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Research Article

Screening Test of Fe_2O_3, ZnO and CuO Metal Oxide towards the Formation of Total Volatile Acids in the Anaerobic Degradation Process of Artificial POME

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Abstract

This study investigated the effect of the addition of metal oxides (Fe_2O_3, ZnO, CuO) as a micronutrient on the formation of total volatile acids (VFAs) and biomass growth in the anaerobic degradation process of artificial waste characterized by Palm Oil Mill Effluent (POME). The biomass used in this research were obtained from POME WWTP sludge and septic tank. Using a circulating bed reactor (CBR) with a variation of the addition of a single metal oxide, as well as a combination of two and three metal oxides in between. The results of the screening test by 2^n factorial method and ANOVA statistical tests (α = 5% and α = 1%) showed that the metal oxides that are significant towards VFAs production are Fe_2O_3 + ZnO & ZnO + CuO. The metal oxides that are significant towards organic compounds removal (sCOD) production are Fe_2O_3 & Fe_2O_3 + ZnO. Meanwhile, the statistical tests validates that there are no combinations of metal oxides that are significantly affecting microbial growth (VSS).

Keywords: *anaerobic digestion, metal oxide, palm oil mill effluent (POME), volatile fatty acids*

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1. Introduction

Currently, Indonesia still holds the title of the world's largest palm oil producing country, with its production reaching 46.5 million metric tons of palm oil (Foreign Agricultural Service United States Department of Agriculture, 2025). The palm oil industry is one of the national strategic sectors that continues to be encouraged by the government, both in terms of productivity and product down streaming. However, along with the increase in palm oil production, there are also environmental problems due to the liquid waste produced from the processing process, known as Palm Oil Mill Effluent (POME). A total of 2.5-3.8 tons of POME is produced for every ton of crude palm oil produced. POME has a high content of organic matter and has the potential to pollute the environment if not handled properly. POME is characterized by thick and brownish color, acidic pH, and also has high concentrations of TSS, BOD, and COD (Prasertsan et al., 2017). Therefore, effective treatment methods are needed to minimize negative impacts on the environment while striving to use waste into value-added products.

POME can be processed through a variety of methods. The commonly used POME processing method is through a biodegradation process by utilizing the activity of microorganisms (Singh et al., 2010). One of them is to use a pool system designed for natural decomposition through biological processes anaerobically and aerobically. In the process of anaerobic degradation, intermediate compounds are produced in the form of total volatile fatty acids (VFA) such as acetic acid, propionic acid, and butyric acid. Volatile fatty acids are fatty acids that contain carboxyl groups (R-COOH), and their carbon chains are no more than six.

VFA has the potential as a precursor in biofuel production as well as a building block in the manufacture of bioplastics (Vázquez-Fernández et al., 2022), so that it can be used in the waste-to-energy concept. Thus, the production of VFA from POME not only contributes to waste management, but also supports the development of renewable energy and the circular economy.

To optimize the production of VFA in the anaerobic degradation process, a strategy is needed that can increase the activity of microorganisms in producing these compounds. One of the strategies that has been known is the addition of micronutrients in the form of metal oxides to the reactor system. These micronutrients function as cofactors for microbial enzymes that play a role in the fermentation pathway (Menon, et al., 2017), so it is expected to increase the accumulation of VFA. Recent studies, however, report varying outcomes regarding the effects of metal-based additives such as Fe-, Zn-, and Cu-derived materials. For example, Fe-based oxides have been shown to enhance hydrolysis and acidogenesis in several organic waste systems, whereas Zn and Cu compounds exhibit more inconsistent influences depending on the substrate, dosage, and reactor operating conditions. Much of the existing evidence remains descriptive, focusing on individual metals or specific process improvements without offering a systematic comparison of their relative catalytic or micronutrient contributions (Wang et al., 2022).

In the context of POME, the role of these metal oxides in directing VFA accumulation has not been clearly established, and studies frequently differ in reactor configuration, metal concentration, or anaerobic conditions, making it difficult to generalize or compare findings across systems. Consequently, the literature does not yet provide a coherent understanding of whether Fe-, Zn-, or Cu-oxide micronutrients offer the most consistent stimulation of microbial acidogenesis during anaerobic POME degradation. Therefore, the problem addressed in this study is the absence of clear, comparative evidence on how different metal oxide micronutrients, specifically Fe, Zn, and Cu oxides, modulate VFA formation in anaerobic POME treatment. This research evaluates these three metal oxides under a unified experimental framework, allowing direct comparison of their catalytic influences on VFA accumulation.

2. Methodology

2.1 Research time and location

The research was conducted from December 2024 to June 2025. POME sampling and POME sludge was carried out at a palm oil industry company, namely PT Condong Garut, West Java. The process of testing characteristics to running was carried out at the Environmental Engineering Water Quality Laboratory, Bandung Institute of Technology.

2.2 Sampling

Samples of POME liquid waste and sludge of WWTP were taken from one of the largest palm oil industry companies in West Java, namely PT Condong Garut. The sampling point for POME liquid waste is located in the fat pit, while the sampling point for WTP sludge is taken in the mixing pond. In addition, domestic waste sludge was also taken from the septic tank located on the campus of the Bandung Institute of Technology. The POME liquid waste taken will be tested for its characteristics to be used as a reference for the manufacture of artificial liquid waste, while sludge from WWTP and septic tanks will be used as biomass mixed cultures.

2.3 Initial characterization of palm oil liquid waste

POME liquid waste needs to be tested for its characteristics first as a reference for the manufacture of artificial liquid waste that will act as a substrate at the seeding stage. The parameters to be tested for the characteristics of POME liquid waste samples are divided into physical parameters and chemical parameters. Physical parameter tests will be carried out directly when at the sampling location, while chemical parameter tests will be carried out at the Environmental Engineering Water Quality Laboratory, Bandung Institute of Technology.

