JURNAL TEKNIK LINGKUNGAN
E-ISSN: 27146715
Journal homepage: www.envengjournal.com

Research Article

Analysis of Seeding and Acclimatization of Indigenous Bacteria for Ethanol to Acetate Conversion from POME In an Anaerobic Process

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Abstract

Indonesia's palm oil industry generates a large volume of Palm Oil Mill Effluent (POME), a wastewater with high organic content. POME has the potential to be anaerobically treated and converted into value-added products such as bioethanol and acetic acid. However, the success of this conversion heavily relies on the preparation of an effective microbial culture. This research aims to analyze the success of a seeding and staged acclimatization process to cultivate an indigenous bacterial culture capable of converting ethanol to acetate. The seeding process was conducted using a mixed culture of POME sludge and septic tank sludge, followed by a three-stage acclimatization with increasing ethanol substrate concentrations (35%, 70%, and 100% (v/v) of the 1000 mg/L target). The success of the process was evaluated based on chemical oxygen demand (COD) and volatile suspended solids (VSS) parameters. The results showed that the seeding process successfully increased the VSS from 1,785 mg/L to 4,351 mg/L. The acclimatization process was also successful, indicated by high COD removal efficiencies of 92% in Stage 2 and 84% in Stage 3, with a stable VSS concentration in the optimal range of 2,000-4,000 mg/L in the final stage. It is concluded that the seeding and staged acclimatization successfully developed a dense, active, and adapted indigenous microbial culture capable of degrading a substrate with an ethanol concentration of 1,000 mg/L.

Keywords: *acclimatization, COD, indigenous bacteria, POME, seeding, VSS*

ARTICLE INFO

Citation: *Tirta, F.A., Handajani, M., & Gumilar, A. (2025). Analysis of Seeding and Acclimatization of Indigenous Bacteria for Ethanol to Acetate Conversion from POME In an Anaerobic Process. Jurnal Teknik Lingkungan, 31(2), 71-89, https://doi.org/10.5614/j.tl.2025.31.2.7*

Article History:
Received 5 Sep 2025
Revised 19 Sep 2025
Accepted 30 Sep 2025
Available online 31 Oct 2025

1. Introduction

The palm oil industry is a leading commodity in Indonesia, with a plantation area reaching 16.8 million hectares, making Indonesia the world's largest producer of palm oil. This large-scale palm oil production is accompanied by a significant amount of wastewater generated from the process of converting palm fruit into Crude Palm Oil (CPO). Every 1 ton of crude palm oil processing can produce 2.5 m^3 of wastewater or palm oil mill effluent (POME). This effluent has complex characteristics, with a very high organic content. The treatment of POME is typically done anaerobically because this method is highly effective in degrading complex organic matter.

Indonesia faces the challenge of increasing energy demand, which drives the transition from fossil fuels to more environmentally friendly renewable energy. One potential alternative is bioethanol, which is ethanol produced through the fermentation of biomass from plantation and agricultural waste. Bioethanol can be used as a fuel blend such as E10 or E85, helping to reduce fossil fuel consumption and greenhouse gas emissions. Bioethanol can also be produced from high-organic wastewater like POME, particularly during the acidogenesis stage of the anaerobic treatment process.

The utilization of anaerobic bacteria in POME treatment yields more than just bioethanol. The acidogenesis process is fundamentally complex, where the decomposition of organic compounds produces various useful

intermediate products. Besides bioethanol, this process also yields a mixture of volatile fatty acids (VFA) such as acetic acid, propionic acid, and butyric acid. Research has shown that through controlled anaerobic fermentation, POME can be efficiently converted into a mixture of VFAs, with acetic acid often being the most dominant component (James & Nachiappan, 2018).

Therefore, the main objective of this research is to analyze and evaluate the success of a series of seeding and acclimatization processes that have been conducted. This analysis focuses on two parameters: the concentration of volatile suspended solids (VSS) as an indicator of biomass growth and stability, and the rate of chemical oxygen demand (COD) reduction as a measure of the microorganisms' effectiveness in consuming the organic substrate.

2. Methodology

2.1 Time and place of research

This research was conducted from November 2024 to July 2024. The wastewater used as a sample in this study was from the palm oil industry, collected from PT Condong Garut. The research process was carried out at the Water Quality Laboratory, Environmental Engineering Study Program, Faculty of Civil and Environmental Engineering, Institut Teknologi Bandung.

2.2 Research design

This study was designed to determine the effect of adding metal oxides (Fe_2O_3, NiO, and CuO) on the conversion of ethanol into acetic acid derived from Palm Oil Mill Effluent (POME). The variations in metal concentrations are presented in the following table.

Table 1. Metal oxide concentration

Sample code	Metal	Concentration (mg/L)	Sources
A	Fe_2O_3	1	(Puteri, 2016)
B	CuO	0.1	(Puteri, 2016)
C	NiO	1.5	(Anggamulia, 2020)

The research design aimed to investigate the effects of variations among trace elements using a screening test method under the following conditions. The experimental design employing a 2^n factorial analysis is presented in the following table.

Table 2. Research design using the screening test method

No	Variaton	A	B	C
1	Control	0	0	0
2	Fe_2O_3	1	0	0
3	CuO	0	1	0
4	$Fe_2O_3 + CuO$	1	1	0
5	NiO	0	0	1
6	$Fe_2O_3 + NiO$	1	0	1
7	CuO + NiO	0	1	1
8	$Fe_2O_3 + CuO + NiO$	1	1	1

Note:

0 = No addition of metal oxide
1 = With the addition of metal oxide

2.3 Seeding and acclimatization

The seeding process was conducted in a 30-liter batch reactor. A total of 4 liters of sludge from POME WWTP and 2 liters of sludge from a septic tank were mixed with 19 liters of distilled water and artificial substrate. This substrate consisted of dextrose monohydrate as a carbon source, along with nitrogen and phosphate sources with a C:N:P ratio of 40:2:1, corresponding to the characteristics of POME. This composition was designed to provide sufficient nutrients for the initial growth of microorganisms. Subsequently, maintenance and monitoring of key parameters such as pH, VSS, and COD were carried out. The seeding stage was considered complete when the COD value stabilized and VSS reached the range of 2,000-4,000 mg/L (Sperling, 2007).

The next process was acclimatization, where the cultivated microorganisms were adapted to the artificial ethanol substrate. The goal was for the surviving microorganisms to be able to efficiently convert ethanol into acetic acid. The microorganism acclimatization process was carried out in stages to provide adaptation time for the microorganisms to the new ethanol-containing substrate. The acclimatization process was divided into 3 stages as follows.

Table 3. Stages of the acclimatization process

Stages	Substrate Concentration
1	35% (v/v) ethanol
2	65% (v/v) ethanol
3	100% (v/v) ethanol

During the acclimatization stages, the measured parameters included VSS and COD. The COD parameter was observed until it reached a stable condition, ranging between 2,000-4,000 mg/L, which indicated that the microorganisms had successfully adapted to the artificial palm oil wastewater.

