. able to adapt and utilize the nutrients contained in the batik effluent substrate optimally. Reductase enzymes induce in the decolorization of dyes indicating their involvement in the decolorization process [43]. Naphtol effluent can be decolorized by ligninolytic enzymes (laccase, lignin peroxidase, and manganese peroxidase) [44,45]. All of the data of this results showed that Pn isolate was the best isolate in this study.

3.4. Analysis of heavy metals

The concentration of the heavy metal on the two effluents used was measured first. The measurement results of Zn, Cd, and Cu metals are presented in Figure 12. These results show that Zn metal is the metal with the highest content in both wastes. Figure 12 showed that the value of the concentration of metal Cd in the Indigosol blue, Naphtol and end of batik effluent, is 0.938, 0.908 and 0.995 ppm, while the Cu metal is 0.33, 0.274; 0.315 ppm, and 290, 3.3, 2,4 for metal Zn.

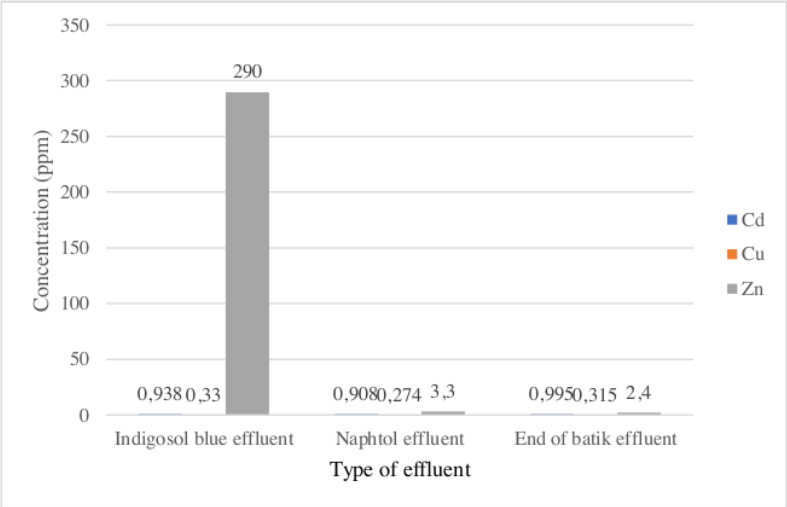

Figure 12. Concentration of heavy metal on batik effluent

The results of the research that has been carried out regarding Zn absorption using fungal mycelium isolated from spent mushroom *P. ostreatus*. It is showed decreased levels of Zn in batik effluent after treatment. The results showed that the average percentage of Zn absorption was above 90%. The highest average percentage of Zn absorption was in treatment using *Penicillium* sp. Pn, while the lowest was in treatment using As1 1 on Naphtol effluent. The complete percentage data is presented in Figure 13. Based on the histogram, the mycelium with the highest reduction in Zn levels was *Penicillium* sp., which was 99.01 If the size of the mycelium pellet is large so that the Zn absorption area is wider. The wider of contact area, the more metal binding sites are available [45]. Biosorption of heavy metal was endothermic reaction [46].

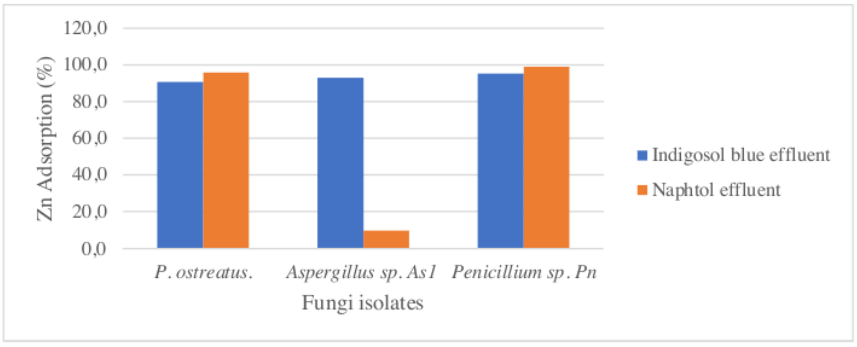

Figure 13. Absorption of Zn on batik effluent

3.5. Toxicity Test

The toxicity test was carried out by testing the viability of tawes fish in liquid media. The liquid medium used was decolorized batik limbar water compared to well water. The survival rate of the fish was observed by counting the number of live fish given to the liquid medium. Presented in Table 2.

These data indicate that color decolorization does not necessarily indicate good water quality for fish living media.

Table 2. Survival Rate Evaluation of Different Medium

Treatment	Time (hours)	Accumulated survival rate of fish with different medium		
		1	2	3
irrigation water	0	10	10	10
	1	10	10	10
	2	10	10	10
Decolorized batik waste	0	10	10	10
	1	10	10	10
	2	10	10	10
Batik effluent	0	10	10	10
	1	0	0	0
	2	0	0	0

4. Conclussion

Based on the results of this study, it can be concluded that the superior microfungi for decolorizing and removing heavy metals from batik effluent is *Penicillium* sp., and its isolate can reduce the toxicity after the treatment results.

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