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Original Article

Exploration of synthetic dyes degrading bacteria from Siak River, Riau Province

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Abstract

The presence of dyes causes a decrease in river quality. Degradation using bacteria could be used as an alternative method to overcome the dyes substances waste. This research aimed to isolate bacteria that can degrade dyes such as congo red (CR) and methylene blue (MB) from Siak River. The result of this study identified a total of 27 isolates of bacteria that can degrade CR with a concentration of 100 ppm in a Minimal Medium with three days of incubation. The ability of bacteria to degrade CR was known by forming clear zones around the colonies. The degradation index ranged from 1.54 to 5.61 after three days of incubation. The isolate CR26 had the highest degradation. Meanwhile, the number of bacteria that degrades the MB in the concentration of 25 ppm are three isolates (MB1, MB2, and MB3) in the degradation index range from 1.32-1.41. One isolate (MB2) was obtained from the concentrations of 50 ppm and 100 ppm with a degradation index of 1.42 and 1.34. Identification results isolate CR 26 as *Bacillus* sp and isolate MB2 as *Enterobacter* sp. This bacteria can be developed to overcome water pollution caused by CR and MB dyes.

Keywords: bacteria, congo red, decolorization, methylene blue, Siak river

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Introduction

Siak River is one of the main rivers in Riau Province that has enormous benefits for all parties, namely as a source of domestic water for communities along the Siak River, industry, fishermen, and transportation. The Siak River has a length of about ± 345 km. The width of the Siak River varies from 20-200 m and has a depth ranging from 6-26 m. Siak River has 43 docks, both small and large. Many industrial plants such as palm oil, rubber, glue, pulp, paper, plywood, molding, sawmill, and tire retreading industries discharge their waste into the Siak River, causing the river to be polluted (Putri et al., 2014). Johar (2019) reported pollution in the Siak River is caused by waste from industries along rivers, shipping, and waste surrounding households. Rivers polluted with high concentrations of waste can adversely affect the environment and health (Irawati et al., 2023).

One of the wastes in the river is liquid waste containing azo dyes in Congo red and thiazine dyes in methylene blue (Hashemi & Kaykhaii, 2022). Dyes are widely used in printing, colour, textile, rubber products, paper and pharmaceutical industries. Synthetic dyes have complex chemical structures, making them difficult to degrade (Abo-State et al., 2017). The presence of dye waste in the waters will cause water pollution that can disrupt the balance of life in aquatic systems (Al-Tohamy et al., 2022), such as discoloration in the waters, and can limit the reoxygenation capacity in aquatic systems to reduce water quality. In addition, if the reoxygenation capacity is disrupted, the decomposition of pollutants in carcinogenic or mutagenic compounds and aerobic biodegradation becomes low (Gita et al., 2017). One of the efforts to tackle pollution caused by dyes is biodegradation using microbes such as bacteria, fungi, and actinomycetes (Patel et al., 2022; Fanny et al., 2018). Bacteria are better at removing dyes because cell division is faster or the growth cycle is shorter than other microorganisms (Eslami et al., 2017; Moyo et al., 2022).

The research that examines the biodegradation of dyes using microbes includes research conducted by Pandey et al. (2007) regarding the decolorization of azo dyes using *Pseudomonas aeruginosa* bacteria which are efficiently able to degrade Congo red dyes with the highest degradation percentage of 87.64% at a concentration of 25 ppm within five days at pH 9. The highest multi-resistant bacterium was *Bacillus* cereus from the Citarum River, West Java, with decolorization rates of 93.04% on 100 ppm Methylene blue (Irawati et al., 2023).

So far, there is no previous report on the Siak River decolorizer of cationic dyes such as Congo red (CR) and Methylene blue (MB), so it is necessary to make efforts to get bacteria that can degrade dyes from the Siak River. This study aims to isolate bacteria that can degrade dyes like Congo red and Methylene blue from Siak River, Riau Province.

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Methods

Study area

Sample was carried out in the Siak River, Riau Province. Siak River water samples were taken from 5 sample points (Sample Point I: N 00°32'27.5" E 101°26'12.7"; II: N 00°32'29.7" E 101°26'17.7"; III: N 00°32'28.3" E 101°26'56.4"; IV: N 00°32'31.1" E 101°26'12.5" and V: N 00°32'22.7" E 101°26'48.3" (Figure 1). Sampling points were taken along the river from upstream to downstream.