2.4 Reactor operation

The reactor was operated with eight variations of metal combinations and two experimental repetitions (duplicates). The reactor had a total volume of 6 liters, with 5 liters of substrate and biomass mixture loaded to allow sufficient headspace. The operation involved a mixture of biomass and artificial ethanol substrate, with a seeding-to-artificial ethanol ratio of 1:4 (1 L of biomass and 4 L of artificial ethanol). The reactor was operated for 72 hours or until the acetogenesis phase was reached, following the preceding acidogenesis phase during which ethanol formation occurs. Sampling was conducted at the 0th and 72nd hours. The metal oxides used in this study were Fe_2O_3, CuO, and NiO.

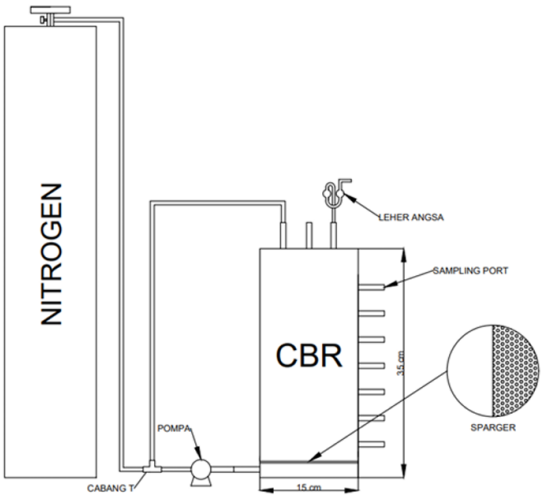

Figure 1. Schematic of the circulating bed reactor used in the study

2.5 Testing parameters

In this study, the key parameters analyzed were pH, VSS, COD, ethanol, and acetic acid. The measurements of these parameters (except for pH) were conducted at the 0th and 72nd hours during the experimental run. The following table presents the parameters, analytical methods, and testing standards employed in this study.

Table 4. Parameters, methods, and testing standards used in the study

No	Parameter	Analysis Method	Testing Standard
1	Dissolved COD	Dichromate COD Spectrophotometry	SNI 6989.2:2009
2	VSS	Gravimetry	APHA 2540 E
3	pH	pH Meter	SNI 06-6989.11-2004
4	Ethanol	Gas Chromatography	ASTM D5501
5	Acetic acid	Gas Chromatography	ASTM D5599

2.6 Data processing

Data analysis in this study was conducted using the 2^n factorial statistical method. The research design was structured as follows:

Factors : Three types of metal oxides, Fe_2O_3, CuO, and NiO
Levels : Presence and absence of metal oxides
Experimental design : Total conditions $2^3 = 8$

The factorial analysis employed two analytical approaches: the table of signs method and the Yates algorithm. The results obtained from both methods were compared and subsequently used for decision-making analysis. The experimental conditions based on this analytical design are presented in Table 5. The factorial experimental approach aims to identify the interaction effects among the tested factors. Under certain conditions, two factors may exhibit synergistic effects (positive interaction) on the response variable. Conversely, in other conditions, the presence of one factor may inhibit the performance of another (negative interaction).

Table 5. Table of sign

Experiment condition								Total effect
1	a	b	ab	c	ac	bc	abc	
+	+	+	+	+	+	+	+	[1]
-	+	-	+	-	+	-	+	[A]
-	-	+	+	-	-	+	+	[B]
+	-	-	+	+	-	-	+	[AB]
-	-	-	-	+	+	+	+	[C]
+	-	+	-	-	+	-	+	[AC]
+	+	-	-	-	-	+	+	[BC]
-	+	+	-	+	-	-	+	[ABC]

Table 6. Calculation using the yates method

Response	[1]	[2]	[3] = Contrast (effect)
1	(1) + a	(1) + a + b +ab	+(1) + a + b + ab + c + ac +bc + abc
a	b + ab	c + ac + bc +abc	-(1) + a - b - ab - c + ac - bc + abc
b	c + ac	a - (1) + ab - b	(1) - a +b + ab - c - ac + bc + abc
ab	bc + abc	ac - c + abc - bc	+(1) - a - b + ab + c - ac - bc + abc
c	a – (1)	b + ab - (1) - a	-(1) - a -b - ab + c + ac + bc + abc
ac	ab - b	bc + abc - c -ac	+(1) - a + b - ab - c + ac - bc + abc

Response	[1]	[2]	[3] = Contrast (effect)
bc	ac – c	ab - b - a + (1)	+(1) + a - b - ab - c - ac + bc + abc
abc	abc - bc	abc - bc - ac + c	(1) + a + b - ab + c -ac - bc + abc

3. Result and Discussion

3.1 Characteristics of palm oil mill effluent

POME characteristics include a thick brownish color, containing oil and grease, and relatively high BOD and COD values. The characteristics of the palm oil mill effluent from the PT. Condong Garut industry are as follows.

Table 7. Characteristics of palm oil mill effluent from PT Condong Garut

Parameter	Unit	Analysis Result
Total COD	mg/L	37.233
Dissolved COD	mg/L	11.283
Total BOD	mg/L	509,5
Dissolved BOD	mg/L	383,6
NTK	mg/L	322
Total phosphate	mg/L	14,7
Ethanol	mg/L	8.397
pH	-	4,54
TSS	mg/L	48.538
VSS	mg/L	47.076
Oil and Grease	mg/L	2.210

The artificial wastewater used in this study was prepared based on the characterization results above. Artificial wastewater was used to match the characteristics of the wastewater at PT. Condong Garut, which contains substrate as a source of metabolism for microorganisms. The artificial wastewater used contained organic compounds such as ethanol with a composition of 4 L of distilled water and 4 mL of ethanol. Based on this artificial wastewater composition, an analysis was then conducted to determine the initial characteristics of the prepared artificial wastewater. The characteristics of the artificial wastewater can be seen in the following table.

Table 8. Characteristics of artificial wastewater

Parameter	Unit	Analysis Result
Dissolved COD	mg/L	1.080
Ethanol	mg/L	897
pH	-	6,11
VSS	mg/L	3.372

The wastewater from the PT. Condong Garut palm oil industry has different characteristics compared to the artificial wastewater. This difference is because the real effluent has gone through various complex palm oil processing stages. Meanwhile, the artificial wastewater is made from synthetic materials, so its components may not necessarily interact with other substances.

3.2 Seeding

Seeding was conducted as the initial stage to cultivate the microorganisms to be used in this research. In this study, the seeding used was a mixture of biomass from the WWTP sludge of PT. Condong Garut, septic tank sludge, and distilled water. The seeding was performed in a batch reactor with a total volume of 25 L under anaerobic conditions. The parameters observed during the seeding process were dissolved COD and VSS. Dissolved COD indicates the amount of organic matter that can be decomposed by microorganisms during

the cultivation process, while VSS indicates the amount of microorganisms that grow or are formed in the process. The following is the dissolved COD and VSS data during the seeding process.

