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Potential of Indigenous Bacteria from Batik Wastewater as Cu Bioremediation Agent

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Abstract -Copper (Cu) is a heavy metal which can cause environmental problems in excessive amount. The presence of indigenous bacteria in a Cu-contaminated-environment can be used to reduce Cu levels in the environment. This study aims to determine the potential of bacteria isolated from batik wastewater as Cu bioremediation agent and to characterize those bacteria. Isolation of bacteria was carried out using the enrichment culture media consist of NB + 100 ppm $CuSO_4$. The copper resistance test was carried out by growing bacterial isolates in NA media containing $CuSO_4$ with concentrations of 200, 300, 400, 500, 600 and 700 ppm. The copper reduction test was determined by atomic absorption spectrophotometer. Bacterial identification was conducted by biochemistry characterization. The results showed that 10 bacterial isolates were able to grow on NA media containing 100 ppm $CuSO_4$. Four bacterial isolates (namely α3, α5, α8, and α9) showed the highest resistance to CuSO4 (700 ppm). Bacterial isolates α3, α5, and α9 were able to reduce Cu by up to 75,03%; 76,73%; and 76,34%. The three bacterial isolates showed similar characteristics to members of the Genus Xanthomonas. Isolate α8 was able to reduce Cu up to 77,05% and was identified as member of the Genus Pseudomonas.

Keywords: indigenous bacteria, batik wastewater, bioremediation, copper (Cu)

Running Title : Potential of Indigenous Bacteria Bioremediation Agent

INTRODUCTION

Heavy metal pollution become a big concern in developing countries, as a result of industrialization. Copper is one of the toxic heavy metals contained in industrial sewage. Copper is essensial trace element for living organism, otherwise in larger amount it can be extremely toxic to living organism (Altimira et al., 2012). Human activities such as industrial production, mining and agriculture release a high amount of copper to soil and aquatic systems (Siska, 2009). Batik wastewater has the potential to contain heavy metal copper, which come from synthetic dyes such as naphtol, indigisol, and copper sulphate used as mordant (Jahan & Datta, 2015). The presence of Copper (II) ions in aquatic systems in large amount, can cause severe damage to aquatic life and biomagnification that can endanger human health (Cahyani et al., 2012). Considering the high risk of copper contaminant to organism, efforts are needed so that the copper concentration in the environment does not exceed the wastewater quality standard.

Conventional method for detoxify and reduce copper as precipitation, ion exchange, oxidation/reduction, membrane filtration and evaporation had several disadvantages such as high cost, low selectivity, incomplete removals, high energy consumption and generation of uneliminated toxic slurries. Of these reasons, bioremediation method using microorganisms has received great deal of attention in recent years. This is because of cheap, economical usage and relatively safe because not produce toxins (Irawati et al., 2017). This method is based on the presence of certain types of microorganisms that are resistant and tolerant to enviromental conditions contaminated with copper (Chojnacka, 2010).

Attention to indigenous microorganisms especially bacteria has increased in recent years due to their potential in bioremediating polluted environments. The potential of indigenous bacteria in heavy metal remediation has been proven in various studies, especially on industrial waste. Some of the bacteria that are often used in copper bioremediation consist of *Bacillus* sp., *Pseudomonas* sp., *Chryseomonas* sp., *Burkholderia* sp., *Citrobacter* sp. and *Kluyvera* sp. which were isolated from secondary waste from various industries (Adel et al., 2014). Dianrevy (2017) succeeded in isolating *Bacillus* sp., *Pseudomonas* sp., and *Zoogloea* sp. from batik wastewater. Based on the result of the study, the bacterial consortium was able to remediate the red naphtol dye batik waste and was able to reduce copper levels in the waste by 74, 63%.