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Figure 1. Map of sampling location in Siak River, Pekanbaru, Provinsi Riau, Indonesia

Isolation and screening of Congo red degrading bacteria

A 10 ml water sample was inoculated into an Erlenmeyer containing 90 ml Mineral Salt Medium (MSM) broth medium containing 1 g KH2PO4, 1 g Na2HPO4, 5 g glucose, 1 L distilled water, and 100 ppm congo red added. The Erlenmeyer was incubated at 30°C for 7 days on a shaker incubator at 150 rpm. At the end of the incubation, the inoculum was grown by spread plate method onto MSM agar + congo red 100 ppm and incubated at room temperature for 24 hours. Bacterial colonies that formed clear zones were selected for further purification (Mahmood et al., 2011).

Purified bacterial isolates were then tested for degradation index. Each bacterial isolate was inoculated by spotting to MSM agar medium + congo red 100 ppm incubated for 3 days at room temperature. Every day, the clear zone formed around the bacterial colony was observed. Degradation index is the ratio between clear zone and colony diameter (Martani et al., 2011).

Isolation and screening of methylene blue degrading bacteria

A 10 ml water sample was inoculated into an Erlenmeyer containing 90 ml MSM broth medium, containing 1 g KH2PO4, 1 g Na2HPO4, 5 g glucose, 1 L distilled water, and 25 ppm Methylene blue was added. Erlenmeyer was incubated on a shaker incubator. After incubation, the inoculum was grown by spread plate method to MSM agar + Methylene blue 25 ppm medium. Bacterial colonies that formed clear zones were selected and purified (Mahmood et al., 2011).

Purified bacterial isolates were then tested for degradation index. Each bacterial isolate was inoculated by spotting to MSM agar + Methylene blue 25 ppm and incubated for 3 days at room temperature. Every day, the clear zone formed around the bacterial colony was observed. Methylene blue degradation ability was carried out with a concentration variation of 25 ppm, 50 ppm, and 100 ppm. Degradation index is the ratio between clear zone and colony diameter (Martani et al., 2011).

Characterization of bacteria

Identification of isolates obtained based on macroscopic characterization, physiology: Gram staining, and biochemical tests: catalase, motilities, hydrolysis casein, starch, gelatin, fermentation of sugar: glucose, sucrose and fructose.

Results

Characterization of bacteria

The temperature range of the five sampling points is 26.7 - 30.6°C and is still considered normal in water bodies and for the life of aquatic biota. The degree of acidity (pH) ranged from 5.48 - 7.63, within the quality standard threshold. However, pH values are outside the threshold (5.48 - 5.65) at sampling points 1-3. The low pH at sampling points 1-3 is due to the presence of domestic waste and also higher population density than 4 and 5. Sharma et al. (2022) reported that neutral pH favors azo dye degradation. The Degradation at pH 5 and 6 were comparatively lower, with 66% and 83.3%, respectively.

Table 1. Characteristic sample and isolation results of Congo red and methylene blue dye degrading bacteria

Sampling Point	Physical and chemical sample					Bacterial Isolate (Congo red)	Bacterial Isolate (Methylene Blue)
	Temp (°C)	pH		Water quality			
			DO (0) (mg/l)	DO 5 (mg/l)	BOD 5 (mg/l)		
I	26.7	5.65	7.0	4.2	2.8	5 isolates (CR3, CR4, CR5, CR6, CR7)	1 isolate (MB1)
II	27.6	5.61	7.4	4.3	3.1	1 isolate (CR8)	1 isolate (MB2)
III	30.6	5.48	8.9	6.1	2.8	6 isolates (CR10, CR12, CR14, CR15, CR16, CR17)	1 isolate (MB3)
IV	29.2	7.63	8.1	3.3	4.8	7 isolates (CR1, CR2, CR11, CR19, CR21, CR24, CR25)	-
V	28.4	6.20	8.6	4.3	4.3	8 isolates (CR9, CR13, CR18, CR20, CR22, CR23, CR26, CR27)	-
Total						27 isolates	3 isolates

Based on the results of the measurement of dissolved oxygen (DO) obtained in the range of 7.0-8.9 mg/ml which is classified as low polluted. The range of Biological Oxygen Demand (BOD) obtained ranged from 2.8-4.8 mg/L and was classified as low polluted.