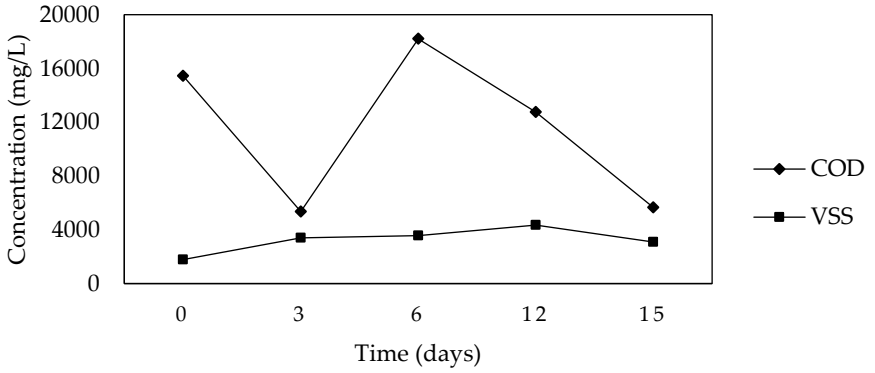

Figure 2. Graph of COD and VSS parameters during the seeding stage

The anaerobic microorganism seeding process was successfully carried out, as evidenced by two key parameters: substrate degradation (COD) and biomass growth (VSS). Initially, the microorganisms rapidly reduced the COD concentration from 15,446 mg/L to 5,356 mg/L within three days. After nutrient supplementation on the sixth day, which increased the COD level to 18,200 mg/L, the culture again demonstrated a strong degradation capability by reducing it to 5,654 mg/L by the fifteenth day. Simultaneously, the biomass concentration (VSS) showed a significant increase from 1,785 mg/L to 4,351 mg/L on the twelfth day, confirming the rapid growth of microorganisms. The combination of high degradation capacity and substantial biomass development indicates that the microbial culture was well adapted and ready for the acclimatization stage with a more specific substrate.

3.3 Acclimatization

Acclimatization was carried out to adjust the microorganisms to new environmental conditions with the aim of enabling them to work efficiently and stably in degrading artificial ethanol as their substrate. Acclimatization was performed in a batch reactor with a total volume of 5 L under anaerobic conditions with a composition of 1 L of seeding and 4 L of artificial ethanol substrate. The microorganism acclimatization process was conducted in three stages based on increasing concentrations of the ethanol-containing substrate. The first stage used a substrate with an ethanol content of 35% (v/v) or 350 mg/L, followed by the second stage with 70% (v/v) ethanol or 650 mg/L, and the final stage using 100% (v/v) ethanol or 1.000 mg/L. This staged approach aims to provide adaptation time for the microorganisms to the new ethanol-containing substrate. The final target of this process is to reach an ethanol concentration of 1.000 mg/L in the final acclimatization stage. During the acclimatization process, COD and VSS values were observed.

First stage

The first stage of acclimatization was carried out using a substrate containing 35% (v/v) ethanol, equivalent to 350 mg/L. The observations obtained from this first-stage acclimatization are presented in the following graph.

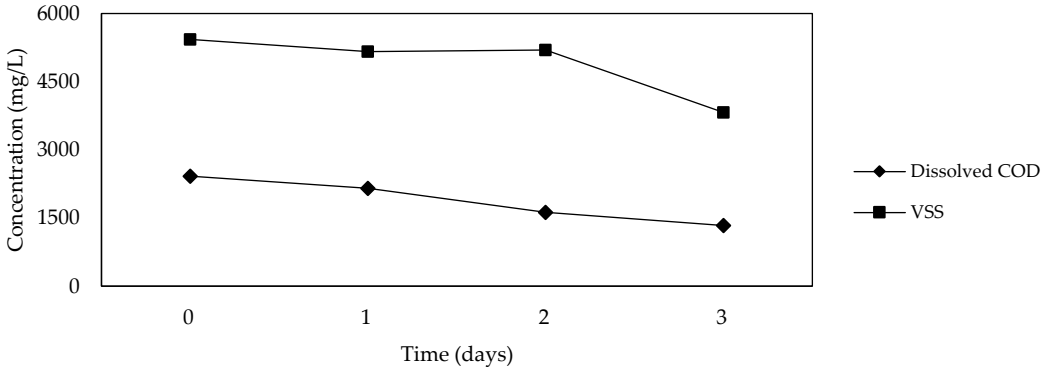

Figure 3. Graph of COD and VSS parameters during stage 1 acclimatization

During the first stage of acclimatization, the microorganisms successfully reduced COD by 44% within three days, decreasing from 2,420 mg/L to 1,334 mg/L, indicating effective substrate utilization. However, the biomass (VSS) exhibited an overall decline. Following an initial adjustment phase that led to partial cell death, the culture entered a stationary phase on the second day before undergoing a significant death phase on the third day, with the specific growth rate dropping sharply to -0.307/day. This reduction in biomass suggests that the microorganisms experienced nutrient limitation from the available substrate; therefore, the acclimatization process needed to proceed to the next stage with a higher substrate concentration.

Second stage

The second stage of acclimatization was carried out using a substrate containing 65% (v/v) ethanol, equivalent to 650 mg/L. The observations obtained from this second-stage acclimatization are presented in the following graph.

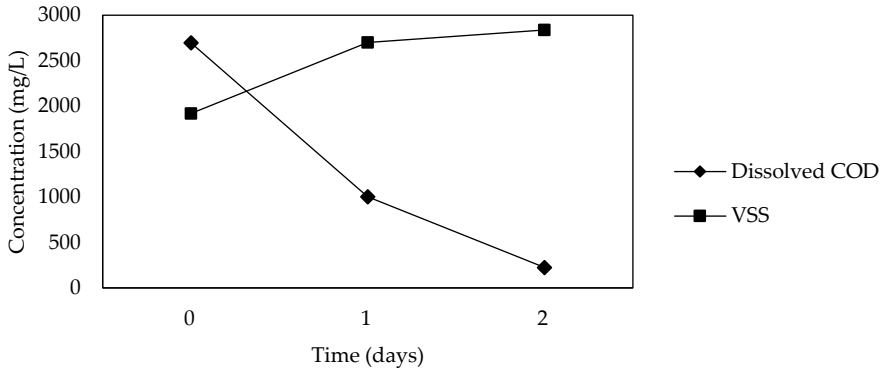

Figure 4. Graph of COD and VSS parameters during stage 2 acclimatization

During the second stage of acclimatization, the microorganisms exhibited excellent adaptation. This was evidenced by a COD removal efficiency of 92%, with concentrations decreasing from 2,696 mg/L to 1,334 mg/L within just two days. Simultaneously, there was a significant increase in biomass (VSS) from 1,919 mg/L to 2,701 mg/L. The growth initially occurred rapidly during the logarithmic phase and then slowed as the culture entered the stationary phase. The combination of high degradation capability and robust biomass growth confirms that the microorganisms successfully adapted and were ready to proceed to the next stage with a higher substrate concentration.

Third stage

The third stage of acclimatization was carried out using a substrate containing 100% (v/v) ethanol, equivalent to 1,000 mg/L. The observations obtained from this third-stage acclimatization are presented in the following graph.

