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Effects of Ethidium Bromide and Sodium Dodecyl Sulfate Concentrations on the Viability of Multidrug-Resistant Bacteria Isolated from River Streams at State University of Malang

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Abstract: This study aimed to analyze the effect on applying various concentration combination EtBr and SDS that have been widely used in plasmid curing to the growth of multidrug-resistant bacterium encoded by MDR1 isolate originated from river stream in State University of Malang. This study is a laboratory experiment including the independent variable is the concentration combination of SDS and EtBr (treatment I: no EtBr and SDS, treatment II: 60 g/L SDS + 35 mL/L EtBr, treatment III: 50 g/L SDS + 50 mL/L EtBr, treatment IV: 40 g/L SDS + 65 mL/L EtBr), the dependent variable is the visible colonies count of the isolate MDR1, and the controlled variables are the bacterial growth media, antibiotics concentration, incubation temperature as well as incubation time. The results for the visible colonies count of isolate MDR1 after being treated with the curing agents were varied. Treatment III showed the best result in lowering the survivability of isolate MDR1 (95.55% inhibition) among the usage of EtBr and SDS, followed by treatment II (39.37% inhibition) indicating that EtBr holds stronger action in eliminating plasmid of the isolate MDR1. Treatment IV however, showed an increase of the visible colonies count of isolate MDR1 in antibiotics containing media suggesting that higher EtBr concentrations may enhance the curing of non-essential while allowing the persistence of more stable resistance determinants, leading to an apparent increase in growth, although further investtigation is mostly needed. Therefore this study contributes to develop standardize method in lowering the survivability of multidrug-resistant bacteria especially isolate MDR1 by eliminating its plasmid using curing agents.
Keywords: Multidrug-resistant (MDR) bacteria; plasmid curing; antibiotics

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INTRODUCTION

Pathogen bacteria are the kind of bacteria that have the ability to infect living organisms and cause health issues (Soni et al., 2024). This is due to the characteristic of the pathogen bacteria possessing the ability to express virulence factors that being within its plasmid encoded by specific gene (Alegbeleye et al., 2018). One of the most common pathogen bacteria is *Escherichia coli* (*E. coli*), able on causing diarrhea and infecting urinary tract infection characterized by polyuria, painful urination, and dysuria (Mane et al., 2023), but not all *E. Coli* falls under the classification as pathogen. *E. coli* is a Gram-negative, rod-shaped (bacillus) bacterium with a length of approximately 1.5 µm and a width of about 0.5 µm (Wei, 2024). *E. coli* can be found in the digestive tract and aquatic environments; when present in normal amounts, it can be beneficial, but an increase beyond normal levels can cause the bacterium to become pathogenic (Unok & Mangawing, 2024). Treatment of diseases caused by pathogenic *E. coli*

typically involves antibiotics such as tetracycline, amoxicillin, and chloramphenicol. However, improper use of antibiotics can trigger the emergence of multidrug-resistant *E. coli* strains, commonly known as superbugs, which are resistant to various types of antibiotics (Arivo & Dwiningtyas, 2019). Genomic analysis and plasmid curing experiments confirm that multidrug resistance in *Escherichia coli* is predominantly encoded on extrachromosomal plasmids rather than the chromosome. When these plasmids, carrying critical genes such as *bla-TEM*, *bla-CTX-M*, and *qnr* are experimentally cured or isolated across diverse strains, the bacteria lose their phenotypic resistance to major antibiotic classes (Azour et al., 2026; Rasool et al., 2023)

Previous study by Sholekah and Listrorini (2023) discovered multi-resistant bacteria isolated form reiver streams in Mathematics and Natural Sciences Faculty, State University of Malang. These study findings include the discovery of 16 isolates of Gram-negative colonies, mainly were found in the form of bacilli suggesting the isolates belong to *E. coli* species based on macroscopic and microscopic observations. The unique characteristic shown by these isolates are their ability to withstand the exposure of antibiotics, such as amoxicillin, tetracycline, and chloramphenicol, indicating that these isolates are multidrug-resistant bacteria (MDR bacteria). Hence, these isolates have the potential to be harmful, especially for the residents residing along the river. Antibiotic resistance might occur due to the horizontal genetic transfer or mutation involving DNA chromosomal or DNA plasmid containing antibiotic resistance genes (Park et al., 2024). Therefore, it is necessary to initiate efforts to weaken or eliminate the bacteria's antibiotic resistance.

