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Acid Mine Drainage Neutralization Effort in Mud Media by *Lactobacillus casei* Bacteria and *Dekkera bruxellensis* Fungi

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ABSTRACT

Mine acid drainage (MAD) is a primary environmental problem caused by mining activity. The main characteristics of MAD are extremely low pH level (1.5–4.0), as well as content of sulfate and a number of heavy metals and metalloids that can destroy vegetation, accelerate erotion, and disrupt land ecosystem balance. The objective of this research was to process MAD by improving pH level and lowering iron and manganese content in MAD by *Lactobacillus casei* and *Dekkera bruxellensis* mixed culture. MAD Neutralization test was conducted on SMSs media with MAD concentration variations of 10, 15, 20 and 25 (%;v/v), and contact time variations of 48, 96, 144, and 192 (hours). The MAD neutralization test by *Lactobacillus casei* and *Dekkera bruxellensis* mixed culture occurred best at 10% concentration (v/v) with contact time of 96 hours. The pH improvement reached up to 6.20 with iron metal efficiency removal at 32.47% and manganese metal up to 24.94%. MAD neutralization test revealed that the best contact time variation is at 96 hours. At this contact time, the pH level was increased to 6.17 with iron metal removal efficiency at 31.17% and manganese metal removal at 25.43%.

Keywords: mine acid drainage, neutralization, iron removal, manganese removal, *Lactobacillus casei*, *Dekkera bruxellensis.*

INTRODUCTION

Coal industry plays an important role in economy development of numerous countries. Despite its positive impact on the economy, coal mining activities are also produce solid waste, air pollutant, and water pollution. Mine Acid Drainage is one of the main environmental problems caused by mining activities (Pondja et al., 2014).

Mine acid drainage (MAD) is a liquid waste produced from mining activities. MAD is considered as a seriously damaging pollutant due to its acidic nature, high toxic metal content (Fe, Zn, Cd, Al, Cu, Pb), dissolved anionic compound (sulfate, nitrate, chloride, arsenic, etc.), hardness, and suspended solids (Dhir, et al., 2018). MAD is a liquid waste formed when sulfide mineral inside rocks reacted with water and oxygen (Candeias et al., 2014). The main MAD characteristics are the extremely low pH level (1.5-4.0), and content of sulfate as well as numerous heavy metals and metalloids (Abinandan et al., 2020, Sihotang et al., 2021; Rinanti et al., 2018).

The acidic nature of MAD can destroy vegetation, accelerate erosion, and disrupt land ecosystem balance. MAD can also absorbed inside aquifer or create waste that merges inside shallow water flow, and pollute ground and surface waters (Favas et al., 2016). Because of this environmental problem, MAD should be processed before being finally discharged into the environment (Wikaningrum et al., 2022). Mine acid drainage processing can be conducted both with active and passive systems. Both methods are effective in lowering acidity through alkalinity improvement, lowered toxic metal and sulfate concentrations. Mine acid drainage processing is conducted through physical-chemical approach. Chemical process includes oxidation, reduction, adsorption, absorption, ion

exchange, complexation, chelation, hydrolysis, and crystallization. On the other hand, physical process includes sedimentation, coagulation and flocculation. MAD processing with physical-chemical methods is expensive because it requires regular maintenance (chemical reagents, mechanical system, worker input) so that the initial operation can be continued. On most of the cases, MAD contains mud as its side product (Dhir et al. 2018).

Biological methods are a successful and efficient alternative technique for treating MAD. Biological methods are carried out by utilizing living cells, namely microbes (bacteria, algae, and fungi), enzymes, dead biomass, or green plants to restore or remediate an environment polluted by MAD (Samal et al., 2020; Siwi et al., 2018). Bioremediation methods have attracted attention due to their low operating and labor costs, easy process design and control, with better sulfate and metal recovery (Rambabu et al., 2020).

Many studies have been carried out to remove heavy metals by utilizing sulfate reducing bacteria (Bwapwa et al., 2017). Sulfate-reducing bacteria are able to produce biogenic H_2S which can react with heavy metals so that metal sulfide deposition occurs. Biogenic alkalinity is a by-product of sulfate-reducing bacteria which is useful for neutralizing MAD (Jamil et al., 2013). Putri *et al.* conducted research in 2021 to neutralize MAD by utilizing a mixed culture of *Pseudomonas aeruginosa* and *Brevibacterium sp.* as a bioremediation agent. The results of the study were a mixed culture of *Pseudomonas aeruginosa* and *Brevibacterium sp.* able to increase the pH of acid mine water synergistically from 2.14 to 5.87 with an optimum contact time of 144 hours and an optimum concentration of MAD as a pollutant (v/v) 10% in liquid SMSs growth media (Putri et al., 2021).