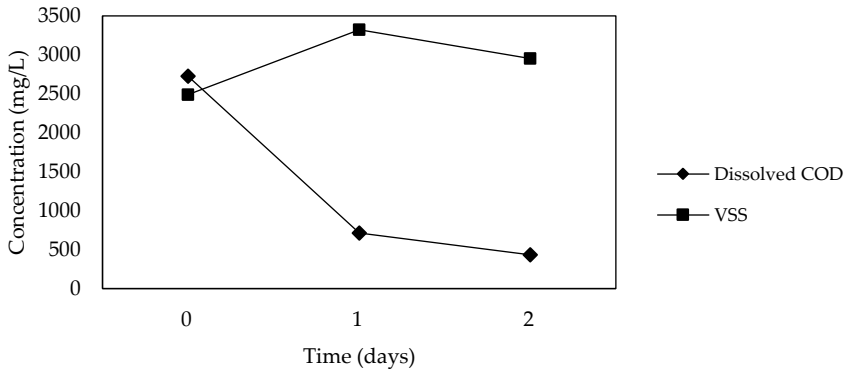

Figure 5. Graph of COD and VSS parameters during stage 3 acclimatization

In the final stage of acclimatization (Stage 3), the microorganisms demonstrated highly effective performance, achieving a COD removal efficiency of 84%, with concentrations decreasing from 2,725 mg/L to 433 mg/L within two days. Biomass growth (VSS) was initially rapid but subsequently entered a death phase as the

substrate was quickly depleted. Nevertheless, the final biomass concentration remained high and stable within the optimal range (2,954 mg/L). Overall, the three-stage acclimatization process successfully enabled the microbial culture to reach a steady-state condition. This was evidenced by the microorganisms' consistent ability to achieve high degradation efficiency, even under varying organic load conditions in stages 2 and 3. Therefore, the adapted culture was deemed ready to be utilized for the degradation of substrates at the target concentration.

3.4 Reactor operation

This study employed a circulating bed reactor with a total capacity of 6 liters, consisting of eight experimental variations, including the addition of three types of metal oxides and one control, all conducted in duplicate. Each reactor was filled with 5 liters of a mixed culture (4 liters of artificial wastewater and 1 liter of biomass) and maintained under anaerobic conditions through nitrogen flushing. The reactors were operated for 72 hours at room temperature to observe acetic acid formation. Key samples, including COD and VSS, were collected at the beginning and end of the experiment, while pH was monitored every six hours.

pH

pH is a crucial environmental factor in anaerobic reactors as it directly influences microbial growth, enzyme activity, and metabolic pathways. Each stage of the anaerobic process operates optimally within a specific pH range to maximize the production of particular metabolites. For instance, acetogenesis, the stage responsible for acetic acid formation, proceeds efficiently within a pH range of 5.5-7.0. Deviations from this optimal range, particularly values below pH 4.5, may shift the metabolic pathway toward the formation of other compounds such as ethanol and butyric acid. Therefore, maintaining precise pH control according to the characteristics of the microbial community and substrate composition is essential to ensure process stability and to direct product formation toward the desired outcome.

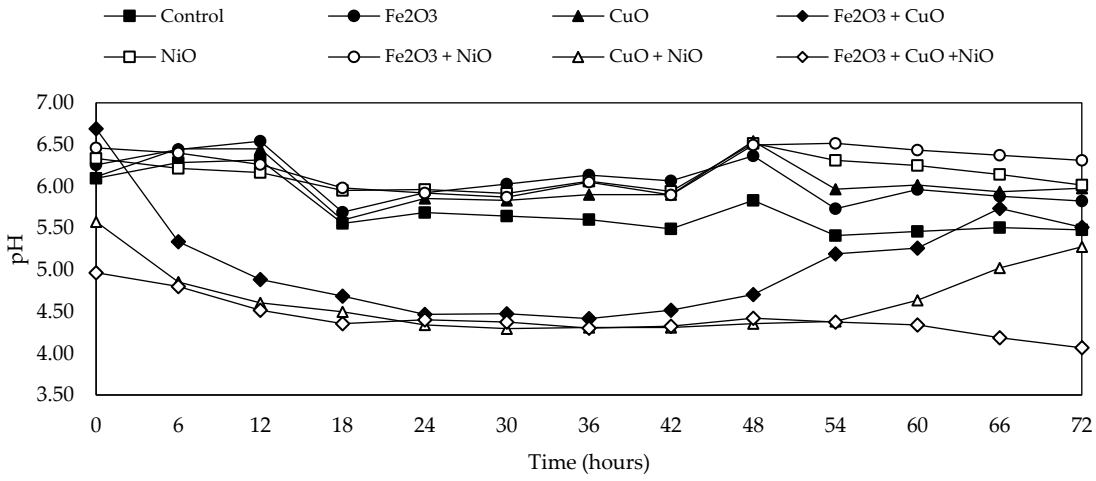

Figure 6. pH profile for each experimental variation

Fluctuations in pH within the reactor indicate the occurrence of various phases of anaerobic fermentation. Although the NiO and Fe_2O_3 + NiO treatments exhibited comparable pH fluctuation patterns within the acetogenic range (pH 6-7), clear differences were observed in the onset of the acetogenesis phase.

The addition of different metal oxides significantly influenced both the timing and duration of the acetogenesis phase (pH 6-7), during which ethanol is converted into acetic acid. For instance, the addition of NiO and the combination of Fe_2O_3 + NiO successfully maintained the acetogenesis phase for extended periods, from the 36th hour and from the start of the experiment, respectively. The addition of NiO alone maintained acetogenesis from the 36th hour onward, indicating that Ni^{2+} mainly contributed to the stabilization and sustained activity of acetogenic microorganisms after the system had passed the acidogenic stage (Khan et al., 2021; Zhang et al., 2023). In contrast, the combined Fe_2O_3 + NiO treatment maintained acetogenesis from the beginning of the experiment, suggesting a synergistic interaction in which Fe^{3+} promoted early redox balance and electron transfer, thereby facilitating the earlier establishment of acetogenic metabolism (Xu et al., 2024). Therefore, despite similar pH trends, the distinct onset times of acetogenesis are

likely attributable to the different functional roles of Fe and Ni in microbial electron transport and enzyme activation.

In contrast, the control reactor (without metal oxides) never reached the appropriate pH range for acetogenesis, while several other metal combinations only achieved it briefly or not at all. In variations that failed to reach ideal conditions, the process remained trapped in a prolonged acidogenesis phase (pH < 5). This condition was caused by the continuous production of volatile fatty acids (VFAs) by acid-tolerant bacteria, which persistently suppressed the pH level.

Due to the excessively acidic environment across all variations, the methanogenesis phase, which requires a higher pH range (6.7-7.4), was not observed within the duration of this study. In anaerobic digestion, methanogenesis generally occurs after the completion of acidogenesis and acetogenesis and is characterized by a slower microbial growth rate (Meegoda et al., 2018). Given the relatively short incubation period of 3 days, it is likely that the system did not reach the metabolic stage at which methanogenic activity could be established. Moreover, pH values below 6.2 are known to inhibit the growth of methanogenic bacteria and substantially reduce the efficiency of organic compound degradation (Revalin, 2024; Syafila, 1997).

Overall, the optimal pH at which ethanol can be converted into acetic acid during acetogenesis depends largely on the type of microorganisms involved in the treatment process. Ethanol conversion occurs most efficiently under near-neutral conditions (Diender et al., 2016). At lower pH levels (below 5.5), some bacteria tend to produce more ethanol, whereas increasing the pH toward neutrality shifts the metabolic pathway toward the production of acetate and other fatty acids (Bertsch & Müller, 2015).