Isolation and test of ability of congo red dye degrading bacteria

A total of 27 bacterial isolates were screened for Congo red dye decolorization from 5 sampling points in the Siak River (Table 1). The degradation index of the test results of the ability of all isolates to degrade Congo red dye at a concentration of 100 ppm can be seen in Table 2. The CR26 isolate is the most potent bacterial strain degradation with a degradation index 5.61.

A clear zone around the colony indicates the ability of bacteria to degrade Congo red dye. All bacterial isolates show differences in the clear zone produced by each isolate. The longer the incubation time, the larger the clear zone. The clear zone produced differs in the incubation time variant (Figure 2). This result is based on the research of (Holey, 2015), that bacterial isolates IS1, IS2, IS3, IS4, IS5, and IS6 isolated from waste soil samples collected from around the dye shops in the Wardha market, India increased the percentage of degradation at a concentration of 100 ppm from day 1 to day 4.

Table 2. Degradation index of bacterial isolates on Minimal Salt Medium + congo red with a concentration of 100 ppm at 3 days incubation

No.	Isolate	Degradation Index			No.	Isolate	Degradation Index		
		Z (cm)	K(cm)	Z/K			Z (cm)	K (cm)	Z/K
1.	CR1	3.92	0.86	4.51	**15**	CR15	0.54	0.35	1.54
2.	CR2	1.52	0.43	3.66	**16**	CR16	1.16	0.59	1.97
3.	CR3	1.37	0.63	2.17	**17**	CR17	1.08	0.51	2.10
4.	CR4	1.37	0.42	3.28	**18**	CR18	0.39	0.21	1.86
5.	CR5	1.68	0.37	4.64	**19**	CR19	0.84	0.41	2.06
6.	CR6	1.83	0.41	4.56	**20**	CR20	1.48	0.43	3.67
7.	CR7	1.10	0.31	3.54	**21**	CR21	0.59	0.36	1.65
8.	CR8	1.70	0.43	4.02	**22**	CR22	2.09	1.12	2.04
9.	CR9	1.47	0.66	2.23	**23**	CR23	0.53	0.28	1.92
10.	CR10	0.93	0.47	1.98	**24**	CR24	1.18	0.48	3.36
11	CR11	1.09	0.57	1.92	**25**	CR25	1.04	0.56	1.85
12	CR12	1.57	0.51	3.24	**26**	CR26	1.71	0.30	5.61
13	CR13	1.50	0.59	2.48	**27**	CR27	1.33	0.60	2.21
14	CR14	2.14	0.48	4.72					

Note: Z: Clear Zone, K: Bacterial Colonies

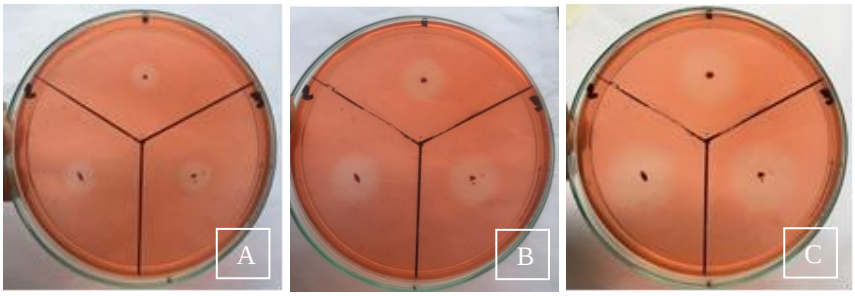

Figure 2. Isolate ability test on CR dye concentration of 100 ppm with variation of incubation time for isolate CR26 A: Day 1, B: Day 3, C: Day 6

Isolation and degrading of methylene blue dye by bacteria

The results of bacterial isolation from 5 different points in the Siak River grown on MSM media with the addition of Methylene Blue (MB) dye can be seen in Table 1. The results of bacterial isolation from 5 different points on the Siak River grown on MSM media with the addition of MB dye obtained three isolates, namely MB1, MB2, and MB3, which can degrade MB dyes at a concentration of 25 ppm.