Fungi have been reported to exhibit significant tolerance/resistance to heavy metals (Kumar and Dwivedi, 2021). The tolerance characteristics and accumulation of heavy metals by fungi are the main criteria in introducing their application in bioremediation processes. The use of fungi in remediation is called mycoremediation (Kumar and Dwivedi, 2021). In addition to their function as decomposers in ecosystems, fungi are also receiving attention because of their potential to remove inorganic pollutants. Fungi are able to tolerate metal concentrations, nutrient availability, pH or very extreme temperatures (Chan et al. 2016). Tolerance of toxic heavy metal concentrations is supported by the ability of fungi to produce extracellular degradative enzymes that reduce heavy metal toxicity when introduced into cells.

In the natural environment, bacteria and fungi live together. Microbes (especially bacteria and fungi) can adapt to heavy metals in the environment (Ye et al., 2020). On the basis of this background, research in the field of environmental biotechnology needs to be carried out to treat MAD that pollutes the environment by utilizing *Lactobacillus casei* bacteria and *Dekkera bruxellensis* fungi as bioremediation agents. This study intends to treat MAD by increasing the pH and decreasing levels of iron and manganese in MAD by mixed cultures of *Lactobacillus casei* and *Dekkera bruxellensis*. The objectives of this study were to determine the growth response, to measure the increase in pH, and to determine the efficiency of removal of iron and manganese contained in MAD by a mixed culture of *Lactobacillus casei* and *Dekkera bruxellensis*.

MATERIALS AND METHODS

Plant material

Materials utilized in this research are *Lactobacillus casei* bacteria and *Dekkera bruxellensis* fungi culture, artificial MAD by mixing $FeSO_4.7H_2O$; $MnSO_4.4H_2O$; and H_2SO_4, Stone Mineral Salt (SMSs) growth media by mixing one liter of aquades with 0.5 gr $CaCO_3$; 2.5 gr NH_4NO_3; 1 gr $Na_2HPO_4.7H_2O$; 0.5 gr KH_2PO_4; 0.5 gr $MgSO_4.7H_2O$; dan 0.2 gr $MnCl_2.7H_2O$, Nutrient Broth (NB), and Nutrient Agar (NA).

Lactobacillus casei and *Dekkera Bruxellensis* Cultivation

Lactobacillus casei bacteria cultivation is conducted on a batch or limited manner by using SMSs media and MAD containing media. Cultivation was conducted by using 80% of the Erlenmeyer capacity. On *Lactobacillus casei* cultivation using SMSs media, the solution composition consists of 80% SMSs media, 10% of *Lactobacillus casei* culture, and 10% of molasses as carbon source. On *Lactobacillus casei* cultivation, the media used contains MAD, the composition of the solution consists of 75% of SMSs media, 10% of *Lactobacillus casei* culture, 10% of molasses as carbon source, and 5% of MAD. The bacteria culture was conducted in 192 hours on 30 °C.

Fungi cultivation was conducted on NB media and media that contains MAD. Cultivation was conducted by mixing 90% of NB media and 105 of *Dekkera bruxellensis* fungi. On *Dekkera bruxellensis* cultivation, the media utilized contains MAD, with composition of 85% NB media, 10% of *Dekkera bruxellensis* culture, and 5% of MAD. Fungi culture was incubated using a shaker incubator for 192 hours on 30 °C temperature and 150 rpm rotation.

Lactobacillus casei and *Dekkera bruxellensis* mixed culture senstivity test on mine acid drainage

Sensitivity test was conducted by exposing *Lactobacillus casei* and *Dekkera bruxellensis* mixed culture on Nutrient Agar (NA) media that have been added with disc paper that contains mine acid drainage.

Mine acid neutralization test using *Lactobacillus casei* and *Dekkera bruxellensis* mixed culture

MAD neutralization test was conducted to reveal Lactobacillus casei and Dekkera bruxellensis mixed culture to increase MAD pH. In this test, two types of control treatment was used: 1) control by adding MAD in SMSs media without adding mixed culture, 2) control with mixed culture inserted into molasses-added SMSs media as carbon source without adding MAD.