2.4 Artificial liquid waste generation

Artificial liquid waste is needed in the next stage, namely seeding and acclimatization. The manufacture of artificial liquid waste is divided into artificial substrates and artificial POMEs.

a. Artificial Substrates
Artificial substrates are used at the seeding stage, as well as as a mixture for artificial POME at the acclimatization stage. The artificial substrate contains dextrose monohydrate, KH_2PO_4, and NH_4Cl, which act as C, N, and P components, respectively. The desired COD target in the artificial substrate is 12,000 COD mg/L.
b. Artificial POME
Artificial POME is used in the acclimatization and *running* stages. The components in artificial POME are tapioca flour which acts as starch, gelatin as protein, and palm oil as oil & fat. The concentration of each of these components refers to Wong & Chin (1985) in Ahmad (2001), namely starch as much as 2 mg/L; protein as much as 3 mg/L; and oil as much as 6 mg/L. The target COD required in an artificial substrate is 2,500 mg/L.

2.3 Seeding

The seeding stage is carried out to multiply existing microorganisms by providing food in the form of artificial substrates. In a 30 L reactor, as much as 25 L of a mixture of sludge and substrate is inserted, while the remaining 5L is in the form of air that acts as a headspace. In the 25 L mixture, as much as 4 L is sludge from anaerobic ponds at PT Condong Garut WWTP and as much as 2 L is sludge containing anaerobic bacteria from septic tanks. Meanwhile, the remaining 19 L of the mixture is a prefabricated artificial substrate. The seeding stage is carried out until the VSS is in the range of 2,000-4,000 mg/L in the reactor and COD shows a constant trend.

2.6 Acclimatization

The acclimatization stage aims to adapt the biomass into an artificial POME substrate gradually from the actual environmental conditions in the field until it can be fully accustomed to the environmental conditions in the laboratory. Acclimatization is carried out in three stages, with the ratio of artificial POME and artificial substrate in the first stage 35%–65%; the second stage 70%–30%; and 100%–0%. At each stage, acclimatization is carried out until the growth curve measured through the VSS parameter shows a stagnant curve trend, which means that the bacteria have entered the stationary phase.

2.7 Running

In this study, a circulating bed reactor (CBR) as shown in Figure 1 with a total capacity of 6 L was used with a working volume of 5 L and a headspace of 1 L. From the working volume of 5 L, the ratio of biomass and artificial POME used was 1:4. The CBR reactor is a modification of the bubble column reactor that utilizes the lifting power of gases to mix particles and liquids. The selection of this reactor was based on its ability to effectively mix materials without damaging the microorganisms inside.

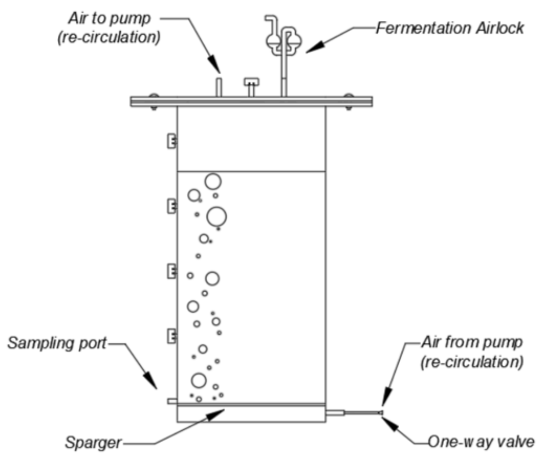


Figure 1. Circulating bed reactor

The variations in metal oxide addition from each reactor are presented in Table 1. The reactor is operated twice (duplo) for each variation of the research design to ensure the results of each *run* are consistent and replicable.

Table 1. Research design matrix

No.	Notation	Fe_2O_3	ZnO	CuO
1	1	0	0	0
2	a	1	0	0
3	b	0	1	0
4	ab	1	1	0
5	c	0	0	1
6	ac	1	0	1
7	bc	0	1	1
8	abc	1	1	1

0 : *Low level* → No addition of metal oxides (0 mg/L for all metal oxides)

1 : *High level* → Addition of metal oxides (10 mg/L for Fe_2O_3; 4 mg/L for ZnO; & 0,1 mg/L for CuO)

There is the addition of cofactors in the form of metal oxides Fe_2O_3, ZnO, and CuO with variations as stated in the research design matrix table above. In the screening test, there was a variation in the presence (high value) and absence (low value) of each metal oxide to determine the effect of its addition. The concentration used is 10 mg/L; 4 mg/L; and 0.1 mg/L for Fe_2O_3, ZnO, and CuO metal oxides, respectively. This selection is also based on previous similar studies that used the addition of Fe, Zn, and Cu metals with a concentration of 10 mg/L each (Gumilar, 2023); 4 mg/L (Deswita, 2016); and 0.1 mg/L (Puteri, 2016).

2.8 Data analysis methods

In analyzing the effect of the addition of metal oxides on biomass growth, the screening test was used with a 2^n factorial statistical test and also an analysis of variance (ANOVA). The screening test aims to select a subset of factors that significantly affect the target parameters. Meanwhile, the 2^n factorial statistical test is designed to quantify the main effects of each factor and its simultaneous interactions, where each factor is tested at two levels (low value & high value) allowing researchers to compare the effect of the combination of treatments on the response (Box et al., 2005). The ANOVA method was then applied to test the statistical significance of the mean difference between treatment data, thus determining whether the difference in the influence of these factors was statistically significant (Kutner et al., 2004).

3. Result and Discussion

3.1 Characteristics of palm oil liquid waste (POME)

Characteristic tests were carried out on POME liquid waste obtained from the sampling results. The data obtained from the physical and chemical parameter testing results are as follows, listed in Table 2 and Table 3. The data from the POME characterization is used for the manufacture of artificial substrates.

Table 2. Results of POME physical parameter characteristics test

No	Parameter	Unit	Result
1	pH		4.26
2	OF	mg/L	5.20
3	Temperature	°C	48.67
4	Conductivity	µS/cm	4690

Table 3. Results of POME chemical parameter characteristics test

No	Parameter	Unit	Effluent Fat Pit
1	COD Total	mg/L	37233.33
2	Dissolved COD	mg/L	11283.33
3	TSS	mg/L	48538
4	VSS	mg/L	1461.99
5	Oil & Grease	mg/L	2210
6	Free Ammonia	mg/L	98
7	NTK	mg/L	322
8	BOD Total	mg/L	15000
9	Dissolved BOD	mg/L	10928.57
10	Orthophosphate	mg/L	10.21
11	Total Phosphate	mg/L	14.77

3.2 Biomass

Biomass is prepared through seeding and acclimatization processes before being used at the running stage. The seeding stage in this study was carried out for 15 days, with checking the COD and VSS parameters which were carried out periodically for 3 days.

