Decoloration of naphthol batik dye by biosurfactant-producing bacteria

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Abstract. The potency of some local isolates, biosurfactant-producing bacteria on decolorization of naphthol was examined. Six isolates designated as AB01, AB02, NF02, NF09, HS06, and HS08 have been tested their abilities to degrade carbofuran. Before the isolated were tested their potencies to decolorize naphthol dye, their abilities to produce biosurfactant was performed. HS08 isolate showed the highest biosurfactant activity with Emulsion Index (EI) was 17. The isolates were grown on Bushnel Haas Agar (BHA) medium amended with 25 ppm of naphthol dye as the sole carbon source. Isolates which were grown on BHA medium with no addition of naphthol served as control. The result showed that all isolates grew much slowly on naphthol-containing agar medium compare to one that grew BHA. HS06 isolates showed the best growth with colony d was 12 mm, while that isolate could reach 30 mm when grew BHA with no naphthol. Based on the highest growth and EI, three isolates; AB01, NF09 and HS06 were chosen for their potencies on naphthol dye decolorization as mixed culture with different conditions which were shaking and static cultures. The result showed that shaking culture results in better decolorization compare to the static one. When applied as consortium, the isolates showed lower percentage of decolorization of dye (33%) compare to the ability as single culture (59%).

1. Introduction
Textile industries, mainly batik industries are very popular in Indonesia including North Sumatra. Among textile industries, batik industries have been developed significantly recently. All students in various levels have batik as their uniform. Moreover, teachers, public servants, and others also wear batik as the formal uniform. In Sumatra, the most popular batik is Batak batik. This cloth shows specific pattern compare to others. One may recognize Batak batik from its pattern and color. Now days, there are many homemade batik industries found in North Sumatra.

Despite of their economic advantages, textile industries cause significant impact to the environment. These industries generate large volume of liquid waste which could deteriorate environment if does not treated properly. The main pollutant in waste is the dyes which are used during dyeing process. There are many types of synthetic dyes available in the market such as: acid dyes, basic dyes, direct dyes, mordant dyes, vat dyes, reactive dyes, disperse dyes, and azo dyes. Azo dyes are mostly applied in textile industries because of their extensive variety in color shade and brilliant color. On the other hand the dyes are recalcitrant and carcinogenic [1]. Dyes decreased significantly photosynthetic activity because of reducing light penetration and threatened some aquatic life due to high content of aromatic compounds, metals, and chloride. Dyes tinctorial value is high; in less than 1 ppm causes obvious coloration [2].
The dye which is frequently used in batik dyeing process is naphtol dyes. Naphtol dyes are insoluble azo dye stuff that could produce very deep orange, red, and scarlet. Due to their complex structure the dyes are very stable in the environment. Many efforts have been developed to reduce the amount of these pollutants in the environment such as: oxonation, photooxidation, adsorption, membrane filter, flocculation, and application of activated carbon [3]. These physicochemical methods have some drawback: less efficient, high operational cost and create other waste. Application of organism to degrade and to reduce the dye color has received a lot of attention since it offers some advantages: low operational cost, easy to be applied, and create no waste, safe for the environment.

Numerous bacterial isolates have been studied for their abilities to degrade various types of hydrocarbon compounds. Many strains of bacteria are able to decolorize a broad spectrum of dyes. Most of them have been identified (Pseudomonas, Bacillus, Klebsiella, Salmonella and others) and determined the mode they attack the dyes, what optimum condition (temperature, pH, nutrition requirement) they work [3,4,5]. We have isolated six local isolates which have been tested for their capabilities of producing biosurfactant and degrading carbofuran. The ability of microorganism to produce biosurfactant is often related to capability of the organism of degrading hydrocarbon compounds. All isolates could degrade completely 100 ppm of carbofuran within 15 days. In this paper, we study the potency of the isolates on naphtol dye decolorization under different nitrogen concentration, pH, glucose supplement, and different culture conditions (static and shaking cultures).

2. Material and Methods

2.1 Bacterial Isolates and Medium

The bacterial isolates were from oil contaminated shore and pesticide contaminated agriculture land. They were designated as; AB01, AB02, NF02, NF09, HS06, and HS08. They were subculture on nutrient Agar for further use. Medium used to study the decolorization of naphtol dye was Bushnell-Hass Broth (BHB) amended with naphtol dye as the sole carbon source. The composition of the medium per L is 1.0g of KH2PO4, 1.0 g of K2HPO4, 1.0 g of NH4NO3, 0.2 g of MgSO4.7H2O, 0.05 g of FeCl3, 0.02 g of CaCl2.2H2O.

2.2 Screening of Biosurfactant Activities

To determine the biosurfactant activities of each isolate, Drop Collapsing Test [17] which has been modified was carried out. The isolates were grown in BHB which has been added by 2% dextrose. The cultures were incubated in shaking incubator at 120 rpm for 15 days. Supernatant was separated from pellet by centrifugation. Four ml of supernatant was added with 4 ml of N-heksan and 2 ml of aquades, mixed well using vortex for 10 seconds. Observe the stable emulsion formed and measure the volume. Emulsion index (EI) was calculated using:

\[
EI (\text{Emulsion Index}) = \frac{\text{Emulsion volume}}{\text{Total volume}} \times 100\%
\]

2.3 Qualitative test of Decolorization

The isolate was spread on BHB agar which amended with 5, 15, and 25 ppm of naphtol [6]. The culture were incubated for 6 days to observe the growth colonies.