The treatment consists of two stages namely Stage I, MAD neutralization test by applying MAD concentration variations; and Stage II, MAD neutralization test by applying contact time variations. On MAD concentration variations, the utilized variations are 10, 15, 20, and 25 (%;v/v) at a temperature of 30 °C as optimum temperature for Lactobacillus casei and Dekkera Bruxellensis growth (Blomqvist, 2011; Śliżewska, 2020), contact time of 96 hours, 10% mixed culture concentration (v/v), and with land: MAD ratio to form slurry condition at 1:1. On contact time variations, the utilized variations are 48, 96, 144, and 192 (hours) at a temperature of 30 °C temperature, contact time of 96 hours, 10% mixed culture concentration (v/v), the best MAD concentration produces pH increase with land: MAD ratio to form slurry condition at 1:1. Every sample was incubated by using shaker incubator at rotation speed of 150 rpm.

Pilot scale design

On the basis of the results of observations on a laboratory scale, a pilot scale design can be carried out by identifying field conditions and data availability, selecting the type of reactor (flow pattern and microbial growth system), and proceeding with a pilot scale design.

Sample analysis

Sample analysis conducted in this study is the analysis of increasing pH and examination of Fe and Mn metals. Analysis of the increase in pH was carried out by measuring pH using a pH meter, while the examination of iron and manganese was carried out using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

Data analysis

The analysis of the data carried out involved bacterial and fungal growth, removal of iron and manganese, as well as the kinetics of the rate of utilization of iron and manganese as a substrate by mixed cultures of Lactobacillus casei and Dekkera bruxellensis. Analysis of bacterial culture growth was carried out using the Total Plate Count (TPC) method with the Spread Plate technique. The dilution was carried out in a test tube by adding 1 ml of inoculum to 9 ml of Nutrient Broth (NB) media. After that, a Petri dish containing Nutrient Agar was added with 1 ml of inoculum from the last dilution and incubated at 37 °C. Furthermore, the number of microbial colonies that grew was counted using the Colony Counter. The number of microbial cells that grow can be calculated using the equation 1.

$$\text{Colony per mL}\left(\frac{\text{CFU}}{\text{mL}}\right) = \\ = \frac{\text{Number of colony per cup}}{\text{Diluent factor}} \quad (1)$$

Fungal growth analysis was carried out using a UV-Vis spectrophotometer which measured absorbance with a wavelength of 600 nm. The amount of light absorbed is proportional to the number of fungal cells. The analysis of the removal efficiency of iron and manganese can be calculated by using equation 2.

$$\text{Removal efficiency (\%)} = \\ = \frac{\text{innitial C (mg/L) - final C (mg/L)}}{\text{innitial C (mg/L)}} \times 100\% \quad (2)$$

RESULTS

Cultivation of *Lactobacillus casei* and *Dekkera Bruxellensis*

Cultivation of *Lactobacillus casei* and *Dekkera bruxellensis* bacteria has been carried out for 192 hours with contact times ranging from 24, 48, 72, 96, 120, 144, 168, and 192 hours in liquid media that does not contain MAD and liquid media that contains MAD. The growth of Lactobacillus casei and Dekkera bruxellensis on media without MAD and with MAD is shown in Figure 1.

On the basis of Figure 1, the growth of *Lactobacillus casei* and *Dekkera bruxellensis* bacterial cultures was more common in liquid media containing MAD compared to liquid media without MAD. The bacterial culture on media that did not contain MAD and on the media that contained MAD, it underwent an exponential phase from 0 hours to 120 hours. At the end of the exponential phase, the number of cells growing on the media that did not contain MAD reached 24,600,000 cells/ml, while on the media containing MAD it reached 28,900,000 cells/ml. The fungal culture on the media that did not contain MAD experienced an exponential phase in the range of 0 hours to 72 hours with a measured absorbance at the end of the exponential phase of 1.640, while on the media containing MAD experienced an exponential phase in the range of 0 hours to 144 hours with The absorbance measured at the end of the exponential phase is 1.939. This results shows that a culture of *Lactobacillus casei* bacteria and *Dekkera bruxellensis* fungi can utilize metal compounds contained in MAD as additional nutrient in it growth process and also proved the finding of Dixit et al. in 2015 that a number of microbes require heavy metal as their essential micronutrient to grow (Rinanti, 2018).

Lactobacillus casei and *Dekkera bruxellensis* mixed culture sensitivity test on mine acid drainage

The sensitivity test was carried out to test the growth ability of the mixed culture of *Lactobacillus casei* and *Dekkera bruxellensis* bacteria in an environment containing MAD which is a xenobiotic compound, which is a foreign compound with a molecular structure that is not recognized by biological systems (Qadir et al., 2017). The test results showed no inhibition zone was formed around the paper disc containing AAT after 48 hours of incubation as shown in Figure 2.