One of the most practical and prominent method to eliminate the antibiotic resistance of MDR bacteria is via plasmid curing (Buckner et al., 2018). This is beacuse a significant number of clinically relevant antibiotic-resistance genes are carried by plasmids and other mobile genetic elements, facilitating their horizontal transfer between bacteria (Castañeda-Barba, et al., 2023). Plasmid curing involve the exposure of plasmid curing agents such as Sodium Dodecyl Sulfate (SDS) and Ethidium Bromide (EtBr) to degrade or inactivate the plasmid containing antibiotic resistance gene of the MDR bacteria (Gulton et al., 2022). EtBr can induce plasmid curing by intercalating into DNA and altering its structure and topology, thereby interfering with plasmid maintenance and replication (Buckner et al., 2018). In contrast, SDS primarily disrupts the bacterial cell envelope and can interfere with plasmid replication and segregation, resulting in plasmid loss during cell division (Sonstein & Baldwin, 1972). Consequently, cells that lose plasmids carrying antibiotic-resistance genes may become susceptible to the corresponding antibiotics However, the effectiveness of plasmid curing agents is highly dependent on the type of bacteria, plasmid stability, and treatment conditions. Beacuse of that, it is difficult to standardize a protocol for the concentration or conditions of curing agents, so curing studies are often conducted using a trial-and-error approach (Aladejana et al., 2024).

Based on the impacts caused by multidrug-resistant bacteria (superbugs) and previous studies, it is necesarry to initiate study to prevent antibiotic resistance through the plasmid curing method. By doing so, the standardize of EtBr and SDS concentration for lowering the antibiotic resistant trait of isolate MDR1 will be scientifically proven and can be used as further reference of the similar research, especially in plasmid curing. The effect of the curing agents will be observed based on the decrease of bacterial cell viability after being treated with SDS and EtBr because they will interfere with the DNA (plasmid) replication. Therefore, this study aims to analyze the effect of concentration vartions of SDS and EtBr as the curring agents for

lowering the resistance to tetracycline, amoxicillin, and chloramphenicol from the isolates found in the river streams of the Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang. Through this research, an alternative method to reduce the resistance of MDR bacteria is provided. This study also contributes to the research of antibiotic resistance bacteria prevention and therefore supports the achievement of Sustainable Development Goals (SDGs) 3.

METHOD

This study was a laboratory-based experimental investigation employing quantitative analysis using the experiment variables as follows; independent variable is the concentration combination of SDS and EtBr, the dependent variable is the visible colonies count of the isolate MDR1, and the controlled variables are the bacterial growth media, antibiotics concentration, incubation temperature as well as incubation time. The research was conducted in Genetic Engineering Laboratory, Biotechnology Study Program, State University of Malang. The purpose of this study Is to investigate the effect of various concentration of curing agents (SDS and EtBr) towards the viability of multidrug-resistant bacterium isolated from river stream in State University of Malang. The bacterium isolate was obtained from the river streams with the coordinate of -7.961144, 112.618699 on October 7th 2024. The isolate that was chosen for this study is based on the highest viability after being inoculated in agar media containing amoxicillin, tetracycline, and chloramphenicol antibiotics from the screening process. This isolate will later be encoded as isolate MDR1 (multidrug-resistant isolate number 1) for this study.

Instruments and Materials

The main instruments used in this study were UV–Visible spectrophotometer (B-ONE Vis DA), light microscope, analytical balance (Ohaus), laminar airflow cabinet (Hitachi), and various glassware. The materials used in this study were MDR1 bacterial isolate, Nutrient Agar (Himedia), Nutrient Broth (Himedia), Gram staining kit (Himedia), Sodium Dodecyl Sulfate (SDS), Ethidium Bromide (EtBr), antibiotics of amoxicillin, tetracycline, and chloramphenicol.

MDR1 Isolate Rejuvenation

The chosen isolate that is isolate MDR1 after preliminary screening was used for rejuvenation. A 100 µL of the culture from the glycerol stock was inoculated in NB media containing antibiotics with the concentration as follows; amoxicillin (32 ppm), tetracycline (16 ppm), and chloramphenicol (32 ppm). The mixture then incubated overnight at 37 °C and 120 rpm until the value of OD_{600} 0,6 was reached.