2.4 Quantitative Test of Naphtol Decolization

Cell suspension (2 mL) with population of 10^8 cell/ml was added into 98 ml of BHB medium which has been enriched with 25 ppm of naphtol dye. One set of culture was incubated in shaking incubator (120 ppm) while the other one was in static condition. The decolorization of naphtol dye in supernatant was measured at 440 nm on day 2nd, 4th, 6th, 8th, and 10th.

\[
\text{Percentage of decolorization} = \frac{\text{Initial absorbance} - \text{Final Absorbance}}{\text{Initial absorbance}} \times 100\%
\]
2.5 Measuring Cell Growth
The growth of cell culture was measured using Standard Plate Count (SPC) on day 5th, 10th, and 15th. As much as 0.1 ml of the culture was spread on plate count agar, incubated for 24 hrs, the growth colonies were counted using colony counter. The total colony is:

\[
\text{Total Colonies} = \text{numbers of colonies} \times \frac{1}{\text{Dilution Factors}} = \text{(CFU/ml)}
\]

2.6 Synergism test among isolates
Before all isolates are applied as a mixed culture, this test should be performed so the culture could work well. Each isolate was streak on agar plate so the three isolates overlap each other as shown by the following illustration image:

![Streak of three different isolates in Synergism Test.](image)

2.7 Naphtol dye Decoloration test by mixed culture
The test was performed by inoculation of 2 ml of mixed culture consisting three different isolates (each isolate has 0.67 ml) into 98 ml of BHB medium amended with 25 ppm of naphtol dye. One set of cultures were incubated at shaking incubator (120 rpm) while another set was in static condition. The decolorization was observed base on absorbance value at 440 nm of spectrophotometer on day 2nd, 4th, 6th, 8th, 10th, and 12th.

\[
\text{Percentage of Decolorization} = \frac{\text{Initial absorbance} - \text{Final Absorbance}}{\text{Initial absorbance}} \times 100\%
\]

3. Result and Discussion
3.1 Biosurfactant activity
All tested isolates showed different levels of biosurfactant activities as shown by Figure 1. The biosurfactant activity was represented by the volume of emulsion formed. The higher the volume and the more stable of emulsion indicated higher activity of biosurfactant. Among 6 isolates, HS06 showed the highest value of EI which was 17% followed by AB01 with 11.1%. Biosurfactant activity may related to how much an isolate secrete the biosurfactant and how effective that biosurfactant work, forming stable emulsion. Extensive studies about the role of biosurfactant on enhancing degradation of hydrocarbon compounds have been reported [7].
The activity of biosurfactant was affected by: 5% salt concentration at pH 8.0 as the best activity, and could be used repeatedly (retained 77%). Although many bacterial species produce biosurfactant: majority were species of Pseudomonas and Bacillus (B. cereus, B. mycoides, B. sphaericus, B. thuringiensis). Previous study of NF02 and NF09 showed higher biosurfactant activities in which the EI were 38% and 42% respectively (8). Biosurfactant activity was also affected by environment factor such as pH medium and salt concentration (9). They found that the highest biosurfactant activity of Pseudomonas aeruginosa ATCC 15442 was 31.02% at pH 6 and 24.0% at 1% of NaCl when the bacterium was grown in 10% of diesel fuel medium in mineral salt solution, when the carbon source was crude oil the highest EI was 52.16% at pH 6 and 33.3% at 1% NaCl.

3.2 Ability of the isolates to grow on BHA amended with naphtol dye

All isolates grew well on Bushnell Haas Agar without any addition of naphtol. The best growth was shown by HS06 isolate with colony diameter (d) reached 29 mm, and this isolate also grew steadily up to day 6th. Meanwhile the slowest growth was shown by NF09 isolate with d only 10 mm. The ability of microorganism to use agar as the carbon source has been well documented (10). Agar consists of two major types of polysaccharides: neutral agarose and charged agaropectin. Agarose is an alternate link of 3-link of β-D galactopyranose residue and 4-linked 3,6 anhydro-α-L-galactosepyranosa residue.

The presence of naphtol at various concentration (5, 15 and 25 ppm) in BHA medium caused all isolates to grow much slowly. It is assumed that naphtol dye is toxic to microorganism and could inhibit the growth of the microorganism. Some research reported that there is a correlation between biosurfactant production and esterase activity. An esterase activity was observed both in the cell-free growth medium and on the cell surface of petroleum-degrading bacterium Acinetobacter calcoaceticus RAG-1(ATCC 31012). It was shown that the enzyme released from the cell surface was either emulsan free or associated with the bioemulsifier [11].
Figure 3 The growth of bacterial isolates on Bushnell Haas Agar with no addition of naphtol dye as a control, 5, 15 and 25 ppm of naphtol.