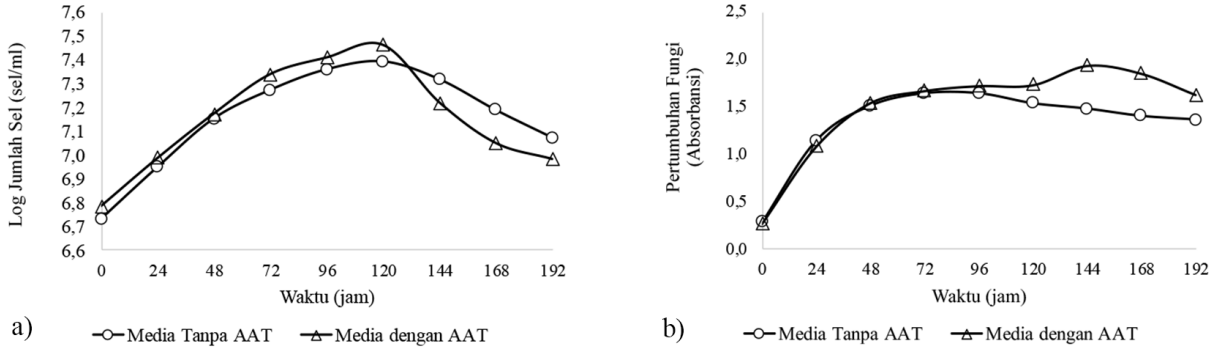

Figure 1. Cultivation curve: (a) *Lactobacillus casei*; (b) *Dekkera bruxellensis*

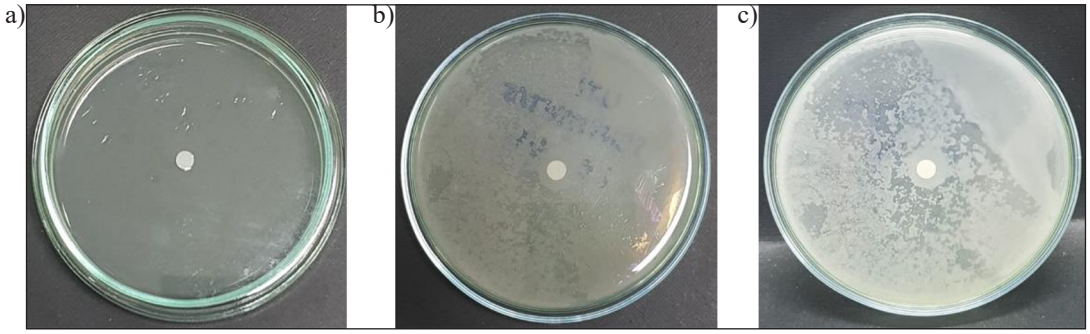

Figure 2. *Lactobacillus casei* and *Dekkera bruxellensis* Sensitivity Test to MAD at: (a) 0 hour; (b) 24 hour; (c) 48 hour

This indicates that the mixed culture of *Lactobacillus casei* and *Dekkera bruxellensis* has high resistance to MAD which is a xenobiotic compound and can utilize the compounds contained in MAD as a substrate for its growth. On the basis of the results of the sensitivity test, the research can be continued by using the bacteria *Lactobacillus casei* and *Dekkera bruxellensis* fungi.

Mine acid drainage neutraliation test by using *Lactobacillus casei* and *Dekkera bruxellensis* mixed culture

Mine acid drainage neutralization test with MAD concentration variations

After testing with AAT concentrations (%; v/v) 10, 15, 20, and 25, the results of the increase in pH were shown in Figure 3a. The best increase in pH that can be achieved during a contact time of 96 hours is at a concentration of 10% (v/v) MAD with pH value of 6.20.

The results of the MAD concentration variation test showed that the higher the MAD concentration, the lower the increase in pH that occurred. This shows that the mixed culture of *Lactobacillus casei* bacteria and *Dekkera bruxellensis* fungi is only able to metabolize dissolved metals in MAD in small amounts. Oves et al. mentioned that at lower concentrations, all metal ions present in the solution can interact with the binding site and thus the percentage of metal removal tends to be higher than the higher ion concentrations (Oves et al., 2013). as found in this study, so it can affect the increase in pH in this research. In addition, pH is an important parameter in the removal of metals by microbes because the pH of the solution affects the nature of microbial binding sites, metal solubility and also causes competition between metal ions and H^+ (Deng and Wang, 2012; Wilan et al., 2019; Widyaningrum et al., 2021; Okeke et al., 2019).