Gram Staining Analysis

The Gram staining procedure is based on the protocol provided within the kit. A thin bacterial smear was prepared on a clean, dry glass slide and allowed to air dry. The smear was subsequently heat-fixed by gentle heating. The fixed smear was flooded with Gram's crystal violet and allowed to stand for 1 minute, followed by rinsing with tap water. The smear was then treated with Gram's iodine solution for 1 minute to facilitate mordant formation, after which it was rinsed with water. Decolorization was performed using Gram's decolourizer (alcohol/acetone) until no further violet colour eluted from the smear, and the slide was immediately rinsed with water. The smear was counterstained with 0.5% (w/v) safranin for approximately 20 seconds, followed by a final rinse with water. The slide was then air-dried or gently blotted dry with absorbent paper. Finally, the prepared slide was examined under an oil immersion objective for microscopic observation and differentiation of bacterial cells.

SDS and EtBr Treatments and Viability Analysis

This procedure is based on Buckner et al. (2018) with modifications. One single colony of isolate MDR1 from triple antibiotics-containing NA was inoculated in triple antibiotics-containing NB and incubated overnight at 37 °C and 120 rpm. After reaching OD_{600} 0,6, 100 µL of the culture then transferred to each of 4 test tubes. To each test tube then added SDS and EtBr as the curing agents in correspondent to Table 1, bring up to 10 mL with NB media and incubated overnight at 37 °C and 120 rpm. For counting of the visible colonies, 100 µL of the culture inoculated in triple antibiotics-containing NA using spread method and analyzed for the next day after incubation with the same manner as the previous incubation conditions. The four variations of treatment (I-IV) are repeated the same way, except for last inoculation in NA, antibiotics-free NA was used instead for comparison.

Table 1. Variation combination of SDS and EtBr concentration

Treatment	Condition
I	None (negative control)
II	60 g/L SDS + 35 mL/L EtBr
III	50 g/L SDS + 50 mL/L EtBr
IV	40 g/L SDS + 65 mL/L EtBr

Data Analysis

Viability analysis of isolate MDR1 after being treated with the curing agents is based on the calculation of visible colonies count on the triple antibiotics-containing NA media. The number of colonies observed then being compared to each various combination of the curing agents concentration as well as to the presence or absence of the antibiotics in the very media. These colonies number then being interpreted to determine which combination of the curing agents gave the least cel viability indicating the most successful of the plasmid lost from the isolate MDR1.

RESULTS AND DISCUSSION

Figure 1 shows that isolate MDR1 is Gram negative bacteria. Morphologically, the bacteria appear rod-shaped (bacilli) with cells arranged in a scattered manner and not forming chains or organized clusters. This pattern of cell arrangement is a common characteristic of Gram-negative bacteria, such as *Escherichia coli*, which are typically found as single cells or in pairs (Nurhasanah et al., 2024), although 16S rRNA anlysis should be performed to accurately identify the species of the isolate. Gram staining plays an important role as an initial step in the identification and characterization of bacteria before further analyses are conducted, such as antibiotic resistance testing and detection of plasmids carrying resistance genes (Silhavy et al., 2025).

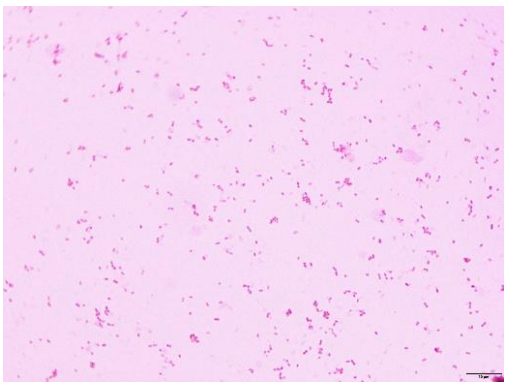

Figure 1. Visualization of isolate MDR1 gram staining under 1000x magnification

The effect of SDS and EtBr exposure to the MDR1 isolate can be seen in Figure 2 and 3 and tabulated in Table 1. After overnight incubation, the visible bacterial counts are mainly reduced in both conditions, that are the presence and the absence of the triple antibiotics. The reduction of the visible bacterial counts is due to the ability of the curing agents in eliminating the plasmid containing antibiotic resistance genes. As a curing agent, EtBr act based on the DNA intercalating mechanism. EtBr will insert itself into plasmid DNA (especially supercoiled DNA) and intercalates between DNA base pairs, by doing so the plasmid will be relaxed and form the open circular DNA. In this form, replication machinery will not function properly and therefore fail to replicate resulting the decrease of the bacterial cell population (Buckner et al., 2018; Letchumanan et al., 2015; Spengler et al., 2006). Meanwhile SDS act based on different mechanism, it will disrupt cell membrane and plasmid-associated protein and destabilizing plasmid maintenance. This process interferes with plasmid attachment and causes the transfer of resistance plasmid between cells reduced. Eventually plasmids are lost during cell divisions (Buckner et al., 2018; Letchumanan et al., 2015).

