

# Studies of *Bacillus subtilis* NAP1 to Degrade BOD, COD, TSS, and pH: The indigenous bacteria in Indonesia Batik Wastewater

*by* Hasminar Rachman Fidiastuti

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# Studies of *Bacillus subtilis* NAP1 to Degrade BOD, COD, TSS, and pH: The indigenous bacteria in Indonesia Batik Wastewater

H. R. Fidiastuti<sup>1\*</sup>, A. S. Lathifah<sup>1</sup>, M. Amin<sup>2</sup>, Y. Utomo<sup>2</sup>

<sup>1</sup>Universitas Tribhuwana Tunggaladewi, Telaga Warna Blok C, Malang, Indonesia

<sup>2</sup>Universitas Negeri Malang, Semarang 5, Malang, Indonesia

hasminarfidi@unitri.ac.id

**Abstract.** Batik is an industry that is quite popular in Indonesia. Batik has been declared as one of Indonesia's cultural heritage and is recognized by UNESCO. Batik production is recorded to always increase every year. Unfortunately, the batik industry in Indonesia is dominated by many small industries with limited fund management. This makes the environmental aspects and waste disposal neglected. Through this research, study and isolation were carried out to explore the potential of indigenous bacterial isolates that can biodegrade dyes in batik along with other physical parameters of waste. A sample that contained wastewater and sediment from batik industry are collected and cultured in 1000 ml Busnall Hass medium which olive oil-enriched and put at the shaker condition at 150 rpm until 6 days at 30°C. Identification of the isolates examined for their morphological, physiological and biochemistry test. *Bacillus subtilis* is one of the indigenous isolates from this research. This study aimed to determine the ability of *B. subtilis* to reduce BOD, COD, TSS, and pH in batik waste and present the results of the DNA analysis of *B. subtilis*. Based on the research that has been done, it can be concluded that the molecular identification of bacteria through analysis of the 16S rDNA gene fragment sequence, the bacteria that play a role in the degradation of dyes is *Bacillus subtilis* strain NAP1 with an average similarity level of 93%. *B. subtilis* NAP1. *B. subtilis* NAP1 isolate offers the potential for future bioremediation of batik wastewater.

## 1. Background

The batik industry in Indonesia is experiencing quite rapid growth and is largely dominated by household-scale industries. The household-scale industry in Indonesia has the characteristics of 1) developing with limited capital, 2) applying simple methods of management, and 3) tending to be environmentally unfriendly because it does not have a further sewage treatment system. Waste generated from the batik industry comes from the process of waxing (experience), coloring, and wax removal (pelorodan) processes [2]. In some cases of handling batik waste, the release of industrial waste is regulated in such a way, while others are because it is not equipped with good waste treatment infrastructure so that it impacts on the condition of the surrounding waters. Industrial textile waste generally has the characteristics of high BOD and COD numbers, which means the wastewater contains a large amount of non-biodegradable organic materials.

The Environmental Protection Agency (EPA) revealed the results of research that only about 10% of industrial waste is safe to be discharged into the environment [12]. Waste migration from landfills will pose a high risk to groundwater sources if not properly managed [1]. So far, several cleansing

methods are known, one of which is biological removal treatment technology, known as bioremediation, and is a profitable alternative because it does not produce dangerous and cost-effective end products [7]. The purpose of biodegradation is to degrade pollutants using microorganisms to restore the environmental conditions to their natural conditions [10].

One of the centers of batik in Indonesia is located in Tulungagung District, East Java, Indonesia. This study aims to isolate, identify, and search for indigenous bacteria isolates that have the potential to reduce BOD, COD, TSS, and pH in batik factory wastewater. Microorganisms were obtained from factory waste through the stages of indigenous bacterial propagation, dilution, bacterial inoculation in the medium, biochemical observation, and measurement of biodegradation activity by isolates. Bacterial species were then identified based on bacterial phenotypes such as gram staining, colony morphology, and enzyme activity. This bacterial isolate was also identified based on 16S rRNA gene sequence homology methods.