An analysis of the efficiency of iron and manganese removal has been carried out in the AAT neutralization test with variations in MAD concentration (%; v/v) as shown in Figure 3b. From the results of the study, the highest removal efficiency of iron reached 32.47% and manganese metal reached 24.94%. The results showed that the higher the MAD concentration, the lower the removal efficiency obtained. Fomina & Gadd (2014), and Verma (2017) stated that at higher metal ion concentrations, the number of binding sites available to microbes was relatively decreased, resulting in a decrease in the metal removal efficiency (Fomina and Gadd, 2014; Verma, 2017).

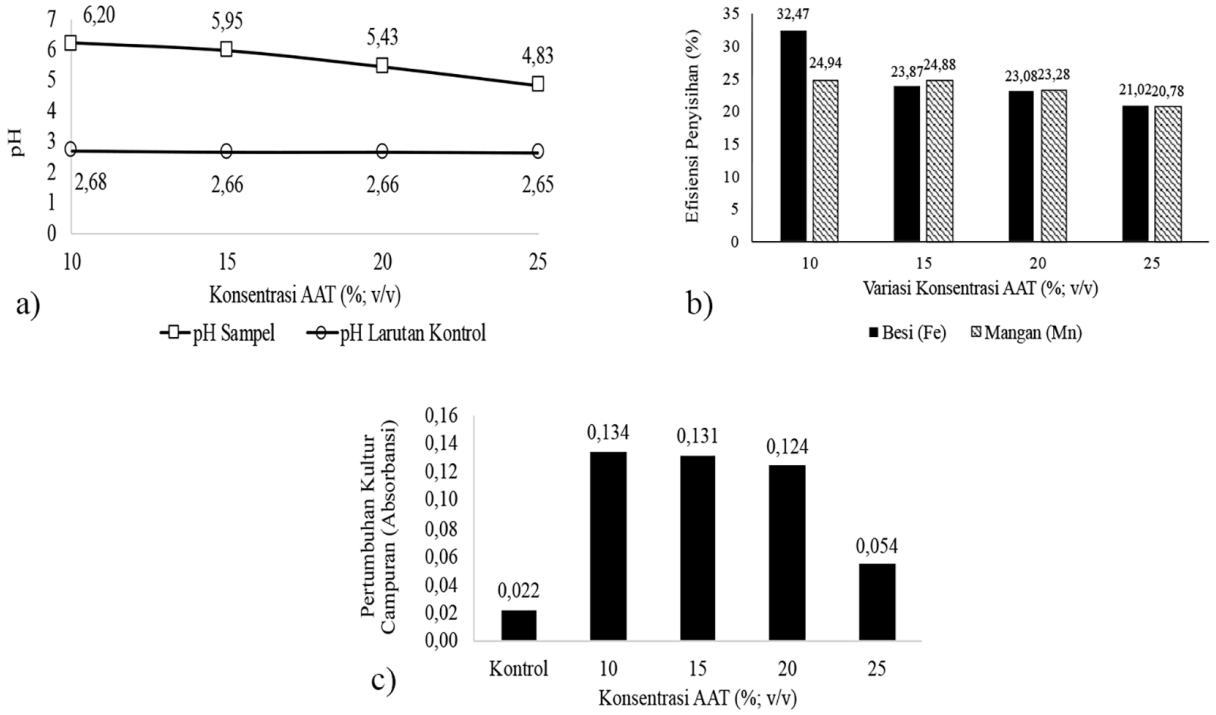

Figure 3. MAD neutralization test with concentration variations (%;v/v): (a) pH improvement (b) Fe and Mn Metals removal efficiency; (c) Amount of mixed culture cell

Analysis of the number of cells was also carried out with the measurement results shown in Figure 3c. The number of cells decreased at a concentration of 25% (v/v) which was 0.054. This indicates that the higher the concentration of MAD, the higher the concentration of dissolved heavy metals so that they are toxic to mixed cultures and cause a decrease in the number of cells. This results support the finding of Hrynkiewicz & Baum (2014) that every metal, at a certain level of concentration, is toxic for microorganisms.

Mine acid drainage neutralization test with contact time variations

After testing with variations in contact time (hours) 48, 96, 144, and 192, the results of the increase in pH were shown in Figure 4a. From the test results, the best increase in pH occurred at a contact time of 96 hours with an increase in pH reaching 6.17. After 96 h, the increase in pH decreased in proportion to the decrease in the number of cells shown in Figure 4c. The decrease in the number of cells occurs because additional nutrients are not given during the contact process, causing cell death. In the control treatment, the number of cells decreased up to 192 hours with a measured absorbance of 0.068. The results of the analysis of the number of cells showed that the contact time of 96 hours was the optimum contact time with the highest number of cells being 0.452 and an increase in pH reaching 6.17. These results indicate that *Lactobacillus casei* and *Dekkera bruxellensis* are acidophilic microbes that can survive under highly acidic conditions, such as MAD.