Effect of metal oxide addition on biomass concentration

To determine the biomass concentration in the reactor, Volatile Suspended Solids (VSS) values were used, obtained by converting optical density (OD) measurements from the spectrophotometer. The VSS parameter serves as an indicator of the microbial biomass concentration. Using the 2^n factorial method, the study aimed to identify which metal oxide had the most significant effect on the biomass growth rate represented by VSS. The hypotheses tested under the factorial 2^n design were as follows:

H_0: The addition of metal oxide *X* has no significant effect on biomass concentration.

H_1: The addition of metal oxide *X* has a significant effect on biomass concentration.

For the control condition (without metal oxide addition), the hypotheses were formulated as:

H_0: There is no change in biomass concentration in the system without metal oxide addition.

H_1: There is a change in biomass concentration in the system without metal oxide addition.

After calculating the F-value (F-calculated) for each variation, the results were compared with the F-table at the 5% and 1% significance levels. The calculation results are presented in Table 9 below.

Table 9. Hypothesis testing of biomass concentration changes

Variation	df	SS	MS	F_{count}	F_{table}		Note
					5%	**1%**	
Control	1	2542,72	2542,72	54,0694	5,59	12,25	H_0 rejected
Fe_2O_3	1	184,24	184,24	3,9178	5,59	12,25	H_0 accepted
CuO	1	36,75	36,75	0,7814	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO$	1	6,47	6,47	0,1376	5,59	12,25	H_0 accepted
NiO	1	66,53	66,53	1,4148	5,59	12,25	H_0 accepted
$Fe_2O_3 + NiO$	1	833,73	833,73	17,7288	5,59	12,25	H_0 rejected
CuO + NiO	1	211,24	211,24	4,4919	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO + NiO$	1	27,58	27,58	0,5865	5,59	12,25	H_0 accepted
Error	7	2295,07	327,87				
Total	15	2064,00					

Subsequently, conclusions were drawn based on the results obtained from the factorial statistical analysis. The findings were then interpreted in relation to the initial hypotheses established as follows.

Table 10. Hypothesis testing of biomass concentration changes

Variation	Notes	Kesimpulan
Control	H_0 rejected	There was a change in biomass concentration in the system without the addition of metal oxides.
Fe_2O_3	H_0 accepted	There was no significant effect of Fe_2O_3 addition on the change in biomass concentration.
CuO	H_0 accepted	There was no significant effect of CuO addition on the change in biomass concentration.
$Fe_2O_3 + CuO$	H_0 accepted	There was no significant effect of Fe_2O_3 + CuO addition on the change in biomass concentration.
NiO	H_0 accepted	There was no significant effect of NiO addition on the change in biomass concentration.
$Fe_2O_3 + NiO$	H_0 rejected	There was a significant effect of the addition of Fe_2O_3 + NiO metal oxides on the change in biomass concentration.
CuO + NiO	H_0 accepted	There was no significant effect of CuO + NiO addition on the change in biomass concentration.
$Fe_2O_3 + CuO +$ NiO	H_0 accepted	There was no significant effect of Fe_2O_3 + CuO + NiO addition on the change in biomass concentration.

Based on the experimental results, the addition of metal oxides tended to reduce biomass growth (VSS) compared to the control. The most detrimental effect was observed in the Fe_2O_3 + CuO combination, which was the only variation that showed a statistically significant decrease in biomass concentration. This finding indicates a synergistic toxic effect, where the combined presence of metals exhibited higher toxicity than their individual components. Although other metal oxide variations did not show statistically significant reductions, a general trend of decreased biomass growth was consistently observed across all treatments.

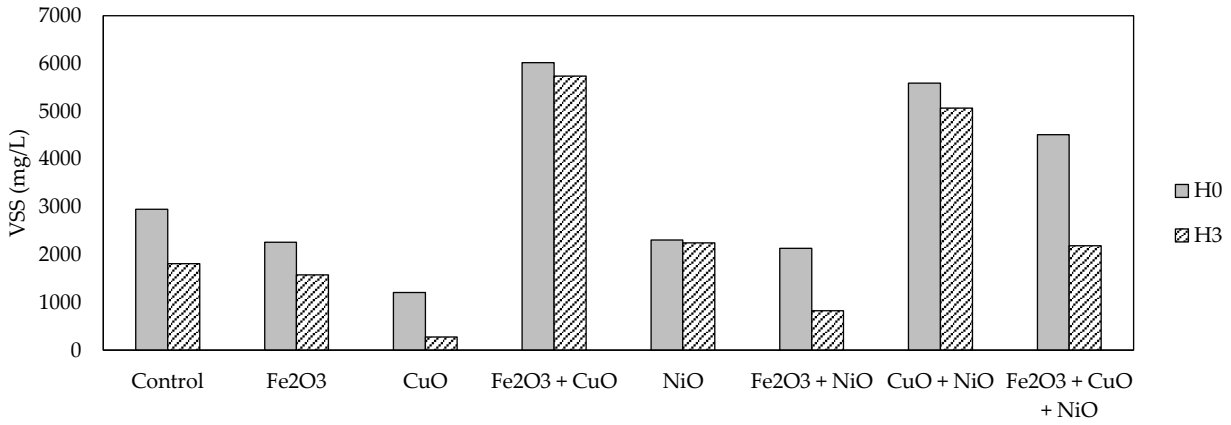

Figure 7. Decrease in biomass (VSS) across each variation

Based on the figure, it can be observed that all variations exhibited a similar trend, namely a decrease in VSS from day 0 to day 3. The control variation, used as a reference, showed a VSS reduction of -1140 mg/L over three days. Two variations experienced a greater decrease than the control: Fe_2O_3 + NiO (-1308 mg/L) and Fe_2O_3 + CuO + NiO (-2330 mg/L) within the same period. Meanwhile, the other variations demonstrated smaller decreases than the control, namely Fe_2O_3 (-687 mg/L), CuO (-933 mg/L), Fe_2O_3 + CuO (-284 mg/L), NiO (-60 mg/L), and CuO + NiO (-521 mg/L) over three days.

The reduction in VSS concentration within the reactors indicates competition among microorganisms and incomplete adaptation to the provided substrate. Ethanol, being a readily degradable substrate, is rapidly converted by acetogenic bacteria into acetate, hydrogen, and CO_2. This rapid acetogenesis process is often not matched by the slower activity of methanogenic bacteria, leading to the accumulation of intermediate

products, particularly VFAs. The buildup of VFAs can result in a drop in pH, which may disrupt or even halt the decomposition process, especially for methanogenic bacteria that are highly sensitive to acidic conditions.

An excessively acidic environment not only inhibits microbial metabolism but can also exert toxic effects, causing cell lysis (rupture of microbial cells). This cell lysis directly leads to a reduction in the number of active biomass cells (Padmono, 2007). However, when these effects are evaluated in relation to the observed pH profiles, cell lysis due to acidic stress cannot fully explain the decrease in volatile suspended solids (VSS) across all variations. Notably, the Fe_2O_3 + NiO treatment exhibited the highest pH values among all variations, yet showed a more pronounced decline in VSS. This indicates that the reduction in biomass in this variation was unlikely to be caused by acid-induced cell lysis. Instead, the decrease in VSS may be associated with enhanced microbial activity, accelerated substrate conversion, or shifts in microbial community structure induced by the presence of Fe and Ni, rather than direct cell damage from low pH conditions (Yao et al., 2020).