The results showed that at a concentration of 25 ppm, the range of degradation index was between 1.32-1.41, with the highest degradation index by isolate MB2 which was 1.41, and the lowest by isolate MB1, which was 1.32. While at concentrations of 50 ppm and 100 ppm, only isolate MB2 can degrade MB dye. So that MB2 isolate is the most potential isolate because it can degrade MB up to a concentration of 100 ppm. At a concentration of 100 ppm, MB2 isolate has a degradation index of 1.34 which is lower than the degradation index at a concentration of 50 ppm, which is 1.42 (Table 3). The ability of bacteria to degrade Methylene blue dye is characterized by the presence of a clear zone around the colony which can be seen in Figure 3.

Table 3. Degradation index in measuring clear zones and bacterial colonies on media containing MB with 100, 50, and 25 ppm concentrations at three days incubation

No	Isolate	Concentration								
		25 ppm			50 ppm			100 ppm		
		Z (cm)	K (cm)	Z/K	Z (cm)	K (cm)	Z/K	Z (cm)	K (cm)	Z/K
1.	MB 1	0.26	0.20	1.32	-	-	-	-	-	-
2.	MB 2	0.79	0.56	1.41	0.97	0.68	1.42	0.71	0.53	1.34
3.	MB 3	0.53	0.40	1.33	-	-	-	-	-	-

Note: Z: Clear Zone, K: Bacterial Colonies.

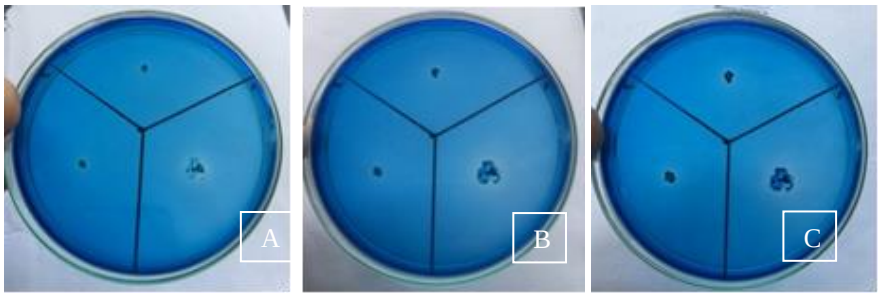

Figure 3. Isolate ability test on MB dye concentration of 100 ppm for isolate MB2 A: Day 1, B: Day 3, C: Day 6

Partial characterization of Congo red and methylene blue dye degrading bacteria

Generally, the isolates obtained in this study have beige color, round colony shape, convex elevation, and entire colony edge. According to Nguyen et al. (2021), growth with sufficient nutrients and favorable environmental conditions for various species of bacteria will sometimes produce distinctive colonies. Some colonies may be colored, some are circular, while others are irregular. The results of macroscopic morphological characterization can be seen in Table 4, while the results of physiological characterization and biochemical tests can be seen in Table 5.

The isolate with the highest degradation of CR is CR26 isolate. This isolate is Gram-positive, basil, and motility negative. Identification results showed isolate CR26 as *Bacillus* sp. The isolates with the highest degradation of MB is MB2 isolate. This isolate is Gram-negative, motility positive, and can ferment glucose and sucrose. Identification results showed isolate MB2 as *Enterobacter* sp.

Table 4. Results of macroscopic characterization of dyestuff degrading bacteria

Isolate	Colony Diameter	Shape	Colour	Elevation	Colony Edge
CR1	Small	Circular	Yellowish white	Raised	Entire
CR2	Moderate	Circular	Cream	Flat	Entire
CR3	Small	Circular	Yellowish white	Flat	Serrate
CR4	Small	Circular	Cream	Convex	Entire
CR5	Small	Circular	Cream	Convex	Entire
CR6	Moderate	Circular	White	Convex	Entire
CR7	Small	Circular	Orange	Raised	Entire
CR8	Moderate	Circular	Cream	Convex	Entire
CR9	Small	Circular	Yellowish white	Flat	Entire
CR10	Small	Circular	Cream	Flat	Entire
CR11	Small	Circular	Cream	Convex	Entire
CR12	Small	Circular	Cream	Flat	Entire
CR13	Small	Circular	White	Umbonate	Entire
CR14	Moderate	Circular	White	Convex	Entire
CR15	Moderate	Circular	White	Flat	Entire
CR16	Small	Circular	Cream	Convex	Entire
CR17	Small	Circular	Cream	Flat	Entire
CR18	Small	Circular	Cream	Convex	Entire
CR19	Small	Circular	Cream	Convex	Entire
CR20	Small	Circular	White	Umbonate	Entire
CR21	Small	Circular	Cream	Flat	Entire
CR22	Small	Circular	Cream	Convex	Entire
CR23	Small	Irregular	Cream	Convex	Entire
CR24	Moderate	Circular	Yellowish white	Flat	Serrate
CR25	Moderate	Circular	Cream	Flat	Entire
CR26	Small	Irregular	White	Flat	Undulate
CR27	Small	Circular	Cream	Convex	Entire
MB 1	Small	Circular	White	Convex	Entire
MB 2	Small	Circular	Cream	Raised	Entire
MB 3	Small	Irregular	White	Flat	Serrate