The analysis continued with the analysis of the removal efficiency of iron and manganese, as shown in Figure 4b. The removal efficiency of iron was 31.17%, while for manganese it was 25.43%. After 96 hours, the removal efficiency of iron and manganese decreased respectively. Similar results were also produced by Kanamarlapudi & Muddada in 2020 who indicated that metal ion accumulation can cause saturated microbes that leads to metal ion desorption inside the solution (Kanamarlapudi, 2020).

This section may be divided into subheadings. It should provide a concise and precise description of the experimental results, their interpretation, and the experimental conclusions that can be drawn.

DISCUSSION

Kinetic calculations have been carried out to explain the relationship between specific growth rates of mixed cultures of *Lactobacillus casei* and *Dekkera bruxellensis* with concentrations of iron and manganese. Figure 5 shows the relationship between specific growth rate and specific substrate utilization rate.

The utilization of iron as a substrate is shown in Figure 5a with specific growth (μ) ranging from

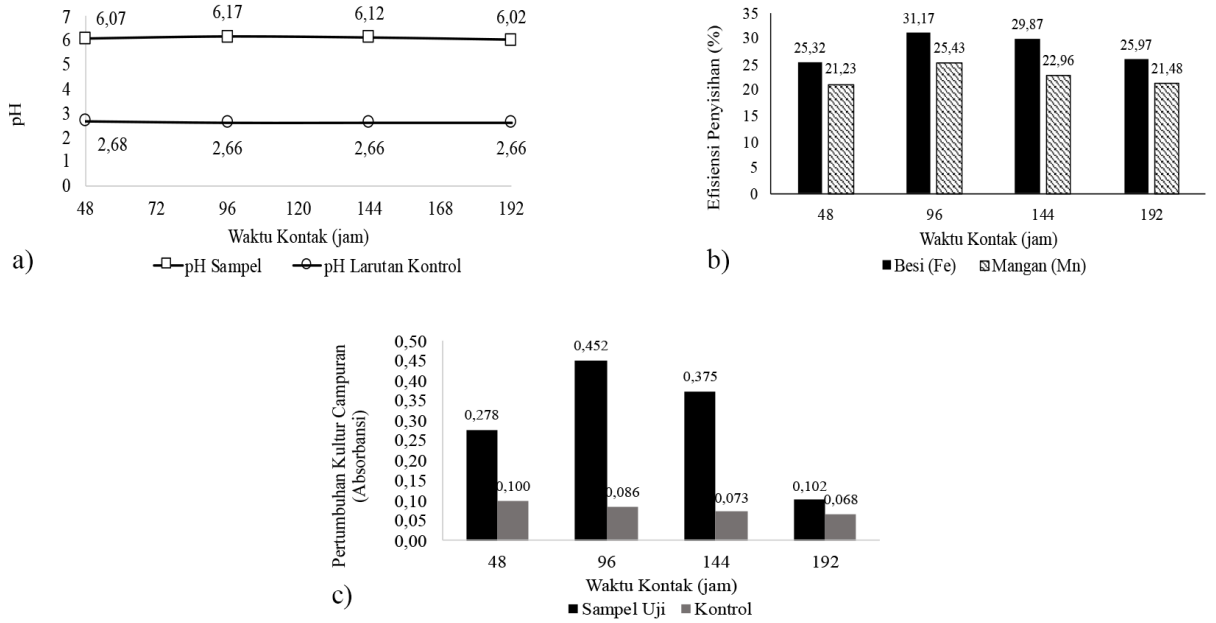

Figure 4. MAD neutralization test with contact time variations (hour): (a) pH improvement; (b) removal efficiency of Fe and Mn; (c) Number of mixed culture cell