Effect of metal oxide addition on organic compound degradation

In this study, organic compounds were represented by COD. The dissolved COD reflects the concentration of biodegradable organic matter, including complex organic compounds such as volatile acids (Tchobanoglous, 1991). In this experiment, dissolved COD represents the amount of substrate available in the reactor, specifically ethanol-containing substrate.

To determine the effect of metal oxide addition on organic compound degradation (expressed as COD), a 2^n factorial analysis was applied. The hypotheses for this factorial analysis were as follows:

H_0: The addition of metal oxide *X* has no significant effect on the degradation of organic compounds.

H_1: The addition of metal oxide *X* has a significant effect on the degradation of organic compounds.

For the control condition (without metal oxide addition), the hypotheses were formulated as:

H_0: There is no degradation of organic compounds in the system without metal oxide addition.

H_1: There is degradation of organic compounds in the system without metal oxide addition.

After obtaining the F-calculated values for each variation, the results were compared with the F-table at the 5% and 1% significance levels. The calculation results are presented in Table 11 below.

Subsequently, conclusions were drawn based on the information obtained from the factorial statistical analysis, as described in relation to the initial hypotheses.

Table 11. Hypothesis testing of organic compound degradation

Variation	df	SS	MS	F_{count}	F_{table}		Note
					5%	1%	
Control	1	268,69	268,69	5,7135	5,59	12,25	H_0 rejected (5%), H_0 accepted (1%)
Fe_2O_3	1	17,24	17,24	0,3666	5,59	12,25	H_0 accepted
CuO	1	5,70	5,70	0,1212	5,59	12,25	H_0 accepted
Fe_2O_3 + CuO	1	2,31	2,31	0,0491	5,59	12,25	H_0 accepted
NiO	1	5,70	5,70	0,1212	5,59	12,25	H_0 accepted
Fe_2O_3 + NiO	1	0,11	0,11	0,0024	5,59	12,25	H_0 accepted
CuO + NiO	1	0,13	0,13	0,0028	5,59	12,25	H_0 accepted
Fe_2O_3 + CuO + NiO	1	12,56	12,56	0,2672	5,59	12,25	H_0 accepted
Error	7	288,72	41,25				H_0 accepted
Total	15	309,25					H_0 accepted

Table 12. Conclusion of hypothesis testing on organic compound degradation

Variation	Note	Conclusion
Control	H_0 rejected	There was a change in biomass concentration in the system without the addition of metal oxides.
Control	H_0 rejected (5%)	There was degradation of organic compounds in the system without the addition of metal oxides at a 95% confidence level, although the effect was not strong enough to meet the stricter 99% significance level.
CuO	H_0 accepted (1%)	There was no significant effect of CuO addition on the change in biomass concentration.
Fe_2O_3	H_0 accepted	There was no significant effect of Fe_2O_3 addition on the change in biomass concentration.
CuO	H_0 accepted	There was no significant effect of CuO addition on the change in biomass concentration.
Fe_2O_3 + CuO	H_0 accepted	There was no significant effect of Fe_2O_3 + CuO addition on the change in biomass concentration.
NiO	H_0 accepted	There was no significant effect of NiO addition on the change in biomass concentration.
Fe_2O_3 + NiO	H_0 accepted	There was no significant effect of Fe_2O_3 + NiO addition on the change in biomass concentration.

In the control variation without metal oxide addition, degradation of organic compounds was observed at the 95% confidence level, although the effect was not strong enough to meet the stricter 99% significance level. The zero-condition experiment can thus serve as a reference point for evaluating the influence of metal oxides at specific concentrations.

Furthermore, the results indicated that there was no significant effect from any of the metal oxide additions, either individually or in combination, on organic compound degradation. However, the absence of a statistically significant effect does not imply the absence of measurable degradation of organic compounds, as represented by COD reduction. As shown in Figure 8 below, each variation still exhibited a measurable degree of organic compound degradation, although the magnitude of the effect was not substantial enough to be considered statistically significant.

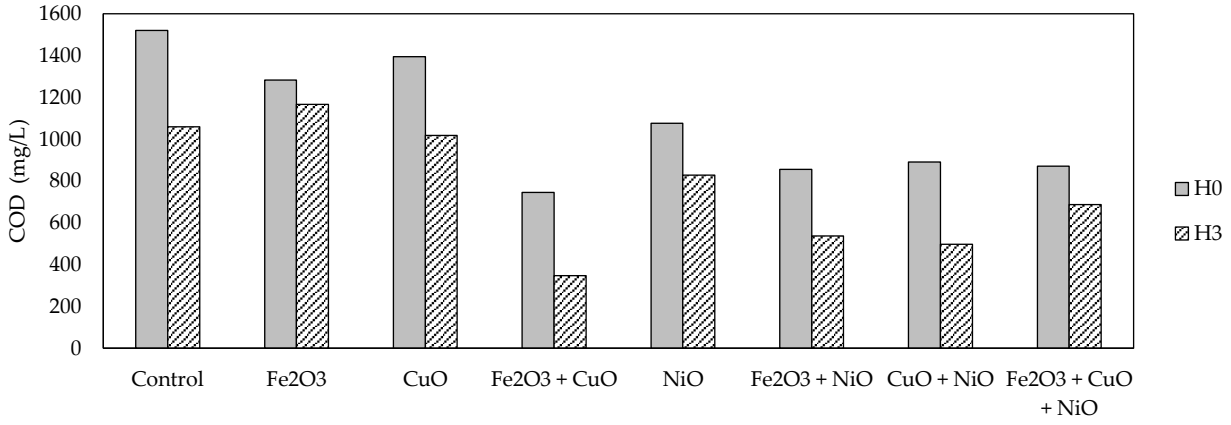

Figure 8. COD observation results for each variation

All experimental variations exhibited a similar trend, namely a decrease in dissolved COD concentration over the three-day observation period. As a reference, the control variation showed a reduction of -460 mg/L. The performance of variations with metal oxide addition, either individually or in combination, varied considerably. Some combinations, such as Fe_2O_3 + NiO (-398 mg/L) and CuO + NiO (-394 mg/L), demonstrated

relatively high COD reductions. Conversely, other variations, including CuO (-117 mg/L) and the three-metal combination (-183 mg/L), showed lower reductions compared to the control.

The observed COD decrease indicates that microorganisms were able to utilize POME as a substrate for metabolic activity. However, the overall reduction in dissolved COD remained relatively low. This can be explained by the fact that COD measures the total amount of oxygen required to chemically oxidize all organic matter present in a sample. The anaerobic process, by contrast, involves a sequence of biochemical conversions of organic compounds from one form to another.

Intermediate products generated during acidogenesis and acetogenesis, particularly volatile fatty acids (VFAs), are still measured as COD. According to Franke-Whittle (2014), VFA accumulation is a key indicator of reactor instability and directly contributes to low COD removal efficiency. When the final step—methanogenesis—is disrupted, intermediate products (VFAs) accumulate. Since VFAs contribute to COD, the overall COD removal efficiency remains low until methanogenesis is re-established.