## 2. Methods

The research is a type of experiment to characterize the indigenous bacteria found in batik wastewater. Samples were taken from one of the centers of the batik industry in the area of Kauman, Tulungagung. The plant that is sampled by waste does not have a specific sewage treatment channel.

### 2.1. Study Area and Period

In January 2019, samples from batik wastewater were collected. Waste samples are taken directly from the last waste collection place before disposal.

### 2.2 Isolation

The collected batik wastewater samples were processed according to the method of Burlage with slight modification [3]. Briefly, a 10% sample was dissolved in an enriched medium (BHM), that contained dye materials.

### 2.3 Propagation

A sample that contained wastewater and sediment from the batik industry are collected and cultured in 1000 ml BH medium which olive oil-enriched and put at the shaker condition at 150 rpm until 6 days at 30°C.

### 2.4 Inoculation

Inoculation was carried out to obtain pure cultures to identify isolates. This stage uses agar nutrient media, it was incubated at 37 for 24 hours.

### 2.5 Find the Potential Isolate

Indigenous bacteria isolates were added to liquid waste in the ratio of 1:10 for 24 hours and placed in a 150 rpm shaker. Physical parameters measured included BOD, COD, TSS, and initial pH before treatment (0H) and after incubation with indigenous bacterial isolates (24H, 48H, and 72H). Testing of BOD levels was measured using the 5<sup>th</sup>-day incubation method, COD was measured by the dichromate oxidation analysis method, TSS was measured by the Gravimetric method, and pH was measured using a pH meter.

### 2.6 Identification of Bacterial Isolates

Identification of the isolates examined for their morphological, physiological and biochemistry test. Bacterial colony identification was classified based on colony color, size, cell arrangement, shape, and staining. Cultural properties included color, form, elevation, margin, surface's colony, and consistency. Physiological and biochemistry test was conducted to evaluate oxidase, indole, catalase, urease, nitrate reduction, citrate utilization, and gelatin. Biochemicals evaluated the ability of the isolates in fermenting sugars, including glucose, rhamnose, sucrose, lactose, arabinose, and raffinose. The isolates were

identified according to the standard description of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The bacterial biochemical tests were performed to determine the ability of microbes for production enzymes, like Indole, etc.

### 2.6 Identification of Bacterial Isolates

Sequence analysis is done by sending the results of bacterial DNA amplification to the service of determining DNA sequences from PT. Genetics Science Jakarta. The molecular identification of the isolates was performed by amplification using universal primers (5'-TGG TTA GTG C[AG]T G[CT]A GT-3') dan (5'-ATC ATC AAA [AG]GA AAC TCGT-3'). The amplified gene products were then purified and sequences were obtained through Kit. The partial sequences of the 16S rRNA gene obtained in this study were analyzed and compared with nucleotide sequence databases in the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment Search Tool (BLAST) program, to confer the percentage sequence similarities. The nucleotide sequences of the isolate were submitted to GenBank NCBI for the accession numbers.

## 3. Results and Discussion

### 3.1 Character of Batik Wastewater

Batik waste has a strong color character when sampling is done. The initial waste character results are shown in Table 1. Table 1 shows that the levels of BOD, COD, TSS, and pH are above the limits set by the government for the textile industry.

**Table 1.** The Early Character of Batik Wastewater. Standard base on East Java Governor's Act on Standard Textile Industry Wastewater No. 72, 2003

Parameters	Unit	Result	Standard*)
BOD	mg/L	377	60
COD	mg/L	568	150
TSS	mg/L	1180	50
DO	mg/L	2	-
pH		10	6-9
Dyes	PtCo Unit	3721	-
Cr <sup>6+</sup>	mg/L	2,3	2,0

### 3.2 Characteristics of Indigenous Bacteria in Batik Wastewater

Based on the biochemical identification results in Table 2, one isolate was obtained from *Bacillus subtilis*. The results of inoculation on the plate media of Figure 1 were then made pure and morphologically observed by observing the shape of the cells as shown in Figure 2.