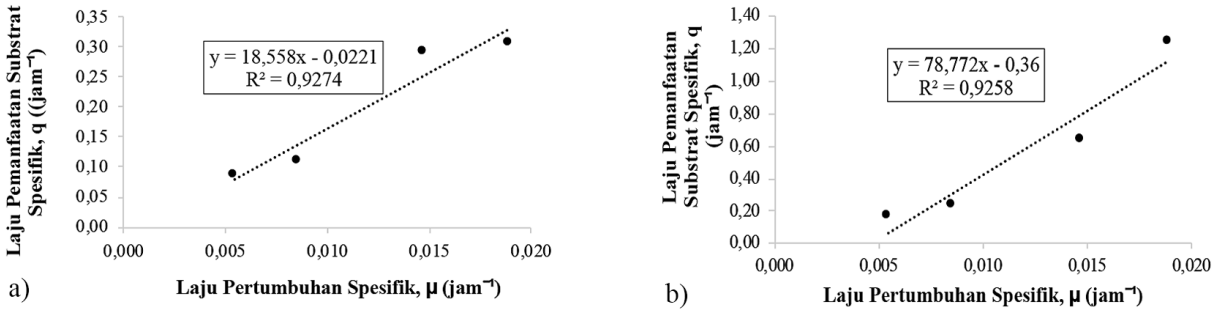

Figure 5. Relationship between specific growth rate and specific substrate utilization rate: (a) iron; (b) manganese

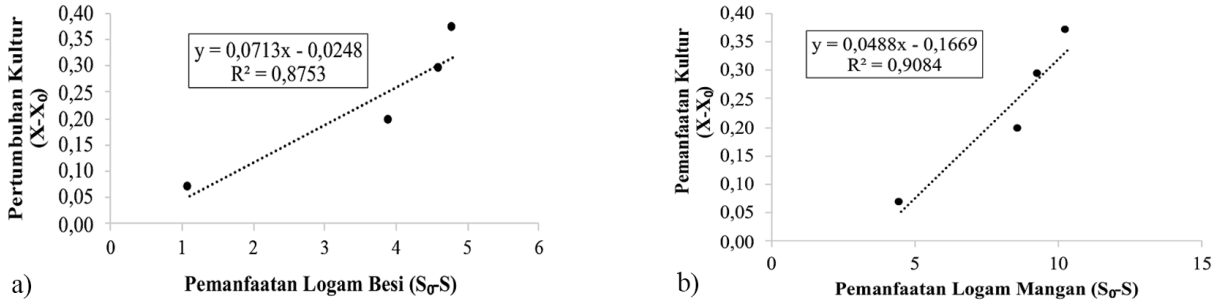

Figure 6. Relationship between mixed culture amount and specific substrate utilization: (a) iron; (b) manganese

0.0054 - 0.0189 h^{-1}, while the rate of utilization of specific substrate (q) ranged from 0.0852–0.3056 h^{-1}. The relationship between μ and q produces YT value of 0.0539 and Kd value of 1.19 × 10-3 hours^{-1}. The utilization of manganese as a substrate is shown in Figure 5b with specific growth (μ) ranging from 0.0054 × 0.0189 h^{-1}, while the rate of utilization of specific substrate (q) ranged from 0.2374–1.25 h^{-1}. The relationship between μ and q produces YT value of 0.0127 and Kd value of 4.57 × 10-3 hours$^{-1.}$

Figure 6 shows the relationship between the amount of mixed culture growth and the utilization of specific substrates. The relationship between the amount of mixed culture growth and the utilization of specific substrates resulted in a linear line with a slope of 0.0713 Yobs for iron and 0.0488 Yobs for manganese.

The relationship between the concentrations of iron and manganese and the specific growth rate (μ) is shown in Figure 7 with the maximum specific growth rate (μ_{max}) obtained which is 0.0189 and half the maximum specific growth rate value ($\mu_{max}/2$) which is at 0.0095. On the basis of Figures 7a and 7b, the value of the saturation constant (*Ks*) is 10.70 mg/L for iron and 30.25

Table 1. Kinetic recapitulation of iron and manganese removal in MAD

Parameters	Calculation results	
	Fe	Mn
YT (hour^{-1})	0.0539	0.0127
Kd (hour^{-1})	1.19 x 10^{-3}	4.57 x 10^{-3}
Yobs (hour^{-1})	0.0713	0.0488
Ks	10.70	30.25