Based on the graph shown in Figure 2, VSS values constantly increased from 1949.56 mg/L on day 0 to 4351.02 mg/L on day 12. On day 15 measurements, VSS was seen to decrease and microorganisms entered the declination (death) phase. In addition, VSS is also at a value of 3091 mg/L, which means that it has met the criteria for MLVSS biomass that is ready to be used.

At the seeding stage, the COD value is expected to tend to be stable, which indicates that the organic compounds in the artificial substrate are no longer used by the biomass to grow. This indicates that the microorganisms are already in the stationary phase. It can be seen that between the 12th and 15th days, the COD value is already in the range of 11,000-13,000 mg/L, where the value has not shown a drastic fluctuation. Based on this, the seeding stage was dismissed to enter the next stage. However, before use, biomass is first adapted to the acclimatization stage using artificial POME.

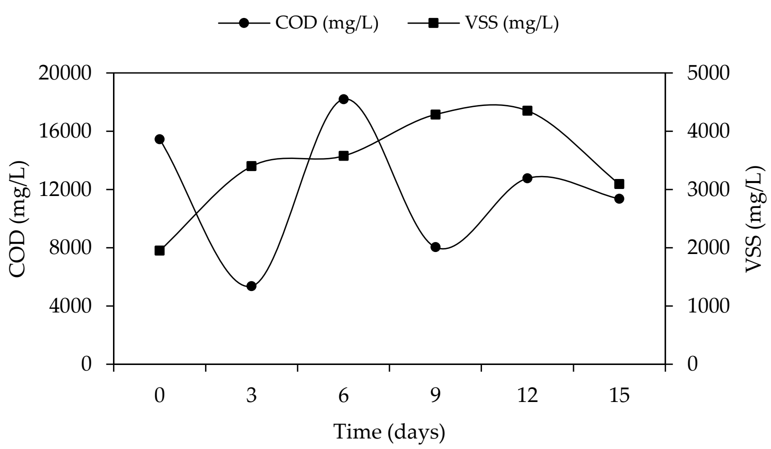


Figure 2. COD and VSS data measurement seeding stage

In the three stages of acclimatization, COD & VSS parameters were also measured. The first stage is a reference for the duration of acclimatization. In Figure 3. (a), the number of microorganisms has decreased significantly and has entered the declination (death) phase. Therefore, the second and third stages of acclimatization were carried out for 2 days.

The adaptation of microorganisms occurs in the lag phase, when microorganisms are still adjusting to new environmental conditions (Chisti & Moo-Young, 2003). A constant VSS value can be an indication that the microorganism has passed the lag phase and has already reached the stationary phase. Meanwhile, the COD value is shown in Figure 3. (b) showed a continuous decrease in all three stages of acclimatization even though the microorganisms were in the stationary phase.

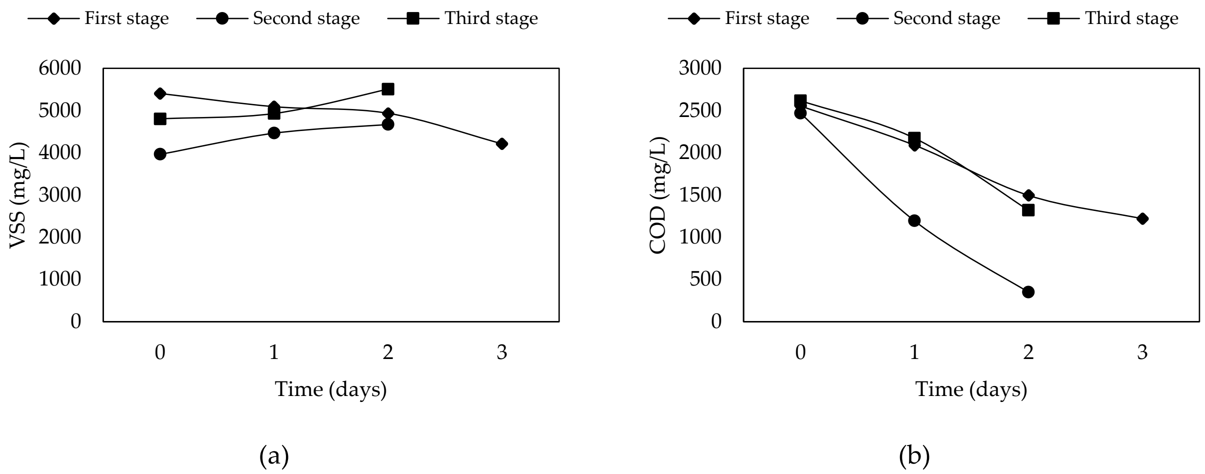
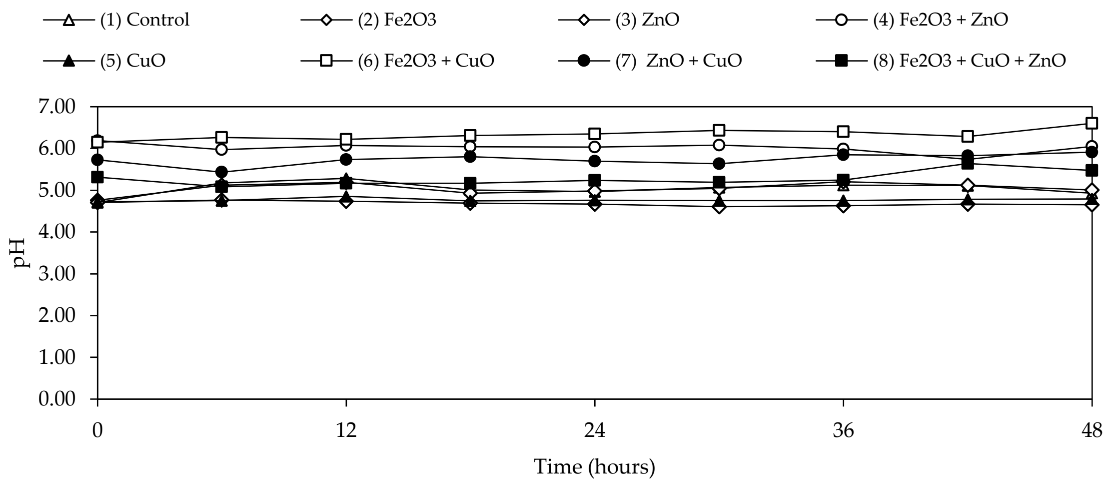