When microorganism grows in environment rich in hydrocarbon, they adjust to the environment through some adaptations. One such adaptation is biosurfactant production; it affects the uptake of hydrocarbon as substrate. The low solubility and high hydrophobicity of many hydrocarbon compounds make them highly unavailable to microorganism. Biosurfactant production helps the hydrocarbon degrading bacterium to gain better access to their hydrophobic substrate as it bring about changing like reduction of surface tension of the environment around the bacterium, reduction of interfacial tension between bacterial cell wall and hydrocarbon molecules, membrane modification like increasing hydrophobicity of cell wall by reducing the lipopolysaccharide content of the wall, enhancing of hydrocarbon dispersing by encapsulation of the hydrocarbon into micelles [11, 12, 13].
3.3 Decoloration of naphtol dye

The ability of isolates to decolorize naphtol dye varied among six isolates as shown by the graph below.

![Graph showing decolorization percentage by bacterial isolates](image)

**Figure 4** Decolorization Percentage by Bacterial Isolates

It showed that AB01 had the highest percentage of decolorization, which reached 60% compared to others. Meanwhile, NF02 isolate showed the lowest percentage of decolorization which was only 30%. In general, decolorization of naphthol dye is better when the isolates were incubated in shaking incubator than in static condition. In static culture, the highest percentage of decolorization was shown by the same isolate, AB01, which was 30%. It means that shaking treatment increases the decolorization twofold. The spontaneous decolorization took place in the control but at very low percentage, less than 10% in both shaking and static cultures. It is assumed that shaking treatment increases oxygen supply into the culture so the rate of metabolism is higher. Furthermore, shaking treatment may enhance the possibility of the cells to bind to naphthol dye and in turn degrade and use it as the carbon and energy sources for their growth. Without agitation it was observed that the naphthol dye tend to settle down at the bottom of the flask.

3.4 Growth of isolates in liquid medium amended with naphthol dye

The growth of 6 isolates in BHB medium containing 25 ppm of naphthol dye under shaking and static condition was measured on day 0, 5, 10, and 15th. The result was shown by the following figure 5.

![Graph showing growth of isolates in liquid medium](image)
From the above figure it was known that all isolated grew well in the medium containing 25 ppm of naphthol dye both in shaking and non-shaking cultures. The initial population of each isolate was $10^6$ cells per ml. Some isolates grew steadily from day 5th to 15th (AB 01, AB 02 and HS 08) while others showed fluctuation. During the first 5 days, the growth was really slowly. Some isolates even decreased in population (NF 02 and HS 06). Those two isolates were not able to adjust and to degrade naphthol dye and used it as the carbon source caused the cells to die. All isolates showed significant increase in population from day 5th to day 10th and 10th. It meant that all isolates were able to degrade naphthol dye and used the degradation products as the carbon source. Numerous genera of bacteria, *Pseudomonas, Flavobacterium, Arthrobacter, Rhodococcus,* and *Bacillus,* have been reported to be able to degrade various types of hydrocarbon compounds. Nunuk (2017) reported that some isolates were able to grow and reduce the carbofuran concentration up to 32% during 15 days of incubation [14]. The ability of those bacteria to degrade carbofuran was closely related to their production of biosurfactant. It has been widely known that biosurfactant enhances the adsorption of hydrocarbon compounds to the cell therefore promote the degradation of the compounds.

3.5 Synergism Test of Selected Bacterial Isolates

The synergism test was performed to AB 01, NF 09, and HS 06 isolates to observe whether they were compatible or not. The result showed that there was no inhibition zone formed when they were streaked overlapping each other on agar plate as shown by the following photograph:

![Synergism test of selected bacterial isolates](image)

It meant that they could be grown together as a mixed culture. According to Deng *et al* (2016) the compatibility of some bacteria is determined by: (i) a member of genera that are not able to degrade certain organic compounds will rely on other members that provide nutrition for it (ii) a member of
genera will protect other members which are sensitive to specific organic compounds by reducing the toxic compounds through producing either specific or nonspecific protective factor [15].

3.6 Decoloration of Batik Naphthol Dye by a Mixed Culture

The three isolates, AB01, NF09, and HS06 were chosen as a mixed culture based on their high capability of naphthol dye decoloration. The result was shown by the following graph:

![Graph showing decoloration percentage by mixed culture under shaking and static cultures](image)

**Figure 7** Decoloration percentage by mixed culture under shaking and static cultures

Based on the above figure, it was shown that the mixed culture under shaking incubation resulted in significantly higher percentage of decoloration (33%) compared to one that is static (20%). These results were much lower than that of single culture. The highest reduction of color by single culture was 60% at shaking culture while the lowest one was 30% at static culture. Many bacterial isolates worked better when they are in a mixed culture. It means that each species within the mixture supports the growth of others. Certain bacteria though show different pattern. They work better when they are as single culture as reported by Pimda, W. and Sumontip B. (2012). Using cyanobacteria *Nostoc hatei* and *Syneccocystis aquatilis* they concluded that *Nostoc hatei* as single culture worked better than as a mixed culture. Meanwhile, *Syneccocystis aquatilis* as a mixed culture worked better than as single culture [16]

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