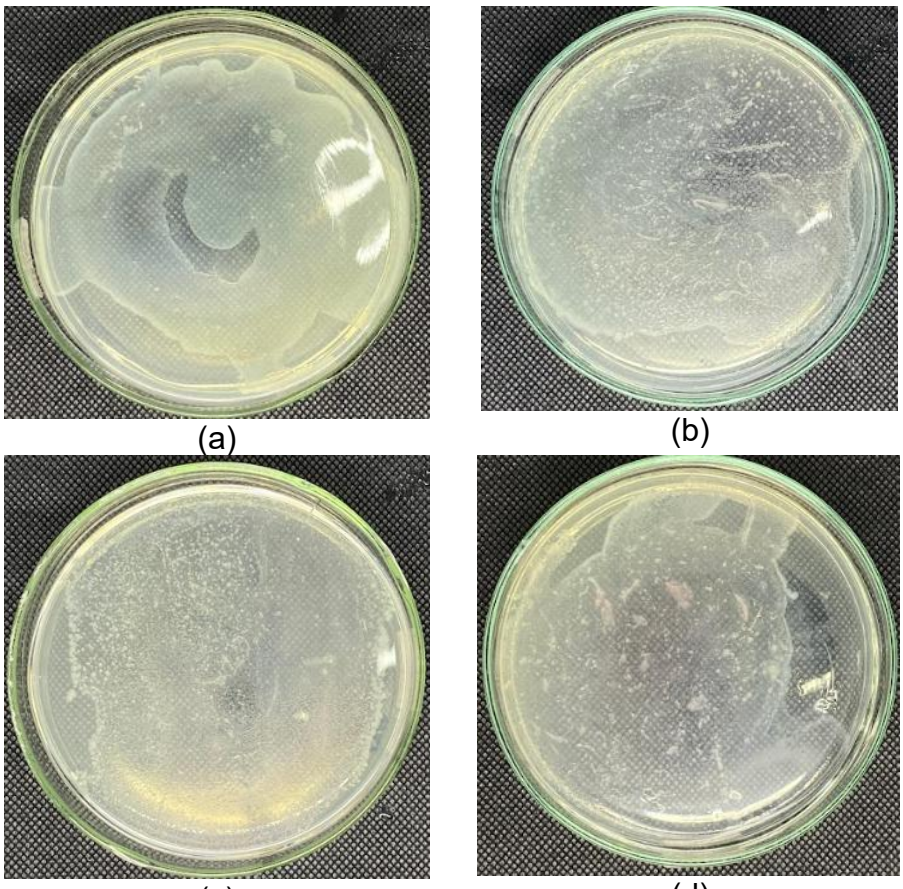

Figure 2. The growth of isolate MDR1 after incubated overnight in agar media without antibiotics: (a) treatment I, (b) treatment II, (c) treatment III, (d) treatment IV