As far as the author's observations, there are bacteria that are able to reduce copper, but information is still needed on indigenous bacteria from batik wastewater that have the ability to reduce copper, given the existence of quite a lot of industrial batik in Indonesia. Based on the results of previous research regarding the ability of indigenous bacteria in heavy metal remediation, this study carried out a resistance ability test and a potential test of bacterial isolates from batik wastewater in reducing copper,so that it can be used as biological agent in remediation of copper from various sources of environmental pollutans. This research is expected to be a development step in the use of bacteria in terms of overcoming environmental waste problems biologically.

MATERIALS AND METHOD

Sampling of batik wastewater

Samples of batik wastewater were obtained from one of the batik industries in Yogyakarta. This research was conducted at the Microbiology laboratory of UIN Sunan Kalijaga Yogyakarta from December 2019 to February 2020.

Procedures

Isolation of copper resistant bacteria.

Isolation was conducted based on Irawati et al (2017), using enrichment culture. A total of 10 mL samples of batik wastewater inoculated onto NB containing $CuSO_4$ 100 ppm, then incubated at 35^{o}C with shaking at 150 rpm for 48 hours. After 48 hours of incubation, samples was serially diluted from 10^{-1} to 10^{-9}. Dillution samples of 10^{-7}, 10^{-8}, and 10^{-9} were inoculated by the pour plate method in NA medium containing $CuSO_4$ 100 ppm and incubated at 35^{o}C for 24-48 hours. The growing colonies on medium were selected followed by purification to obtain single colony.

Copper resistance test.

Testing the resistance of bacteria to copper was adopted from the research of Saikia et al. (2015). Bacterial isolates were grown on NA medium containing $CuSO_4$ with concentrations of 200, 300, 400, 500, 600, and 700 ppm. Each treatment was repeated three times. Testing the resistance of bacteria to copper was carried out by the streak plate method. Bacterial isolates that are able to grow at the highest copper concentration after 24 hours of incubation at 35^{o}C were then used for further tests.

Copper reduction test (Biosorption of heavy metal).

Bacterial cells were grown in 150 mL NB containing 100 ppm $CuSO_4$. Cell culture was incubated at 35^{o}C with shaking at 150 rpm (Anggriany et al., 2018). After cell growth decrease, sampling was carried out to measure the reduction in copper levels.

Samples (100 mL) was centrifuged at 5000 g for 20 min at 4^{o}C to separate supernatant from the pellet. The supernatan was digested with HNO_3. The copper content was determined by using an atomic absorption spectrofotometer at 324,7 nm. Copper reduction efficiency was calculated as (Ci-Cf/Ci)x 100% where Ci is the initial concentration and Cf is the remaining copper concentration in the solution (Mokhtar et al., 2011).

Characterization of bacteria

The selected isolates were studied for their phenotypic and biochemical characters. The results of characterization were used for identification using profile matching analysis of selected isolates based on Bergey's Manual of Determinative Bacteria (Buchanan & Gibbons, 2003) and Bergey's manual of Systematic Bacteriology (Boone et al., 2010).

Data analysis

The data obtained were presented in tabular for, then analyzed descriptively qualitatively to show which isolates were able to reduce highest copper levels.

RESULTS AND DISCUSSION

Bacterial isolation

Ten copper toleran bacteria were successfully isolated from batik wastewater, namely: α1, α2, α3, α4, α5, α6, α7, α8, α9, and α10. The isolates were differentiated based on colony morphology, including: color, shape, edge, elevation and colony diameter. Colony color is white (dominant) and a few green glowing yellow; generally circular and irregular; has entire (dominan), undulate and filiform edges; flat elevated and diameter 0,2-0,9 cm. Ten bacteria isolates that were able to grow on NA media containing 100 ppm $CuSO_4$ were then tested further to determine the high level of resistance to copper.