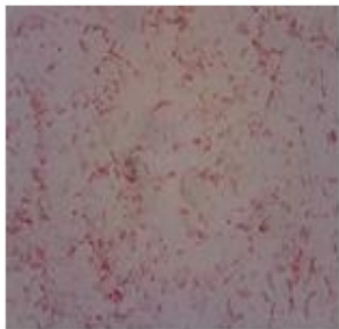
**Table 2.** Biochemical Test Results for indigenous bacteria in batik wastewater

Parameters	Result
Spora	+
Oksidase	+
Motility	-
Nitrate	+
Lysin	-
Ornithine	-
H <sub>2</sub> S	-
Glukosa	+
Mannitol	+

Xylose	+
ONPG	+
Indole	-
Urease	-
V-P	+
Sitrate	-
TDA	-
Gelatin	-
Malonate	-
Inositol	-
Rhamnose	-
Sukrose	-
Lactose	-
Arabinose	+
Adonitol	-
Raffinose	-
Salicin	-
Arginine	-
Catalase	+
Coagulase	-
Hemolisa	beta
Uji Sensitive	No
Novobiocin	
Strach hydrolysis	+
Casein hydrolysis	+
Species	<i>Bacillus subtilis</i>

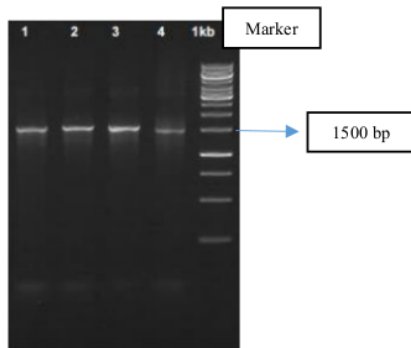


**Figure 1.** Innoculation results on plate media.



**Figure 2.** Observation results of cell shapes.

In addition to biochemical testing, qualitative testing of *B. subtilis* DNA was also carried out. Tests were carried out using agarose gel or horizontal and UV-induced fluorescent electrophoresis gel electrophoresis methods. *B. subtilis* qualitative test results can be seen in Figure 3 (codes 3 and 4). Based on the DNA band image, it is known that the DNA obtained has a length of 1500 bp (base pair).

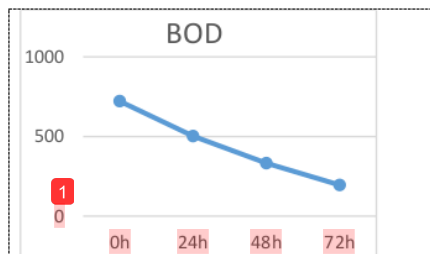


**Figure 3.** Horizontal electrophoresis DNA type.

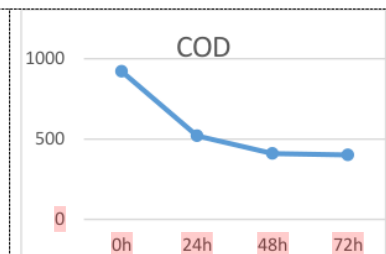
After an analysis using BLAST, the nucleotide base sequence in *B. subtilis* was 92% similar in the first test and 94% in the second test with the bacterial gene *Bacillus subtilis* NAPI strain. Research conducted by Phulpoto states that the bacterium *B. subtilis* NAPI strain can degrade fat [11]. *Bacillus* group can survive at high temperatures, do not produce secondary metabolites, and have the ability to produce large amounts of extracellular protein, so that it is widely used in industrial applications [14].