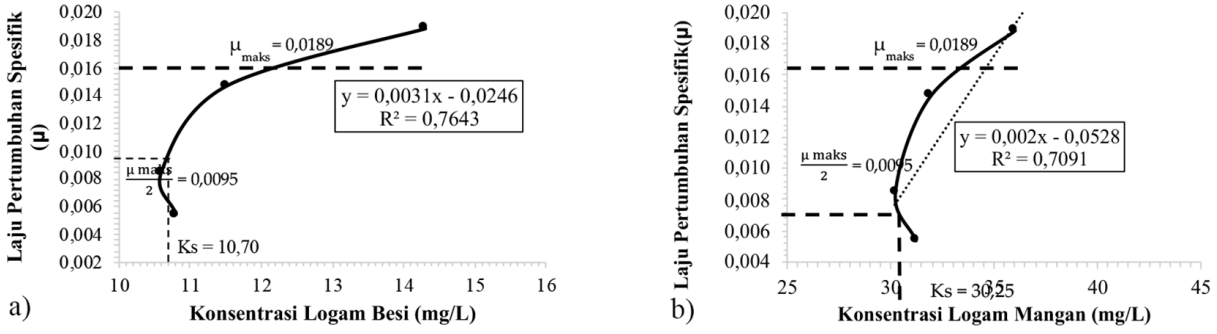

Figure 7. Relationship between metal concentration and specific growth rate (μ): (a) iron; (b) manganese

Table 2. Reaction order

Reaction order formula						
	Iron			Manganese		
Order	0	1	2	0	1	2
R^2	0.6758	0.6965	0.7173	0.6307	0.6367	0.6421
Slope (a)	0.0587	0.0048	0.0004	0.0845	0.0026	0.00008
Intercept (b)	1.922	0.1367	0.0747	5.7611	0.1547	0.0289

mg/L for manganese metal. The recapitulation of the removal kinetics of iron and manganese in MAD can be seen in Table 1 and the reaction order can be seen in Table 2.

After calculating the kinetics of the iron and manganese removal rate, it was obtained that R^2 was close to 1 with iron metal as a substrate of 0.7173 at a reaction rate of order 2 with the equation $Y = 0.0004x + 0.0747$ and manganese metal as a substrate was obtained R^2 which close to 1 which is 0.6421 at the rate of reaction of order 2 with the equation $Y = 0.00008x + 0.0289$. The recapitulation of the pilot scale design calculations can be seen in Table 3 and the reactor design sketch can be seen in Figure 8.

Table 3. Pilot scale design calculation recapitulation

Component	Value	Unit
Number of reactor	8	Pieces
Reactor height	4	m
Reactor diameter	8.3	m
Effective height	3.2	m
Freeboard	0.8	m
Paddle diameter	4	m
Paddle width	0.8	m
Range between paddle and tank base	1	m
Paddle stirring speed	1.8	rpm
Mud room height	0.51	m
Mixed culture volume	17.12	m^3
Growth media volume	119.83	m^3

CONCLUSIONS

On the basis of on the results of this research, *Lactobacillus casei* and *Dekkera bruxellensis* growth response on MAD containing media shows that mixed culture possesses high resistance to MAD. The best MAD pH level improvement and iron and manganese removal occurred at 10% concentration (v/v) with contact time of 96 hours, with final pH level of 6.17. The obtained iron removal efficiency was at 32.47% and 24.94% for manganese. Iron removal kinetic in MAD utilized reaction order 2, which produced equation $Y = 0.0004x + 0.0747$ and R^2 value of 0.7173. Meanwhile, manganese metal removal by using reaction order 2 produced equation $Y = 0.00008x + 0.0289$ and R^2 value of 0.6421. Pilot scale design will be implemented by using a Sequencing Batch Reactor (SBR) unit fitted with paddle impeller with reactor diameter of 8.3 m and reactor height of 4 m.

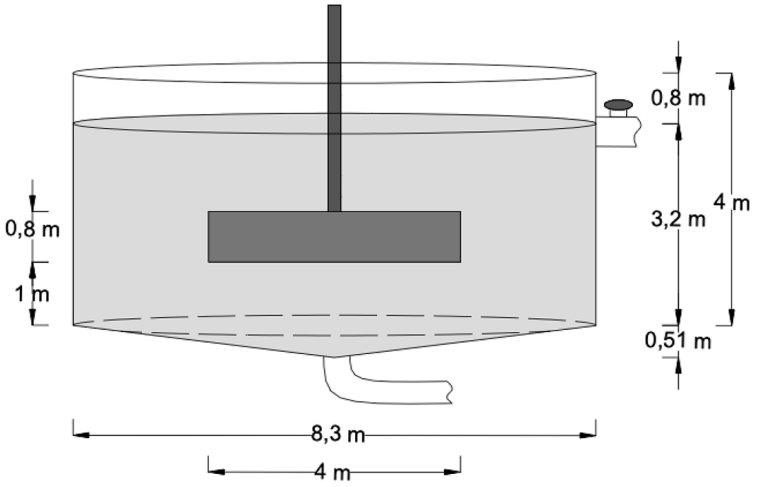

Figure 8. Reactor design sketch

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