Discussion

Synthetic color degradation bacteria from Siak River, Riau Province, was explored at five sampling points. Based on the results of the measurement of dissolved oxygen (Dissolved Oxygen) was classified as low polluted because a lot of incoming waste causes dissolved oxygen levels to decrease. The range of BOD (Biological Oxygen Demand) was classified as low polluted. The highest BOD value is at point 4 because the area at point 4 has many residential areas, so the activities that occur in the river are increasing. This high BOD value indicates that the greater the amount of organic matter decomposed using a certain amount of oxygen in the waters (Sholihah et al., 2019). High BOD values cause receiving water bodies to lose DO content, creating anoxic conditions that harm aquatic systems and negatively affect both flora and fauna (Kishor et al., 2021). Bacteria can degrade, decolorize, detoxify and mineralize various pollutants by using different metabolic pathways.

Table 5. Results of physiological characterization and biochemical test of dyestuff degrading bacteria

Isolate	Carbohydrate Fermentation Test			Gram Staining	Cell Morphology	Hydrolysis			Motility Test
	Glu	Suc	Lac			Starch	Gelatin	Casein	
CR1	+	+	-	Positive	Basil	-	-	-	-
CR2	+	-	-	Positive	Basil	+	+	+	+
CR3	+	+	-	Negative	Basil	+	-	+	+
CR4	+	-	-	Positive	Coccus	+	-	-	-
CR5	+	+	+	Positive	Coccus	-	-	-	+
CR6	+	+	+	Negative	Coccus	-	-	-	+
CR7	+	+	-	Positive	Coccus	+	+	+	+
CR8	+	+	+	Negative	Coccus	-	-	-	+
CR9	+	+	+	Positive	Basil	-	-	-	+
CR10	+	+	+	Negative	Coccus	+	-	-	+
CR11	+	+	-	Negative	Coccus	-	+	-	+
CR12	+	+	-	Positive	Coccus	-	-	-	+
CR13	+	+	+	Negative	Coccus	-	-	-	+
CR14	+	+	+	Positive	Basil	-	-	-	+
CR15	+	+	+	Negative	Basil	-	-	+	-
CR16	+	+	+	Positive	Coccus	-	+	+	+
CR17	+	+	+	Negative	Coccus	+	+	-	+
CR18	-	-	+	Positive	Basil	+	-	+	-
CR19	-	+	-	Negative	Basil	+	+	+	-
CR20	+	+	+	Positive	Coccus	+	+	+	-
CR21	+	-	-	Negative	Coccus	-	-	+	-
CR22	+	+	+	Positive	Coccus	-	+	+	+
CR23	+	+	+	Positive	Basil	+	-	+	-
CR24	+	-	-	Positive	Basil	+	-	+	-
CR25	+	+	-	Positive	Coccus	-	-	-	+
CR26	+	-	+	Positive	Basil	-	+	+	-
CR27	+	+	+	Positive	Coccus	+	+	+	+
MB 1	+	-	-	Positive	Coccus	-	-	-	-
MB 2	+	+	-	Negative	Coccus	-	-	-	+
MB 3	+	-	-	Positive	Basil	+	-	+	+

Bacteria in the early stages require glucose in Minimal Salt Medium (MSM) as a cosubstrate and carbon source to accelerate the metabolism process. One example is *P. aeruginosa*, proven to degrade azo dye methyl red in the presence of glucose under aerobic conditions on a Mineral Salt Medium (Ikram et al., 2022). Glucose is a substrate needed in glycolysis and reacts with the dehydrogenase enzyme to produce nicotinamide adenine dinucleotide (NADH). NADH is an electron carrier for the degradation process of azo dyes (Hopp et al., 2019).