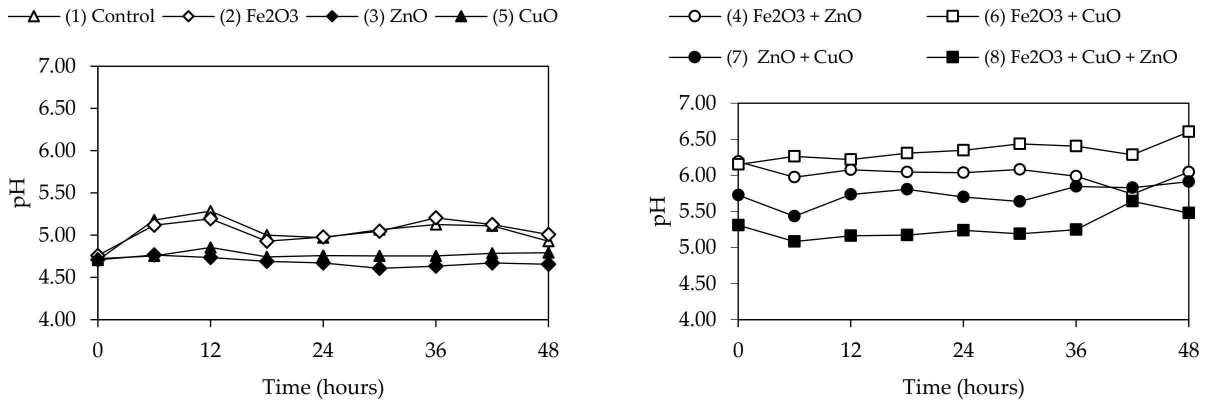
Figure 3. (a) VSS data of the acclimatization (b) COD data of the acclimatization

3.3 pH measurement

The pH parameter is a very important parameter because it has a big impact on the activity of microorganisms in anaerobic degradation of organic compounds. In this study, sample for pH is tested every 6 hours to help determine the pathways of the anaerobic degradation process of the reactor. The pH profile not only reflects the acidogenesis and methanogenesis phases but also the metabolic responses of microbial consortia to trace metal additives. Trace metals can serve as redox-active cofactors in enzymatic electron transfer, influencing microbial metabolism and pathway selection. For instance, iron (Fe) in its oxidized (Fe^{3+}) and reduced (Fe^{2+}) states participates in dissimilatory reduction reactions that facilitate the transfer of electrons during acidogenic and methanogenic processes, thereby stabilizing redox conditions conducive to VFA conversion and methanogenesis (Wang et al., 2025).



(a)



(b) ©

Figure 4. pH measurement results (a) all variations (b) single metal oxide variations (c) combination metal oxide variations

In the control reactor variation, the pH decreased at the 0 to 12th hour indicating the acidogenesis phase, then increased significantly at the 12th to 24th hour. This increase proves the transition to the phases of acetogenesis and methanogenesis. With Fe_2O_3 alone, pH remained relatively stable without a marked upward trend, suggesting that Fe_2O_3 supported redox buffering that prevented excessive acid accumulation, likely due to its involvement in electron transfer reactions that sustain microbe-mediated VFA consumption (Wang et al., 2022). ZnO displays a similar stagnant pattern, suggesting inhibition of metanogenesis and accumulation of acids potentially due to metabolic stress or competition for essential cofactors. Zn^{2+} is known to influence central metabolic pathways and enzyme activities in bacteria, potentially triggering stringent responses that reorient metabolism toward maintenance rather than growth (Luche et al., 2018).

In contrast, CuO caused a consistent pH decline across the time, which can be attributed to inhibitory effects on both acidogenic and methanogenic microbes, since copper at elevated levels has been shown to suppress organic acid biotransformation and hydrogen metabolism in anaerobic systems (Kong et al., 1994). This suggests toxic inhibition of key enzymatic pathways involved in phase transition. In the Fe_2O_3-ZnO combination, the downward shift followed by a moderated pH trend reflects a synergistic interaction in which Fe facilitates electron flow while Zn modulates enzyme stability, together promoting acidogenesis and acetogenesis (Shi et al., 2016). Conversely, combinations with CuO (Fe_2O_3-CuO, ZnO-CuO) showed persistent low pH, indicating that the inhibitory effects of Cu dominate, overriding any potential benefits from Fe or Zn. The triple metal mixture (Fe_2O_3–ZnO–CuO) resulted in stagnant pH for 48 h, supporting the hypothesis that Cu toxicity antagonizes the beneficial redox interactions between Fe and Zn.

These observations align with general findings that trace elements can exert both stimulatory and inhibitory effects on anaerobic metabolism depending on concentration and speciation. Iron (Fe) often enhances microbial electron transport and organic degradation at moderate doses, whereas copper (Cu) can be inhibitory at relatively low concentrations due to its complexation with thiol groups and interference with key redox enzymes (Liu et al., 2014). Thus, CuO acts as an inhibitor of phase transition in anaerobic degradation, while the Fe_2O_3-ZnO is the only configuration that promotes pH profiles favorable to progression toward methanogenesis. These mechanistic interpretations link the observed pH dynamics to underlying microbial metabolism and metal redox interactions, directly addressing the reviewer's request for biochemical explanation.

3.4 Microorganism growth

The number of microorganisms contained in the research reactor is expressed using the Volatile Suspended Solid (VSS) parameter in mg/L. In this study, the measurement of VSS parameters is represented by optical density (OD) parameters to facilitate measurement. As a first step, a correlation curve is made between the VSS and OD parameters so that a linear equation is obtained. The OD absorbance of the sample measured on the spectrophotometer is then fed into the equation to obtain a representative VSS value.