Copper resistance

Bacterial resistance test was carried out to determine the level of tolerance of bacteria to copper. According Saikia et al. (2015), the level of tolerance of bacteria can be seen based on their growth in NA media containing $CuSO_4$ with concentration from 200 to 700 ppm. The results of resistance testing on 10 bacterial isolates showed that the α3, α5, α8, and α9 bacterial isolates were able to grow on NA media containing $CuSO_4$ 700 ppm.

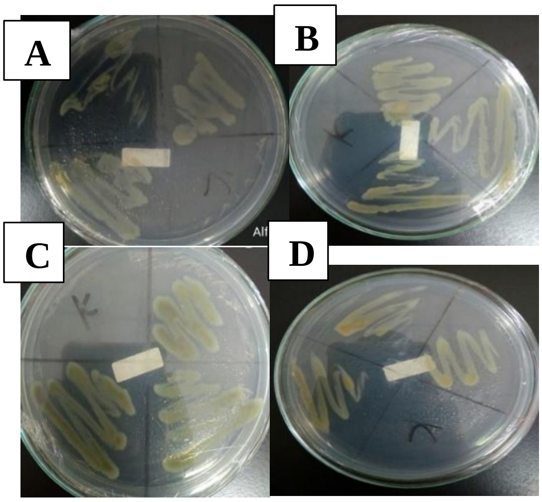

Figure 1. Bacterial resistance test. (A) Isolate α3, (B) Isolate α5, (C) Isolate α8, (D) Isolate α9.

Copper reduction test

The bacteria most resistant to copper were then selected for the reduction test. Bacterial isolates that have a copper resistance mechanism are known to reduce levels of heavy metal pollutans, especially copper in the environment. This is a results of research by Irawati et al. (2017) who proved that bacteria that were able to survive (resistant) on media with copper were also proven to reduce copper levels by a percentage of 82,53%.

Table 1. The percentage of copper reduction by the four isolates of indigenous batik wastewater

Bacterial Isolates	Initial Cu Levels (ppm)	Final Cu Levels (ppm)	Persentage Reduction
α3	100	24,97	75,03%
α5	100	23,27	76,73%
α8	100	22,95	77,05%
α9	100	23,66	76,34%

Characterization of bacteria

The four isolates that have the potential as copper bioremediation agent show various phenotypic characters, as shown in Table 2.

Table 2. Phenotypic characters of copper reducing bacterial isolates from batik wastewater.

No	Characters	α3	α5	α8	α9
A	**Colony morphology**				
	Media agar plate				
1	Form	Circular	Circular	Irregular	Irregular
2	Edge	Entire	Entire	Undulate	Filiform
3	Color	Green glows yellow	White	White	White
4	Elevation	Flat	Flat	Flat	Flat
5	Diameter	0,3 cm	0,5 cm	0,3 cm	0,5 cm
	Media slant agar				
1	Growth	Thin	Thin	Thin	Thin
2	Growth pattern	Spiny	Spiny	Spiny	Effuse
B	**Cell morphology**				
1	Gram	-	-	-	-
2	Cell shape	Rod	Rod	Rod	Rod
3	Arrangement of cells	Single	Single	Single	Single
4	Endospore	-	-	-	-
5	Motility	+	+	+	+
C	**Effect of temperature factor**				
1	4	-	-	-	-
2	35	+	+	+	+
3	50	+	+	+	+
D	**Effect of pH factor**				
1	5	+	+	+	+
2	7	+	+	+	+
3	9	+	+	+	+
E	**Effect of NaCl (%)**				
1	3	+	+	+	+
2	6,5	+	+	+	+
3	15	-	-	-	+