### 3.2 Isolate Ability in Reducing Batik Wastewater Parameters

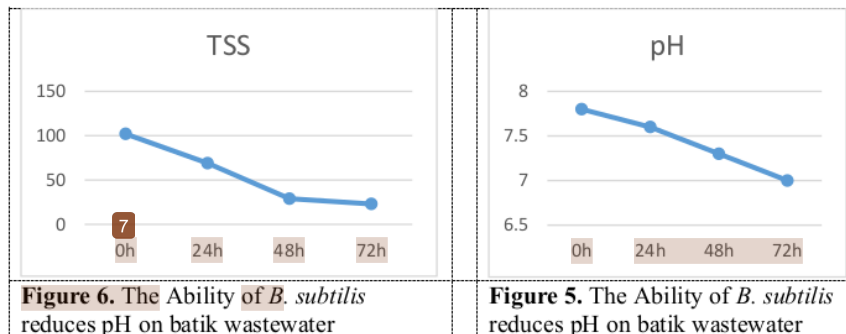
*B. subtilis* was inoculated into batik waste with a concentration ratio of 1:10. In measuring the ability of biodegradation, *B. subtilis* can reduce the number of BOD (Figure 4), COD (Figure 5), TSS (Figure 6), and pH (Figure 7) within 72 hours. Figure 4 to 7 shows that the removal efficiency for a single culture. The highest removal increase was found in 48h. The decrease of BOD and COD was greatly affected by the microorganism activity; thus, longer exposure duration means greater eliminated organic matter, which is called the biodegradation mechanism [5]. Biodegradation is part of the bioremediation process. As most people know, bioremediation has been applied throughout the world in overcoming the problem of ecosystem pollution both on land and in water [1]. Bioremediation utilizes biological processes in the waste treatment process using microorganisms to break down complex substances [6]. Among the community of organisms that have been tested and have been shown to have a high success rate as bioremediation including algal groups, protozoa, bacteria, archaea and fungi [9]. Among all groups of organisms, bacteria are the most successful bioremediation because they have advantages in terms of modes of reproduction and nutrition [8].



**Figure 4.** The Ability of *B. subtilis* reduces BOD on batik wastewater



**Figure 5.** The Ability of *B. subtilis* reduces COD on batik wastewater



**Figure 6.** The Ability of *B. subtilis* reduces pH on batik wastewater

**Figure 5.** The Ability of *B. subtilis* reduces pH on batik wastewater

The isolated *B. subtilis* NAP1 in this activity could withstand the concentration of pollutants in the form of high levels of organic matter. These pollutant concentrations are used as isolates as a single source in the supply of carbon and energy sources for their survival. It was also reported that *B. subtilis* NAP1 also can effectively degrade waste oil-based paints from the paint warehouses [11].

The product of *Bacillus subtilis* gene *azoR1* is annotated as a putative azoreductase [9] which is responsible for reducing the level of coloring material which in this case is contained in the waste. Azoreductase catalyzes the NAD(P)H-dependent reduction of azo compounds to the corresponding amines, which involves cleavage of the azo linkages (-N=N-), resulting in azo dye degradation [4]. Further studies need to be done, whether in the decolorization process a product is produced in the form of aromatic amine compounds. As it is known that aromatic amine compounds are toxic.

#### 4. Conclusions

Isolated *Bacillus subtilis* NAP1 can tolerate increased concentration of dyes in batik waste. This causes the *Bacillus* species to dominate other bacterial species that inhabit the liquid waste of batik factory which are rich in color material. Isolated *B. subtilis* uses dyes and organic matter content in the waste as a source of carbon and energy. The presence of *B. subtilis* becomes important in the process of biodegradation of dyes in waste. *B. subtilis* has promising potential in reducing chemical dyes in waste and has been shown to reduce its concentration in vitro conditions. This study becomes important to be applied on a broader scale, especially its application in the field.

#### 11 Acknowledgement

This research was funded by Kemenristekdikti Indonesia through a collaborative research scheme between universities, Tribhuwana Tungadewi University and Malang State University. All authors read and approved the manuscript.

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