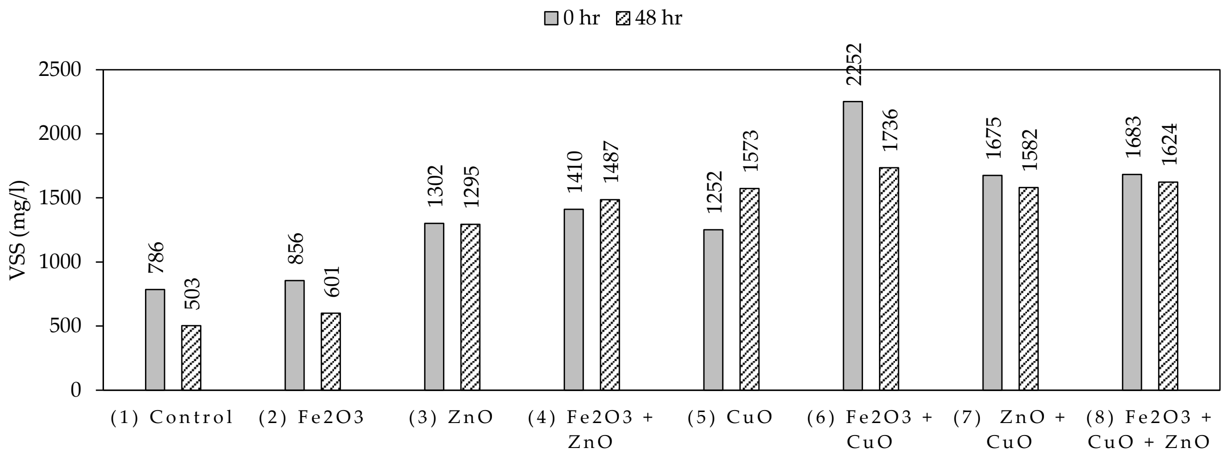


Figure 5. Average results of VSS data measurement replication

Based on the results of the average graph of VSS in Figure 5 showed varying increases and decreases in each treatment with the addition of metal oxides. In general, an increase in VSS indicates that microbial biomass is growing and active, indicating that the reactor environment supports metabolic activity and substrate degradation. Conversely, a decrease in VSS can mean cell lysis, metal toxicity, or nutrient limitations, resulting in biomass degradation. Trace metals such as Fe can serve in enzyme complexes that catalyze carbon flow through key pathways (e.g., in acetyl-CoA synthase, hydrogenase, and methanogenesis enzymes), whereas high concentrations of Cu may disrupt membrane integrity and electron transport chains, leading to decreased viability (Wang et al., 2025; Liu et al., 2014).

In the control reactor, the decrease in VSS reflects natural decay in the absence of micronutrient support. Treatment with a single Fe_2O_3 also showed a decrease in VSS, although the decrease was smaller. This suggests that the addition of Fe_2O_3 does not significantly promote microbial growth. Meanwhile, treatment with a single ZnO showed relatively stable VSS. This shows that ZnO is able to maintain biomass stability. A single CuO treatment recorded the highest increase in VSS, indicating that CuO in low concentrations was able to stimulate microbial activity that was adaptive to the metal. However, prolonged exposure likely leads to inhibition due to copper's interaction with cellular thiol systems and inhibition of key enzymes (Liu et al., 2014).

The combination of Fe_2O_3-ZnO resulted in an increase in VSS, indicating a synergy between the two metals that supports the active growth of microbes. However, the combination of Fe_2O_3-CuO produced a different result, i.e. VSS decreased quite significantly. This decline, although initially very high, indicates that this combination may exert toxic pressure on the microbial community, or lead to cell lysis. ZnO-CuO and Fe_2O_3-ZnO-CuO treatments also showed a decrease in VSS, but in relatively small amounts. This indicates that the microorganism may still be able to survive or adapt, even if it does not develop significantly.

To accurately quantify the effect of the addition of metal oxide on the growth of microorganisms in this study, a statistical test was carried out using a factorial 2^n. The factorial analysis method in this experiment has the following hypothesis:

H_0 : No significant effect of the addition of metal oxide on biomass growth

H_1 : There is a significant effect of the addition of metal oxide on biomass growth

Meanwhile, for the treatment variation without the addition of metal oxides, the hypothesis is as follows:

H_0 : No significant biomass growth in the system without the addition of metal oxides

H_1 : There is a significant biomass growth in the system without the addition of metal oxides

VSS (mg/L) data of each variation measured at the 0th and 48th hours was then processed to obtain data on the biomass growth rate per day using the formula as shown below.

$$Biomass\ Growth\ Rate\ (mg/L/day) = \frac{VSS\ T_{48}\ (mg/L) - VSS\ T_0\ (mg/L)}{Running\ duration\ (day)} \quad (1)$$

The biomass growth rate was calculated for both replications at each variation. It aims to adjust the data format to the calculation method using a 2^n factorial statistical test. The results of the calculation of biomass growth rate are listed in the Table 4.

Table 4. Treatment and biomass growth rate (mg/L/day)

Metal Oxide (mg/L)			**Biomass Growth Rate (mg/L/day)**	
Fe_2O_3	ZnO	**CuO**	**Replicate 1**	**Replicate 2**
0	0	0	-118.13	-165.31
10			-80.63	-174.38
0	4		-23.44	16.25
10			-27.50	104.38
0	0	0,1	140.00	180.94
10			-385.63	-130.31
0	4		-177.19	84.06
10			17.50	-76.25

Then, biomass growth rate data for all variations was calculated using the ANOVA statistical test which took into account the degree of freedom (d_f), sum of squares (SS), mean square (MS), and also F-Ratio (F_{calc}). After calculating the components of the ANOVA, the F-Ratio of the calculation results (F_{calc}) is compared with the F-Ratio of the statistical component table of the Critical Values of the F-Distribution (Dougherty, 2002), as seen on the Table 5. The significance value used is α = 5% and α = 1%.