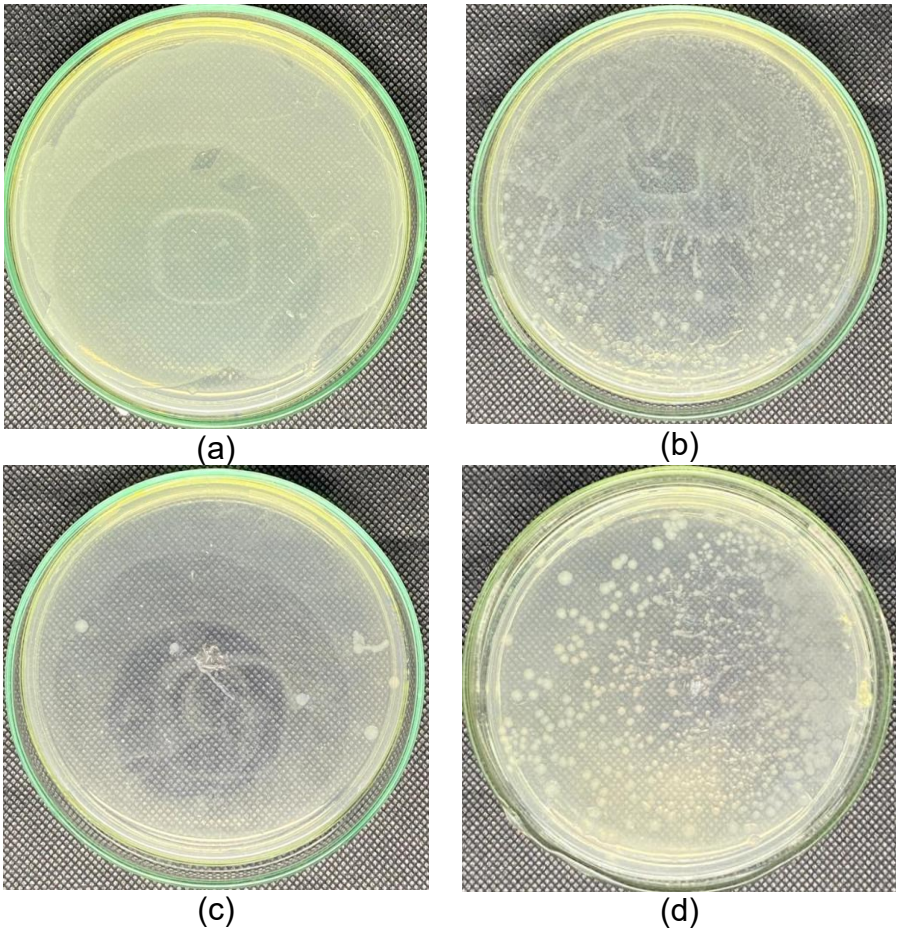

Figure 3. The growth of isolate MDR1 after incubated overnight in agar media with triple antibiotics; (a) treatment I, (b) treatment II, (c) treatment III, (d) treatment IV

Table 2. The visible colony counts of isolate MDR1 after incubated overnight in agar media

Treatment	Visible Colony Counts		Percent Inhibition (%)
	Without antibiotics (Figure 2)	With triple antibiotics (Figure 3)	
I	57	1	98.24
II	287	174	39.37
III	135	6	95.55
IV	80	158	N/A

Based on the visible colony counts, the isolate MDR1 grew better (more colonies observed) after being treated with SDS and EtBr (treatment II, III, and IV) compared to the negative control (treatment I) in both conditions that are with and without triple antibiotics in the agar media. The reason for this phenomenon is the fact that plasmid actually is a metabolic burden, since the bacterial cells will need more energy and resources to do plasmid replication (Millan & MacLean, 2017). Plasmid carriage is known to impose a fitness cost on bacterial cells by requiring additional energy and resources, thereby reducing growth rate and competitiveness, making the strongest growth inhibiton up to 98.24% (Wang & Joffre, 2025). Consequently, the elimination of plasmids enhances bacterial growth and viability, thus when isolate MDR1 lost its plasmid because of the exposure of SDS and EtBr, the cells growth will increase.

The comparison of the visible colonies counts for the treatment I when inoculated in agar media containing triple antibiotics shows great reduction (98.24%). This phenomenon is somewhat unnatural since the isolate was not treated with the curing agents and should retain its survivability in the presence of antibiotics. This occurance can be attributed to the fitness cost associated with antibiotic resistance. Resistance mechanisms require additional energy for gene expression and maintenance, which reduces bacterial growth rate (Vogwill & MacLean, 2015). Furthermore, antibiotics may still exert sublethal stress on resistant cells, leading to slower growth compared to optimal conditions without antibiotics (Andersson & Hughes, 2010). So the growth of isolate MDR1 in the antibiotics-free media was still better than in the agar media containing triple antibiotics.