Table 2. Continue

No	Characters	α3	α5	α8	α9
F	**Acid producing**				
1	Glucose	+	+	-	+
2	Mannitol	-	-	-	-
3	Sucrose	-	-	-	-
4	Maltose	-	-	-	-
5	Lactose	-	-	-	-
G	**Gas producing**				
1	Glucose	-	-	-	-
2	Mannitol	-	-	-	-
3	Sucrose	-	-	-	-
4	Maltose	-	-	-	-
5	Lactose	-	-	-	-
H	**Biochemistry characterization**				
1	Catalase	+	+	+	+
2	H_2S production	-	-	-	-
3	Nitrate reduction	-	+	+	-
4	Starch hydrolysis	+	+	+	+
5	Gelatin hydrolysis	-	-	-	-
6	Citrate hydrolysis	+	+	+	+
7	Indol	-	-	-	-
8	MR	-	-	-	-
9	VP	-	-	-	-
10	Oxigen requirements	Aerob	Aerob	Aerob	Aerob
I	**Clear zone to antibiotics (cm)**				
1	Amoxilin	0,9	0,8	0,8	1,2

2	Cefixisme	0,9	0,7	1	0,8
3	Tetrasiklin	0,7	2,1	0,8	0,7

Information:
+: the isolate gave a positive result on the test
-: the isolate gave a negative result on the test

Table 3. Profile matching of isolates α3, α5, α8, and α9 with reference genus based on *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 2003) and *Bergeys's Manual of Systematic Bacteriology* (Boone, et al., 2010).

Characters unit	Genus *Pseudo-Monas*	α8	Genus *Xantho-monas*	α3	α5	α9
Pigmentation	*Non-/* Yellow-green Pigmented	Green	Yellow	Yellow	Yellow	Yellow
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram	Negative	Negative	Negative	Negative	Negative	Negative
Motility	Motile/ Non-motile	Motile	Motile	Motile	Motile	Motile
Endospore	-	-	-	-	-	-
Arrangement of cells	Single	Single	Single	Single	Single	Single
Catalase	+	+	+	+	+	+
H_2S-production	NA	-	+	-	-	-
Indole-formation	NA	-	-	-	-	-
Nitrate-reduction	+/-	+	+/-	-	+	-
Starch-hydrolysis	+/-	+	NA	+	+	+
Gelatin-hydrolysis	+	-	NA	-	-	-
Citrate-hydrolysis	+	+	NA	+	+	+
Metyl red	-	-	NA	-	-	-
Voges-proskauer	-	-	NA	-	-	-
Gas producing:						
Glucose	+/-	-	NA	-	-	-
Sucrose	NA	-	NA	-	-	-
Maltose	NA	-	NA	-	-	-
Acid producing						
Glucose	+/-	-	+	+	+	+
Sucrose	+/-	-	+	-	-	-
Maltose	-	-	+	-	-	-
O_2 requirements	Aerob	Aerob	Aerob	Aerob	Aerob	Aerob
Growth at temperature:						
4ºC	-	-	-	-	-	-
35ºC	+	+	+	+	+	+
50ºC	NA	+	NA	+	+	+

Information : NA – *not applicable*

Discussion

From the results of the resistance test, four bacterial isolates that had high tolerance to copper were obtained, namely isolates α3, α5, α8, and α9 bacterial isolates which were able to grow on NA media containing $CuSO_4$ 700 ppm.

The bacterial isolates were able to survive in high copper concentrations due to resistance mechanism that they passively (biosorption) and actively (bioacumulation) display in response to the presence of heavy metals in their environment (Bae et al., 2003). In principle, biosorption is the binding of metal ions to the cell structure of microorganisms, especially in the cell wall. Functional groups responsible for the attraction and sequestration of pollutans include carboxylate, hydroxyl, carbonyl, carboxyl, imine, imidazole, sulfonate, sulhydryl, thioether, phenolic, amide, amine, phosphate, and phospodiester groups within the cell-wall components, such as polysaccharides,lipids and proteins (Hansda et al., 2015). The biosorption mechanism is a complex process, that no single mechanism is able to describe the copper absorption. The binding occurs by any one or a combination of the physicochemical attractions between the metal ions and functional groups present on the cell wall surface. It includes, coordination, complexation, ion exchange, physical adsorption or inorganic microprecipitation (Sud et al., 2008).