Table 5. Hypothesis testing on biomass growth

Variation	**df**	**SS**	**MS**	F_{calc}	F_{table} **5%**	F_{table} **1%**	**Information**
1	1	8803.30	8803.30	4.57	5.59	12.25	H_0 is accepted
a	1	5602.37	5602.37	2.91	5.59	12.25	H_0 is accepted
b	1	4636.61	4636.61	2.41	5.59	12.25	H_0 is accepted
ab	1	13024.75	13024.75	6.77	5.59	12.25	H_0 is accepted
c	1	10633.68	10633.68	5.53	5.59	12.25	H_0 is accepted
ac	1	9409.33	9409.33	4.89	5.59	12.25	H_0 is accepted
bc	1	11905.71	11905.71	6.19	5.59	12.25	H_0 is accepted
abc	1	10340.35	10340.35	5.37	5.59	12.25	H_0 is accepted
Error	7	13470.73	1924.39	-			
Total	15	-	-	-			

The H_0 and H_1 hypotheses have been defined in the previous section. If the calculation obtained is smaller than the F-Table, hence the hypothesis H_0 will be accepted. Conversely, if the calculation obtained is greater than the F-Table, so the hypothesis H0 will be rejected. From the calculations in Table 5, all variations show that the H_0 hypothesis is acceptable. This means that the entire treatment of each variation, whether the metal oxide reactor or the control reactor, has no significant effect on the growth rate of the microorganisms.

Table 6. Conclusion of hypothesis testing on biomass growth

Variation	**Hypothesis (α = 5%)**	**Conclusion**
1	H_0 is accepted	No significant biomass growth occurs in the process without the addition of metals
a	H_0 is accepted	No significant effect of Fe_2O_3 metal oxide on biomass growth
b	H_0 is accepted	No significant effect of ZnO metal oxide on biomass growth
ab	H_0 is accepted	No significant effect of Fe_2O_3 and ZnO metal oxide interactions on biomass growth
c	H_0 is accepted	No significant effect of CuO metal oxide on biomass growth

Variation	Hypothesis (α = 5%)	Conclusion
ac	H_0 is accepted	No significant effect of Fe_2O_3 and CuO metal oxide interactions on biomass growth
bc	H_0 is accepted	No significant effect of ZnO and CuO metal oxide interactions on biomass growth
abc	H_0 is accepted	There is no significant effect of Fe_2O_3, ZnO, and CuO metal oxide interactions on biomass growth

Microorganisms did not show significant growth either in reactors with added micronutrients in the form of metal oxides, or those without added micronutrients. This is likely caused by microorganisms that have reached a dormant state or a stationary phase of the seeding and acclimatization stages so that they no longer use organic compounds in the substrate to grow.

3.5 Dissolved organic compound degradation (sCOD)

Chemical Oxygen Demand (COD) shows the level of oxygen required for the organic compounds present in the sample to be chemically degraded. COD parameter testing can represent the large number of substrate organic compounds present in the reactor. In the process of anaerobic degradation, decreased dissolved COD indicates the organic compounds contained in the substrate are used by microorganisms for metabolic activity. Organic compounds that are measured as COD can be monomers or oligomers that have been dissolved as a product of the hydrolysis stage, as well as fermentation products of acidogenesis and acetogenesis in the form of volatile acids, ketones, and alcohols. If the dissolved COD in the reactor is reduced, it means that the organic compounds that were originally dissolved in the reactor have changed phase into non-liquid products, such as CO_2 and CH_4 gas from the products of the methanogenesis stage.

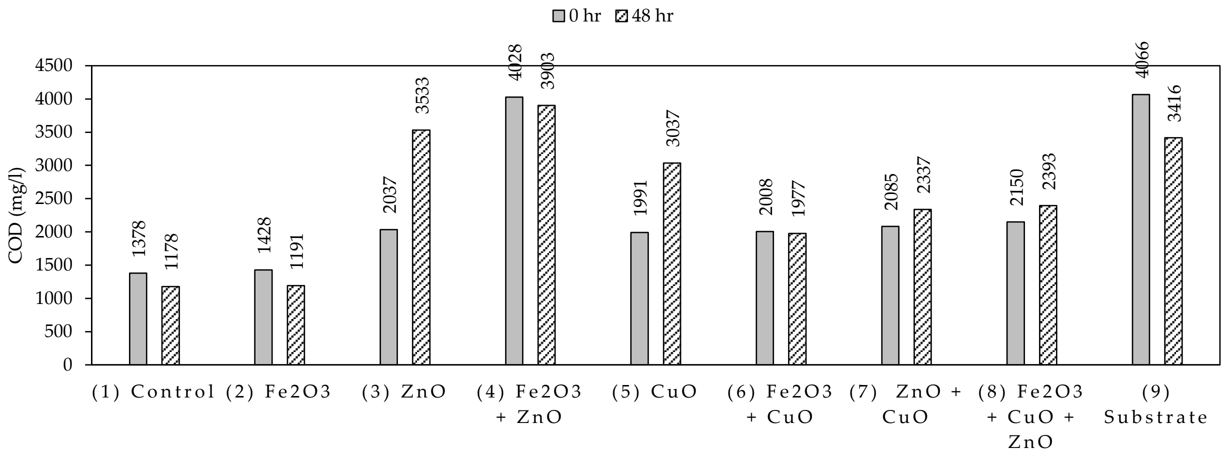


Figure 5. Average results of sCOD data measurement replication

However, an increase in dissolved COD does not mean that organic compounds are not used in the metabolic activity of microorganisms. The increase in COD indicates the accumulation of products, both products resulting from the hydrolysis stage and acid products from fermentation at the acidogenesis and acetogenesis stages. This may also indicate an inhibition at the stage of methanogenesis that converts dissolved organic compounds into gaseous phases (Marañón, et al., 2012). To accurately quantify the effect of the addition of metal oxides on the dissolved COD allowance in this study, a statistical test was conducted using a factorial 2^n. The factorial analysis method in this experiment has the following hypothesis:

H_0 : There was no significant effect of the addition of metal oxide on soluble COD removal

H_1 : There is a significant effect of the addition of metal oxides on soluble COD removal

Meanwhile, for the treatment variation without the addition of metal oxides, the hypothesis is as follows:

H_0 : No significant dissolved COD dispensing in the system without the addition of metal oxides

H_1 : There is significant dissolved COD dispensing in the system without the addition of metal oxides COD (mg/L) data for each variation measured at the 0th and 48th hours was then processed to obtain data on the COD removal rate per day using the formula as stated below.

$$sCOD\ Removal\ Rate\ (mg/L/day) = \frac{sCOD\ hour{-}48\left(\frac{mg}{L}\right) - sCOD\ hour{-}0\ \left(\frac{mg}{L}\right)}{Running\ duration\ (day)} \quad (2)$$

The dissolved COD removal rate is calculated for both replications on each variation. The results of the calculation of COD removal rate are listed in Table 7 below.

