Production of Biogas from Artificial Substrates (oil, protein, and cellulose) by Indigenous Anaerob Bacteria

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Abstract. Indigenous bacteria was isolated from POME, and the bacteria was applied to degrade oil, protein, and cellulose in an anaerobic condition and to produce biogas from artificial substrates, namely: oil, protein, and cellulose. Degradation process of the substrate was conducted in a bioreactor with degradation time of 3, 6, 9, 12 and 15 day. Stenotrophomonas rhizophila strain e-p10 as lipolytic bacteria dan Bacillus toyonensis strain BCT-711 as proteolytic and celulolytic bacteria were used in the degradation process. Specific growth rate, µ = 4.57 day⁻¹ was obtained for lipolytic bacteria; 4.73 day⁻¹ for proteolytic and 4.42 day⁻¹ for cellulolytic bacteria. The optimum time for degradation of the substrate was 12 days. The production of methane achieves 47.04 % for lipolytic bacteria, 19.67% for proteolytic and 51.40% for celulolytic bacteria.

1. Introduction
CPO waste or commonly known as Palm Oil Mill Effluent (POME) is categorized as liquid waste. POME has the content of COD, BOD, protein, and lipids and total liquid waste is 56 to 67% of each processing of palm fruit into palm oil [1]. POME of palm oil mill contains BOD 1018,57 mg / l, COD: 5928,40 mg / l, Total Solids (TS): 428 mg / l, acidity degree (pH) 6.1, oil and fat 1869 mg / l and NH₃-N 100,9 mg/l. Large BOD and COD content show high organic and inorganic substances in POME. If POME is not processed prior to discharge into the environment, it causes environmental pollution of water bodies. Some will settle, break down slowly, consume dissolved oxygen, cause turbidity, emit a sharp odour and can damage aquatic ecosystems [2] and no less its role in environmental damage is the presence of methane gas (CH₄) and carbon dioxide (CO₂) degradation process potentially damaging the ozone layer.

In every palm oil plant, the wastewater treatment has been generated by applying the lagoon system (pond), but the lagoon system operation has not been optimized until the outlet has not met the liquid waste quality standard. Less optimal POME processing systems such as processing systems that require wider land, time consuming and release of methane directly into the atmosphere from the decomposition of organic substances that occur in anaerobic ponds, required a reliable processing system, especially the using of the true type of bacteria able to degrade liquid waste component well. POME contains many carbon sources such as hemicellulose, proteins, organic acids and oil residues.

The existence of these components to managed and required bacteria capable of degrading...
cellulose, protein, and oil. Indigenous bacteria has the capability to degrade fats and oils (lipolytic bacteria), cellulose (cellulolytic bacteria) and proteins (proteolytic bacteria).

A lot of POME research has been done, the quality of biogas from Empty Fruit Bunches (EFB), Palm Press Fiber (PPF) and Decanter Cake (DC) by using an anaerobic boiler to produce 53-65% methane in biogas at F / I 2: 1 [3]. The POME processing is done by process of using ozone, the comparative kinetic parameter of hydrogen production from raw POME and given treatment of ozone. POME (102.78 mg COD mg-1 ozone) in concentrations ranging from 5,000-37,000 mg L-1, POME ozonation concentrations of 30,000 mg L-1 gave maximum hydrogen production. Potential 349.8 mL and maximum hydrogen yield of 182.3 mL g-1 COD [4]. POME treated with partial ozonation increases hydrogen production by up to 60% higher than raw POME [5]. Pre treatment of ozonation from POME develops biome-degradability of constituent POME and kinetics of biomethane production. Biomethane yield increased by 54% when POME was treated with partial ozonation [6].

The integration of microalgae biogas from POME will bring additional benefits to the palm oil industry, local communities, and the environment. Thus, sustainable production in the palm oil industry can be established [7]. Methane extracted from POME should be a liability of the biodiesel and palm oil industries [8]. The UASB was used for processing POME to produce biogas from 0.3 to 3.3 l daily [9]. Methane productivity was 256 mL/g VS resulted in shear-loop anaerobic contact stabilization (SLACS) system [10].

POME processing can be done under anaerobic conditions. POME fermentation with the help of anaerobic indigenous bacteria will produce products such as CH\(_4\) (Biogas), CO\(_2\), H\(_2\)S and H\(_2\)O. Treatment of POME in anaerobic conditions can be performed well if it involves microorganisms already adaptable to the environment in which the waste is located [11], since the environment is a habitat for the bacteria so that indigenous bacterial isolation (lipolytic, cellulolytic and proteolytic) is needed. The isolated indigenous bacteria required tests related to their level of ability to degrade the liquid waste. Required values of indigenous bacterial kinetics parameters (lipolytic, cellulolytic and proteolytic) which include the specific rate of growth (\(\mu\)) [13].