The comparison of the visible colonies counts for the variation curing agents concentration treatments II and III when inoculated in agar media containing triple antibiotics shows great reduction. The reduced bacterial growth observed on antibiotic-containing media compared to antibiotic-free media after treatment with plasmid curing agents can be explained by the combined effects of antibiotic stress and the fitness cost of resistance. Although resistant, bacterial cells must expend additional energy to maintain resistance mechanisms, such as efflux pumps or antibiotic-degrading enzymes, which reduces their growth rate. This burden becomes more pronounced in the presence of antibiotics, where cells must actively counteract their effects (Wang & Joffr, 2025). Treatment II showed lower inhibition (39.37%). This lower rate suggests incomplete curing, where a majority of the population retained resistance plasmids. Similar concentration-dependent variability was reported by Letchumanan et al. (2015) in *Vibrio* species, where sub-optimal chemical ratios failed to eliminate resistant phenotypes effectively

In addition, curing agents such as SDS and EtBr may cause sublethal damage to bacterial cells, further decreasing their viability. EtBr acts as a DNA-intercalating agent that inserts between adjacent base pairs of DNA and can alter DNA conformation and topology, therfore interfering with the replication and stable maintenance of plasmid DNA, resulting in the loss of plasmids during subsequent bacterial cell division (Buckner et al., 2018). SDS is an anionic detergent that primarily affects the bacterial cell envelope and membrane that induce cellular stress and interfere with plasmid replication, segregation, and maintenance, increasing the likelihood that daughter cells lose the plasmid during cell division (Sonstein & Baldwin, 1972). As a result, only a subset of resistant and robust cells can grow on antibiotic-containing media, and their growth remains suboptimal. In contrast, antibiotic-free media provide more favourable conditions, allowing surviving cells to grow more efficiently and produce higher colony counts.

Treatment III shows greater reduction (95.55%) of visible colonies count compared to treatment II indicate that higher concentration of EtBr. Exposure to higher concentrations of EtBr exerts stress on the bacterial population, resulting in the survival of only the most robust cells. When these surviving cells retain antibiotic resistance, either through stable genetic mechanisms or incomplete plasmid elimination (Andersson & Hughes, 2010). Isolate MDR1 may exhibit improved growth due to reduced metabolic burden combined with maintained resistance capability. Consequently, these selected cells are able to grow more efficiently on antibiotic-containing media compared to populations treated with lower EtBr concentrations. This aligns with findings by El-Mansi et al. (2000), who observed that chemical curing agents, such as EtBr and SDS interfere with supercoiled plasmid replication and cell membrane integrity, resulting in high loss of plasmid-mediated resistance markers

The comparison of the visible colonies counts for the variation curing agents concentration treatments IV when inoculated in agar media containing triple antibiotics shows increasement instead of reduction. This phenomenon may be attributed to selective enrichment of resistant subpopulations, where only the most viable and fit resistant cells survive under antibiotic pressure. Excessive concentrations of curing agents frequently lead to cell lysis, viable-but-non-culturable (VBNC) states, or paradoxical survival artifacts due to stress-induced envelope alterations or efflux pump up-regulation rather than true plasmid curing (Liu et al., 2012). In contrast, non-selective media may allow the growth of both damaged and plasmid-cured cells, resulting in lower overall colony counts (Andersson & Hughes, 2010). Additionally, the effects of curing agents such as SDS and EtBr may reduce the viability of a portion of the bacterial population. Subinhibitory concentrations of antibiotics or experimental variation may also contribute to this observation (Davies et al., 2006).

CONCLUSION

This study succesfully showed that EtBr and SDS in various concentrations affect the growth of multidrug-resistant bacterium namely isolate MDR1 with the presence of antibiotics (amoxicillin, tetracycline, and chloramphenicol). Based on this study, the strongest inhibitor for isolate MDR 1 growth was the concentration of 50 g/L SDS and 50 mL/L EtBr (treatment III), reducing the colonies growth up to 95.55%, suggesting possible alteration of plasmid-associated resistance characteristics. Hence, this study provides an initial assessment on how to lower the antibiotic resistance characteristic for multidrug-resistant bacterium, especially isolate MDR1 originated from river stream of State University of Malang even though further molecular analysis is required to confirm plasmid elimination and resistance gene loss.

RECOMMENDATION

For future studies, it is highly recommended to investigate the identity of isolate MDR1 using 16S rRNA analyisis, as well as the phatogenic characteristic of isolate MDR1 needs to be investigated using blood agar method. Also, quantification of the plasmid concentration and the plasmid quality of isolate MDR1 prior and post-treatment using plasmid curing agent need to be investigated to strengthened the claim of the plasmid lost due to the exposure of SDS and EtBr.

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