Table 7. Conclusion of hypothesis testing on COD removal rate

Metal Oxide (mg/L)			**COD Removal Rate (mg/L/day)**	
Fe_2O_3	**ZnO**	**CuO**	**Replicate 1**	**Replicate 2**
0	0	0	41,67	158,33
10			8,33	229,17
0	4		-420,83	-1075,00
10			183,33	-58,33
0	0	0,1	-666,67	-379,17
10			-33,33	64,58
0	4		-152,08	-100,00
10			-137,50	-106,25

Then, COD removal rate data for all variations was calculated using the ANOVA statistical test which took into account the degree of freedom (df), sum of squares (SS), mean square (MS), and also F-Ratio (Fcalc). After calculating the components of the ANOVA, the F-Ratio of the calculation results (Fcalc) is compared with the F-Ratio of the statistical component table of the Critical Values of the F-Distribution (Dougherty, 2002), as seen on the Table 8.

Table 8. Hypothesis testing on organic compounds degradation (sCOD removal rate)

Variation	**df**	**SS**	**MS**	**F_{calc}**	**F_{table} 5%**	**F_{table} 1%**	**Information**
1	1	373244,62	373244,62	8,11	5,59	12,25	H_0 is rejected
a	1	470510,25	470510,25	10,23	5,59	12,25	H_0 is rejected
b	1	103939,07	103939,07	2,26	5,59	12,25	H_0 is accepted
ab	1	304221,19	304221,19	6,61	5,59	12,25	H_0 is rejected
c	1	20814,07	20814,07	0,45	5,59	12,25	H_0 is accepted
ac	1	254709,47	254709,47	5,54	5,59	12,25	H_0 is accepted
bc	1	131784,12	131784,12	2,86	5,59	12,25	H_0 is accepted
abc	1	239182,12	239182,12	5,20	5,59	12,25	H_0 is accepted
Error	7	321822,64	45974,66	-			
Total	15	-	-	-			

The H_0 and H_1 hypotheses have been defined in the previous section. If the calculation obtained is smaller than the F-Table, hence the hypothesis H_0 will be accepted. Conversely, if the calculation obtained is greater than the F-Table, so the hypothesis H_0 will be rejected.

Table 9. Conclusion of hypothesis testing on organic compounds degradation (sCOD removal rate)

Variasi	**Hipotesis (α = 5%)**	**Summary**
1	H_0 is rejected	There is significant dissolved COD allowance in the process without the addition of metals
a	H_0 is rejected	There is a significant effect of Fe_2O_3 metal oxide on dissolved COD elimination

Variasi	Hipotesis (α = 5%)	Summary
b	H_0 is accepted	There was no significant effect of ZnO metal oxide on dissolved COD elimination
ab	H_0 is rejected	There is a significant effect of Fe_2O_3 and ZnO metal oxide interactions on the elimination of soluble COD
c	H_0 is accepted	No significant effect of CuO metal oxide on dissolved COD dispensing
ac	H_0 is accepted	There was no significant effect of Fe_2O_3 and CuO metal oxide interactions on COD allowance
bc	H_0 is accepted	There was no significant effect of ZnO and CuO metal oxide interactions on dissolved COD elimination
abc	H_0 is accepted	There was no significant effect of Fe_2O_3, ZnO, and CuO metal oxide interactions on dissolved COD settling

Based on Table 9, metal oxides that have a significant effect on the dissolved COD elimination rate are Fe_2O_3 and Fe_2O_3 + ZnO with a significance level of 5%. However, no metal oxide is classified as very significant with a 1% confidence level because all F_{calc} obtained are smaller than 1% F_{table}.

3.6 Total volatile acid (VFA) production

The total volatile acid test is carried out by the distillation method so that all measured volatile acids are read as acetic acid. The results of this total volatile acid test provide an indication of the stages in the anaerobic pathway. If the total volatile acid increases, there is a possibility of acid buildup, which indicates that the microorganisms in the reactor have not yet entered the methanogenesis phase. In the phases before methanogenesis occurs, namely acidogenesis and acetogenesis, the monomers of the products of the hydrolysis stage are converted into volatile acids, such as acetic acid, propionic acid, and butyric acid. In contrast, if the total measured volatile acid decreases, the microorganism is assumed to be in the stage of methanogenesis.

Trace elements influence VFA production through their roles in microbial metabolism: iron-based compounds can accelerate hydrolysis and enrich acidogenic microbes, resulting in higher VFA yields, as demonstrated in earlier studies where Fe_3O_4 enhanced VFAs production by up to 160% in anaerobic fermentation of food waste through enriched hydrolytic and acidogenic bacterial populations and improved electron transfer (Wang et al., 2022).

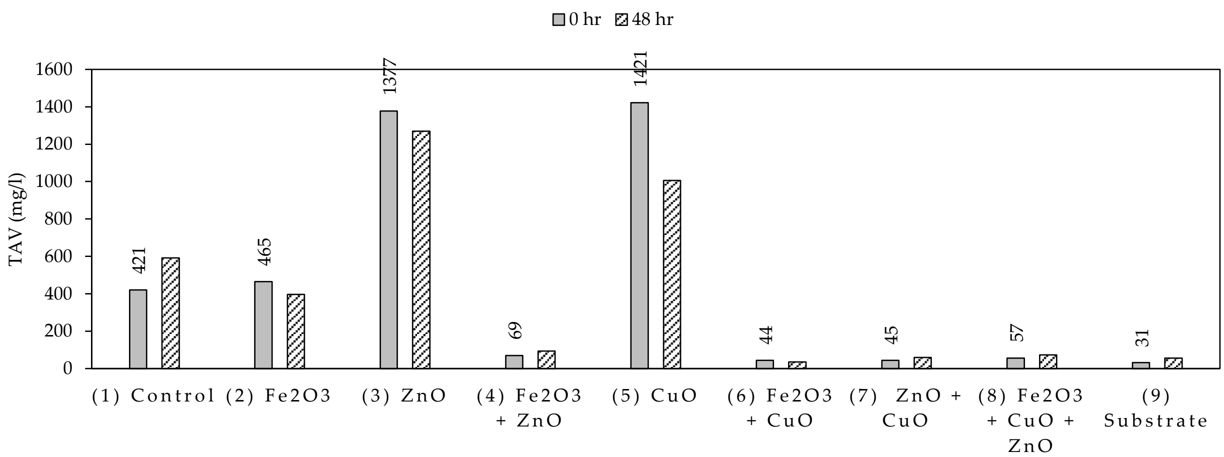


Figure 6. Average results of VFA data measurement replication

To accurately quantify the effect of the addition of metal oxides on the production of VFA in this study, a statistical test was carried out using a factorial of 2^n. The factorial analysis method in this experiment has the following hypothesis:

H_0 : No significant effect of the addition of metal oxide on VFA production

H_1 : There is a significant influence of the addition of metal oxides on VFA production

Meanwhile, for the treatment variation without the addition of metal oxides, the hypothesis is as follows:

H_0 : No significant VFA production in the system without the addition of metal oxides

H_1 : There is significant VFA production in the system without the addition of metal oxides

VFA data (mg/L) of each variation measured at the 0th and 48th hours was then processed to obtain data on the VFA production rate per day using the formula as shown below.

$$VFA\ Production\ Rate\ (mg/L/hari) = \frac{VFA\ hour{-}48\ (mg/L) - VFA\ hour{-}0\ (\frac{mg}{L})}{Running\ duration\ (day)} \quad (3)$$

The VFA production rate is calculated for both replications on each variation. The results of the calculation of the VFA production rate are listed in Table 10 below.

Table 10. Treatment and production rate of VFA (mg/L/day)

Metal Oxide (mg/L)			VFA Production Rate (mg/L/day)	
Fe_2O_3	**ZnO**	**CuO**	**Replicate 1**	**Replicate 2**
0	0	0	144,65	25,16
10			31,45	-100,63
0	4		-25,16	-81,76
10			42,81	-17,93
0	0	0,1	-138,36	-276,73
10			-10,31	0,93
0	4		0,00	14,85
10			18,87	-3,14

Then, VFA production rate data for all variations was calculated using the ANOVA statistical test which took into account the degree of freedom (df), sum of squares (SS), mean square (MS), and also F-Ratio (F_{calc}). After calculating the components of the ANOVA, the F-Ratio of the calculation results (F_{calc}) is compared with the F-Ratio of the statistical component table of the Critical Values of the F-Distribution (Dougherty, 2002). The significance value used is α = 5% and α = 1%.

Table 11. Hypothesis testing on VFA production

Variation	df	SS	MS	F_{calc}	F_{table} 5%	F_{table} 1%	Information
1	1	8803,30	8803,30	4,57	5,59	12,25	H_0 is accepted
a	1	5602,37	5602,37	2,91	5,59	12,25	H_0 is accepted
b	1	4636,61	4636,61	2,41	5,59	12,25	H_0 is accepted
ab	1	13024,75	13024,75	6,77	5,59	12,25	H_0 rejected
c	1	10633,68	10633,68	5,53	5,59	12,25	H_0 is accepted
ac	1	9409,33	9409,33	4,89	5,59	12,25	H_0 is accepted
bc	1	11905,71	11905,71	6,19	5,59	12,25	H_0 rejected
abc	1	10340,35	10340,35	5,37	5,59	12,25	H_0 is accepted
Error	7	13470,73	1924,39	-			
Total	15	-	-	-			

The H_0 and H_1 hypotheses have been defined in the previous section. If the calculation obtained is smaller than the F-Table, hence the hypothesis H_0 will be accepted. Conversely, if the calculation obtained is greater than the F-Table, so the hypothesis H_0 will be rejected.

Table 12. Conclusion of hypothesis testing on VFA production

Variations	Hypothesis (a = 5%)	Conclusion
1	H_0 is accepted	No significant VFA production in the process without the addition of metals
a	H_0 is accepted	There is no significant effect of Fe_2O_3 metal oxide on the total production of volatile acids
b	H_0 is accepted	There is no significant effect of ZnO metal oxide on the total production of volatile acids
ab	H_0 rejected	There is a significant influence of the interaction of Fe_2O_3 and ZnO metal oxides on the total production of volatile acids
c	H_0 is accepted	There is no significant effect of CuO metal oxide on the total production of volatile acids
ac	H_0 is accepted	There was no significant effect of the interaction of Fe_2O_3 and CuO metal oxides on the total production of volatile acids
bc	H_0 rejected	There is a significant influence of the interaction of ZnO and CuO metal oxides on the total production of volatile acids
abc	H_0 is accepted	There was no significant effect of the interaction of Fe_2O_3, ZnO, and CuO metal oxides on the total production of volatile acids

Based on Table 12. Conclusion of Hypothesis Testing on VFA Production, metal oxides that affect the rate of total production of volatile acids (VFA) are variations of metal oxides Fe_2O_3 + ZnO and ZnO + CuO with a significance level of 5%. However, no metal oxides are classified as very significant with a 1% confidence level because all the F_{counts} obtained are smaller than the $F_{of\ the}$ 1% table. This supports the concept that synergistic redox effects of Fe and Zn may promote VFA formation by stabilizing metabolic electron flows and enhancing enzyme activity, whereas Cu's inhibitory effects can limit these pathways if dominant (Kadam et al., 2022).

4. Conclusion

The conclusion of this study is that the addition of metal oxides Fe_2O_3, ZnO, and CuO that significantly affect the production of VFA are Fe_2O_3 + ZnO and ZnO + CuO. Metal oxides that have a significant effect on the degradation of organic compounds (sCOD) are Fe_2O_3 and Fe_2O_3 + ZnO and ZnO + CuO. Meanwhile, the addition of metal oxides Fe_2O_3, ZnO, and CuO and their interactions with each other did not have a significant effect on biomass growth. This can occur due to microorganisms that have reached a tuberous condition or a stationary phase from the seeding and acclimatization stages so that they no longer use organic compounds in the substrate to grow.

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