The isolate with the highest degradation of Congo red is *Bacillus* sp. Several *Bacillus* strains have been reported to degrade dyes. *Bacillus* could be used as a good microbial source for wastewater treatment, specifically in the aerobic biological degradation of textile dye effluent (Hanis et al., 2020). B. velezensis CR-502T can degrade Congo red was proven through the lignin peroxidase efficiency and accumulation in the biomass of the living cells (Pham et al., 2022). B. albus has a strong potential to decolorize and detoxify MB dye for environmental safety. The findings of the toxicity assessment revealed that the toxicity of the bacteria-treated dye solution was significantly reduced, enabling 90% seed germination of mung bean (Kishor et al., 2021). B. thuringiensis has an efficient degradation effect on MB dye. The strain showed the highest decolorization rate for MB at pH 6.0, temperature 30 °C, rotation speed 140 rpm, and 10–50% salinity (Wu et al., 2022).

The process of CR dye degradation by bacterial isolates is due to breaking azo bonds in the dye. According to Misal & Gawai (2018), the enzyme azoreductase breaks the azo bond, breaking CR into diamino naphthyl sulfonic acid and benzidine. CR dye, a class of azo dyes, has a threshold in the waters of 5 ppm, according to the Decree of the Minister of Environment No. 51 of 1995. If these compounds exceed the threshold, the presence of these compounds will cause environmental pollution, such as decreased river water quality (Yuningrat et al., 2017). Isolates isolated in this study can degrade CR up to 100 ppm so that these isolates may be developed to overcome pollution caused by Congo red dye waste.

The difference in degradation index depends on the carbon source. The high degradation index at 50 ppm concentration is because of the optimum concentration in degrading MB by *Enterobacter* sp.. *Enterobacter* strain TS1L using a PHA medium containing 50 mg/l of MB, can degrade methylene blue (MB) at 92.57% and produce polyhydroxyalkanoate (PHA) at 56.02% so that this bacteria can be a solution to industrial dye-contaminated wastewater problem (Rakkan et al., 2023). *Enterobacter* sp. is rapid microbial degradation of textile dyes, can degrade synthetic dyes such as MB (percentage of degradation after 48 hours 67.30%), and may be used in overcoming industrial waste (Govindasamy et al., 2018). Cells of *Enterobacter hormaechei* were reduced azo bonds enzymatically and used as a biocatalyst for the degradation of azo dyes (Thangaraj et al., 2021). *E. aerogenes* PP002 effectively degrades the azo dyes DG 28 and DB 71 (Sudha et al., 2018).

Degradation or the formation of clear zones makes long-chain aromatic compounds (dyes) split into short-chain aromatic compounds due to the enzyme

azoreductase (Patel et al., 2020). Degradation can be seen based on the decrease in color intensity on the media or the formation of a clear zone. It can be used as an early indicator of the dye degradation process. According to Wang et al. 2012 in Valerie et al. (2018), the enzyme laccase is the enzyme that plays a role in the degradation process of malachite green. Malachite green belongs to the same group as MB when classified according to the similarity of its structure, namely the cationic dye group. So it is suspected that the enzyme that plays a role in MB dye degradation is the laccase enzyme (Ghobadi Nejad et al., 2019). Mechanisms degradation of dye proceeds via three different mechanisms: (1) absorption and concentration of the dye, (2) intracellular absorption and subsequent degradation of the dye, and (3) extracellular degradation of dye (Ghribi et al., 2016).

The present study showed the ability of several bacterial strains to degrade CR and MB dye. The results showed that the degradation depends on the incubation time. The *Bacillus* sp. isolate is the most potent bacterial strain that degraded CR dye, and *Enterobacter* sp. isolates are potent bacterial degraded MB dye. The isolated bacteria can be developed to overcome water pollution caused by dangerous dyes from the Siak River, Riau Province.

Conclusion

Total 27 bacterial isolates were screened for Congo red dye decolorization from 5 sampling points in the Siak River. The degradation index of the test results of the ability of all isolates to degrade Congo red dye at a concentration of 100 ppm. The *Bacillus* sp. CR 26 isolate is the most potent bacterial strain were degradation with degradation index 5.61. Total 3 bacterial isolates were screened for Methylene blue dye. The *Enterobacter* sp. MB 2 is the most potent bacterial strain were degradation with degradation index 1.41. This bacteria can be developed to overcome water pollution caused by CR and MB dyes. The two isolates need to be identified more specifically based on molecular markers.

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