The form of bacterial resistance to copper also controlled by genes on chromosomal and plasmids (Irawati et al., 2016). Bacterial resistance to copper has been described in groups of Gram negative bacteria such as *Pseudomonas syringae*, *Xanthomonas*, and *E. coli*.

Molecular analysis of the copper resistance on *P.syringae* pv. *tomato* revealed an operon Copper (*Cop*) located in plasmid , namely structural gene *CopA, CopB, CopC,* and *CopD* whose expression is controlled by two regulatory genes, namely *copR and CopS*. E.coli resistance controlled by the conjugative plasmid pRJ1004 which is composed of six structural genes, namely *pcoA, pcoB, pcoC, pcoD, pcoR,* and *pcoS* (Silver, 1996 & Cooksey, 1994).

Based on the results of the copper reduction test, it was found that there was a descrease in copper concentration by bacteria. Data in table 4 show that bacterial isolates from batik wastewater can reduce copper levels. From the results above, it can be seen that the largest percentage reduction was in bacterial isolate α8 with a reduction percentage of 77,05%. Isolate α3 had the lowest reduction ability with a percentage 0f 75,03%, while isolates α5 and α9 had almost the same reduction ability, with a percentage of 76,73% for isolate α5 and 76,34% for isolate α9.

The reduction percentage of the four bacterial isolates showed the effectiveness of the bacteria in reducing the copper concentration. The copper concentration in batik wastewater was 0,0130 mg/L, while the copper concentration used in this study was 100 mg/L, meaning that the bacterial isolates was able to reduce copper levels higher than the original environment.

Based on the results of profile matching analysis on table 3, isolates α3, α5, and α9 were thought to be members of the Genus Xanthomonas because they showed several similarities in characters, including having yellow pigmentation, rod-shaped cell, Gram negative, motile, aerobic and catalase positive. Isolates α8 showed several characters including, green pigmentation, rod-shaped cell, Gram negative, motile, does not form endospores, aerobic, and able to reduce nitrate. The results of the profile matching analysis of isolate α8 showed had similar characters to the Genus Pseudomonas.

Several previous studied stated that bacteria form the Gram negative group were resistant to copper, including form the Genus Pseudomonas and Zoogloea which were isolated from batik wastewater (Dianrevy, 2017), besides that there were also bacteria from the Genus Xanthomonas (Silver, 1996). Meanwhile, bacteria from the Gram positive group, namely from the Genus Bacillus, were isolated from batik wastewater and electroplating industrial waste (Dianrevy, 2017; Rani et al., 2010). Based on this research, the reduction percentage of copper using these bacteria can reach 60 to 80% by paying attention to supporting parameters such as pH, Copper concentration and incubation time.

The bacterial isolates obtained in this study had the ability to reduce copper in the range of 75 to 77%, which means that they had the same reduction ability as the bacterial isolates obtained in previous studies. The ability of bacterial isolates to reduce copper is influenced by the activity of each microorganism so that it is necessary to optimize temperature, pH, nutrition, incubation time and other environmental factors to increase the ability of bacteria to reduce copper.

CONCLUSSION

There are four bacterial isolates from batik wastewater that have high tolerance to copper, namely α3, α5, α8 and α9. Isolates α3, α5, and α9 were able to reduce copper by 75,03%; 76,73%; and 76,34% respectively. The three bacterial isolates showed similar characteristics to members of the Genus Xanthomonas. Isolate α8 was able to reduce Cu up to 77,05% and was identified to be similar to member of the Genus Pseudomonas. This research is a preliminary study to obtain bacterial isolates as bioremediation agents for copper from batik wastewater. It is necessary to do research on optimization of temperature, pH, nutrition, incubation time and other environmental factors to increase the ability of microorganisms to reduce copper. In addition, it is necessary to identify bacteria molecularly to confirm the identification results with biochemical tests.

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