Effect of metal oxide addition on ethanol

This part of the study aimed to determine the extent to which ethanol was converted into acetate during the acetogenesis phase. Therefore, a substrate containing ethanol was used to measure the conversion efficiency. The influence of metal oxide addition on this process was analyzed using a 2^n factorial analysis. The hypotheses for this factorial method were as follows:

H_0: The addition of metal oxide *X* has no significant effect on ethanol concentration change.

H_1: The addition of metal oxide *X* has a significant effect on ethanol concentration change.

For the control condition (without metal oxide addition), the hypotheses were formulated as:

H_0: There is no change in ethanol concentration in the system without metal oxide addition.

H_1: There is a change in ethanol concentration in the system without metal oxide addition.

After obtaining the F-calculated values for each variation, the results were compared with the F-table at 5% and 1% significance levels. The results of the analysis are presented in Table 13 below.

Table 13. Hypothesis testing of ethanol concentration changes

Variation	df	SS	MS	F_{count}	F_{table}		Notes
					5%	1%	
Control	1	4227,60	4227,60	0,7081	5,59	12,25	H_0 accepted
Fe_2O_3	1	13788,80	13788,80	2,3094	5,59	12,25	H_0 accepted
CuO	1	9145,44	9145,44	1,5317	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO$	1	86,21	86,21	0,0144	5,59	12,25	H_0 accepted
NiO	1	737,80	737,80	0,1236	5,59	12,25	H_0 accepted
$Fe_2O_3 + NiO$	1	12206,40	12206,40	2,0444	5,59	12,25	H_0 accepted
CuO + NiO	1	287,01	287,01	0,0481	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO + NiO$	1	369,26	369,26	0,0618	5,59	12,25	H_0 accepted
Error	7	41795,01	5970,72				H_0 accepted
Total	15	85249,38					H_0 accepted

Subsequently, conclusions were drawn based on the results obtained from the factorial statistical analysis. The findings were then interpreted in relation to the initial hypotheses established as follows.

Table 14. Conclusion of hypothesis testing on ethanol concentration changes

Variation	Notes	Conclusion
Control	H_0 accepted	There was no change in ethanol concentration in the system without the addition of metal oxides

Variation	Notes	Conclusion
Fe_2O_3	H_0 accepted	There was no significant effect of Fe_2O_3 addition on the change in ethanol concentration
CuO	H_0 accepted	There was no significant effect of CuO addition on the change in ethanol concentration
$Fe_2O_3 + CuO$	H_0 accepted	There was no significant effect of Fe_2O_3 + CuO addition on the change in ethanol concentration
NiO	H_0 accepted	There was no significant effect of NiO addition on the change in ethanol concentration
$Fe_2O_3 + NiO$	H_0 accepted	There was a significant effect of the addition of Fe_2O_3 + NiO metal oxides on the change in ethanol concentration
CuO + NiO	H_0 accepted	There was no significant effect of CuO + NiO addition on the change in ethanol concentration
$Fe_2O_3 + CuO +$ NiO	H_0 accepted	There was no significant effect of Fe_2O_3 + CuO + NiO addition on the change in ethanol concentration

A lack of significant effect on ethanol concentration does not imply that no change in ethanol concentration occurred at all. As shown in Figure 9 below, each variation still exhibited measurable changes in ethanol concentration; however, the magnitude of these changes was not substantial enough to be considered statistically significant.

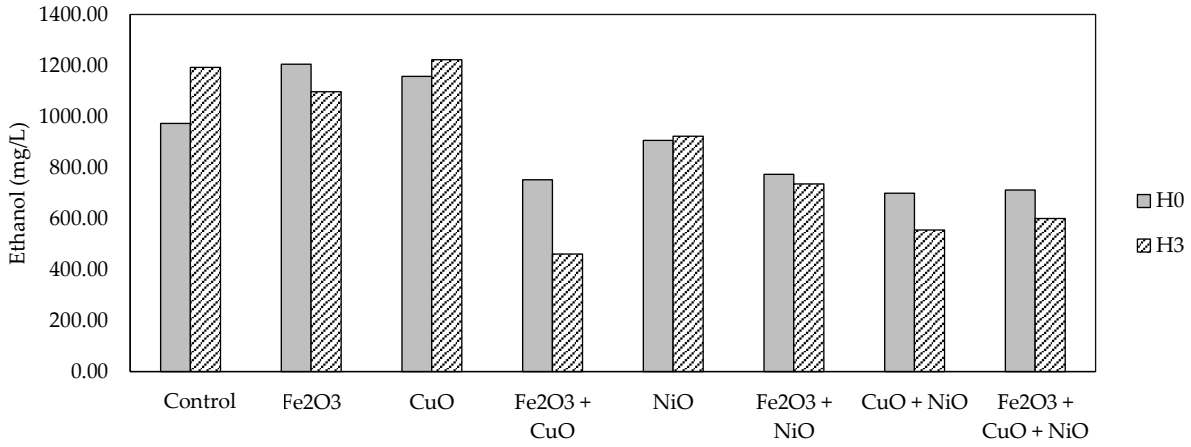

Figure 9. Observation results of ethanol concentration in each variation

Changes in ethanol concentration showed different trends among experimental variations. In several reactors, such as those containing Fe_2O_3 and all mixed-metal variations, a decrease in ethanol concentration was observed. This decrease indicates that the process proceeded as expected, having passed the acidogenesis phase and entered the acetogenesis phase, during which alcohols such as ethanol are consumed and converted into other products, such as acetic acid.

Conversely, in the control reactor, CuO, and NiO variations, an increase in ethanol concentration occurred. This increase indicates that the acidogenesis process, which produces ethanol, was still ongoing. The carbon source for this new ethanol production likely originated from dead biomass that underwent lysis (cell rupture). The released cellular components, such as complex carbohydrates, were then fermented by other microorganisms into more ethanol. The products of biomass cell lysis can indeed serve as substrates for subsequent biological processes (Gosset & Pavlostathis, 1985).

In the NiO-added variation, although the pH condition was within the acetogenesis range (where ethanol should have decreased), its concentration instead increased. This is likely due to selective inhibition, where NiO inhibits acetogenic bacteria (ethanol consumers) more strongly than acidogenic bacteria (ethanol producers). As a result, the rate of ethanol production exceeded its consumption rate, a concept supported by

the fact that acetogenic bacteria are more sensitive to heavy metal inhibition compared to acidogenic bacteria (Kida et al., 2001).

Effect of metal oxide addition on acetic acid formation

In this experiment, a 2^n factorial analysis was used to determine the effect of metal oxide addition on acetate formation. The hypotheses for the factorial analysis in this study are as follows:

H_0 = There is no effect of adding metal oxide X on acetic acid formation
H_1 = There is an effect of adding metal oxide X on acetic acid formation

Meanwhile, the control condition (without metal addition) used as a reference has the following hypotheses:

H_0= There is no acetic acid formation in the system without metal oxide addition
H_1 = There is acetic acid formation in the system without metal oxide addition

After obtaining the calculated F-value for each variation, the results were compared with the F-table values for 5% and 1% significance levels. The calculation results are presented in Table 15.

Table 15. Hypothesis testing of acetic acid concentration changes

Variation	df	SS	MS	F_{count}	F_{table}		Keterangan
					5%	1%	
Control	1	4227,60	4227,60	0,7081	5,59	12,25	H_0 rejected
Fe_2O_3	1	13788,80	13788,80	2,3094	5,59	12,25	H_0 accepted
CuO	1	9145,44	9145,44	1,5317	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO$	1	86,21	86,21	0,0144	5,59	12,25	H_0 accepted
NiO	1	737,80	737,80	0,1236	5,59	12,25	H_0 accepted
$Fe_2O_3 + NiO$	1	12206,40	12206,40	2,0444	5,59	12,25	H_0 accepted
CuO + NiO	1	287,01	287,01	0,0481	5,59	12,25	H_0 accepted
$Fe_2O_3 + CuO + NiO$	1	369,26	369,26	0,0618	5,59	12,25	H_0 accepted
Error	7	41795,01	5970,72				
Total	15	85249,38					

Subsequently, conclusions were drawn based on the results of the factorial statistical experiment. These results provide explanations regarding the initial hypotheses that had been established as follows.

Table 16. Hypothesis testing of acetic acid concentration changes

Variation	Notes	Conclusion
Kontrol	H_0 rejected	There is no acetate formation in the system without the addition of metal oxides
Fe_2O_3	H_0 accepted	There is no effect of Fe_2O_3 metal oxide addition on acetate formation
CuO	H_0 accepted	There is no effect of CuO metal oxide addition on acetate formation
$Fe_2O_3 + CuO$	H_0 accepted	There is no effect of Fe_2O_3 + CuO metal oxide addition on acetate formation
NiO	H_0 accepted	There is no effect of NiO metal oxide addition on acetate formation
$Fe_2O_3 + NiO$	H_0 accepted	There is no effect of Fe_2O_3 + NiO metal oxide addition on acetate formation
CuO + NiO	H_0 accepted	There is no effect of CuO + NiO metal oxide addition on acetate formation
$Fe_2O_3 + CuO + NiO$	H_0 accepted	There is no effect of Fe_2O_3 + CuO + NiO metal oxide addition on acetate formation

In this study, the aim was to determine the concentration of acetic acid that could be formed from ethanol substrate during the acetogenesis phase in the reactor operation process. Based on the screening test conducted, all variations of metal oxide addition showed no significant effect on acetate formation. However, this does not mean that there was no change in acetate concentration at all. As shown in Figure 10 below, each variation still exhibited changes in acetate concentration, although the effect was not substantial enough to be considered statistically significant.

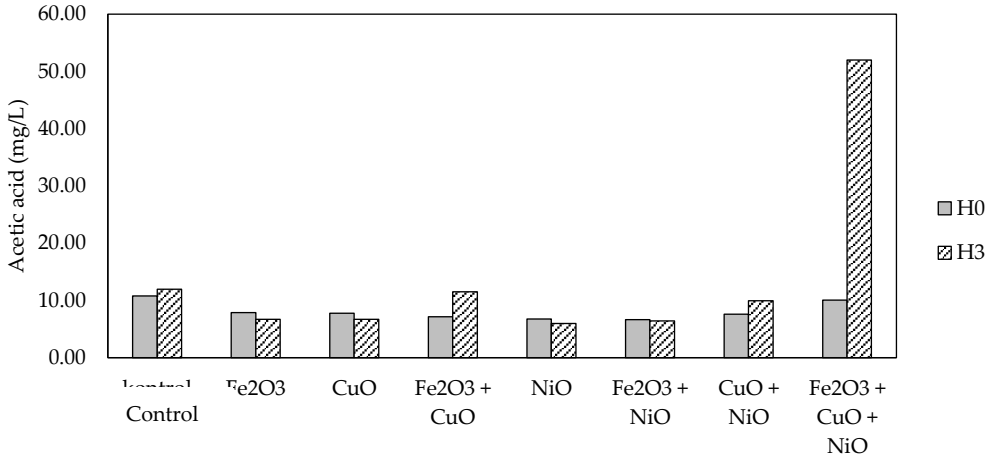

Figure 10. Graph of acetic acid changes in each variation

Acetate formation exhibited varying trends across different experimental variations. In the control reactor, Fe_2O_3, Fe_2O_3+NiO, and the three-metal mixture, there was an increase in acetate concentration. This aligns with the observed decrease in ethanol concentration in the same variations, indicating that the anaerobic process successfully entered the acetogenesis stage, where ethanol was converted into acetic acid.

Conversely, several other variations showed a decrease in acetate concentration. In the Fe_2O_3+CuO mixture, although ethanol decreased, acetate did not accumulate. This was likely due to Fe_2O_3 enhancing electron transfer, causing intermediate products to be consumed rapidly without accumulation (Baek et al., 2018). Meanwhile, in the CuO and NiO variations, both ethanol and acetate did not show a measurable increase, suggesting that although the system may have entered the acetogenic pH range, the acetogenesis process was not kinetically effective in producing or accumulating acetate (Muñoz-Duarte et al., 2025). This observation does not contradict the pH analysis, which indicated that NiO was able to maintain conditions favorable for acetogenesis. Rather, it suggests that under NiO treatment, acetogenic activity may have been limited or balanced by concurrent acetate consumption, low conversion rates, or microbial adaptation constraints (Aghtaei et al., 2022; Pilarski et al., 2025). Consequently, the process in the NiO variation appears to have been partially inhibited at the metabolic level, despite the maintenance of acetogenic pH conditions.

The failure observed in the CuO variation may be associated with the potential inhibitory effects of copper oxide on microbial activity, as Cu-based compounds are known to damage cell membranes and interfere with essential enzymatic functions in acetogenic bacteria (Chen et al., 2011). Although the exact toxic threshold concentration of CuO was not explicitly determined in this study, previous reports have shown that even relatively low concentrations of soluble copper species can exert inhibitory effects on anaerobic microorganisms (Chen et al., 2008). Therefore, the observed process inhibition in the CuO variation suggests that the applied CuO concentration may have approached or exceeded the tolerance limits of acetogenic bacteria under the experimental conditions, rather than indicating definitive acute toxicity. These results differ from the findings of Rabbani (2024), who reported increased acetate production in more metal variations after 24 hours. In contrast, the present study showed that after 72 hours, only certain metal combinations exhibited positive results compared to the control.

The discrepancy with previous studies may be explained by two main factors. First, the difference in substrate sources, this study used only ethanol as the acetate precursor, meaning that if the conversion pathway was inhibited, no alternative carbon source was available. Second, the longer operational time (72 hours vs. 24

hours) allowed for more complex processes to occur, such as acetate re-consumption by other bacteria or inhibition caused by product accumulation over time.

4. Conclusion

Based on the analysis of the effects of metal oxides Fe_2O_3, NiO, and CuO on the conversion of ethanol into acetate, it can be concluded that statistically, there was no significant effect from the addition of these metal oxides. However, descriptively, the mixed variation of Fe_2O_3+CuO+NiO exhibited the highest acetic acid production compared to the control reactor after 72 hours. The analysis of environmental parameters provides context for these findings, showing that pH fluctuations, which represent anaerobic fermentation phases—were often hindered by the accumulation of volatile fatty acids (VFA). Furthermore, a general decrease in biomass (VSS) was observed, indicating cell lysis. Although dissolved COD degradation occurred in all reactors, demonstrating ethanol utilization by microorganisms, the overall degradation efficiency remained relatively low.

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