Research aimed at isolation of bacteria from various industrial wastes as bacterial agents for waste treatment process continues to be developed by looking at the level of kinetic parameters that occur. The bacteria isolated from the lactic acid-producing proteolytic colony resulted in kinetic parameters of \(\mu_m = 1.104\) day\(^{-1}\), \(K_s = 4.324\) g / l, \(k_d = 0.031\) hours\(^{-1}\), \(Y_g = 0.182\) mg MLVSS / mg COD [13]. Isolation of bacilli bacteria in glucose medium tested kinetic parameter yield value \(\mu_m = 0.148\) days\(^{-1}\), \(K_s = 0.413\) g / l, \(k_d = 0.442\) hour\(^{-1}\), the highest \(Y = 0.8665\) mg MLVSS / mg COD [13]. Bacterial isolation and kinetic parameter testing with \(\mu_m = 0.372\) days\(^{-1}\), \(K_s = 3,362.1\) g / l, \(k_d = 0.132\) hours\(^{-1}\), the highest \(Y = 0.9114\) mg MLVSS / mg COD [14] and also produced \(\mu_m = 0.102\) days\(^{-1}\), \(K_s = 0.555\) g / l, \(k_d = 1.37\) x 10-16 hours\(^{-1}\), the highest \(Y = 0.4583\) mg MLVSS / mg COD [15].

From previous studies, the isolation of indigenous bacteria was carried out only partially to the availability of the bacterial species present in the waste. Comprehensive efforts need to be made for the development of POME processing by conducting scientific research on the ability of indigenous bacteria. So it needs to be done indigenous bacteria isolation process (lipolytic, cellulolytic and proteolytic) which function for the degradation of oil, protein, and cellulose. The isolated indigenous bacteria need to be tested by the kinetics parameter by giving the substrate according to the type of indigenous bacteria as an important factor in measuring the level of bacteria reliability in conducting the degradation process of liquid waste (POME) with fermentation reactor system.

The objective of this study was to determine the parameters of the degradation kinetics of the liquid waste component (POME) which includes the average growth rate for lipolytic, proteolytic and cellulolytic bacteria, \(\mu\) (day\(^{-1}\)), degradation rate (ppm/day) and product formation rate % mole) over the growth span within the bioreactor.

If the value of the kinetic parameter of bacterial growth at the recommended number, then the cell doubling speed is constant and maximum. Every cell in the population splits into two cells at the same rate. The variation in the degree of bacterial growth in this exponential phase is strongly influenced by nutrient levels in waste media or subsilation concentration (VS), suitability of microorganism type,
hydrodynamic system (stirring system) process in the reactor, incubation temperature, and pH conditions.

Types of indigenous bacteria produced in this study as one of the factors that determine the success of POME waste component overhaul in the future. Processing of palm oil mill effluent can be done under anaerobic and aerobic conditions. With these two conditions in addition to processing waste so as to meet the quality standards of waste to be discharged into the environment, can also produce methane gas as biogas and organic fertilizer. The process begins with the separation of suspended mud or solids, and then liquid waste is pumped into an anaerobic reactor for the reshuffling of organic matter into biogas.

2. Materials and methods

2.1 Preparation of mineral medium (MM), the substrate of oil, protein, and cellulose

The chemicals used for the preparation of minerals were 450 ml with the composition MgSO$_4$·7H$_2$O 0.09 g, CaCl$_2$ 0.009 g, CaCl$_2$ 0.009 g, KH$_2$PO$_4$ 0.45 g, K$_2$HPO$_4$ 0.45 g, NH$_4$NO$_3$ 0.45 g, FeCl$_3$ 1 drop, sterile aquadest 450 ml, pH 7–7.2; beef and yeast. Preparation of oil substrate was conducted by preparing 4,500 ml of MM, and it was added to 90 ml of vegetable oil. Protein substrate was prepared by adding 4,500 ml of MM with skim milk of 90 g. Cellulose substrate was prepared by adding 4,500 ml of MM with CMC (Carboxymethyl cellulose) of 90 g.

2.2 Preparation of lipolytic, proteolytic, and cellulolytic bacteria inoculum

The lipolytic bacterial inoculum was prepared by adding 450 ml of MM with 9 ml vegetable oil and Stenotrophomonas Rhizophila strain e-p10 of suspension of 1 ose was added to the mixture. The number of cells was observed using haemocytometer and Stenotrophomonas rhizophila strain e-p10 was added continuously until the bacterial population reached 10$^6$. Preparation of the proteolytic bacteria inoculum was held by adding 450 ml of MM with skim milk of 9 g and Bacillus toyonensis strain BCT-7112 suspension of 1 ose. A number of cells were observed using haemocytometer and Bacillus toyonensis strain BCT-7112 until the bacterial population reached 10$^6$. The cellulolytic bacteria inoculum was prepared by adding 450 ml MM with CMC (carboxymethyl cellulose) of 9 g and Bacillus toyonensis strain BCT-7112 suspension of 1ose then the cell number was observed using haemocytometer. The Bacillus toyonensis strain BCT-7112 was added until the bacterial population achieved 10$^6$.

2.3 Substrate degradation process

Substrates of vegetable oil and mineral medium of 4.5 litres were added with an inoculum of lipolytic bacteria of 450 ml, and the mixture was fed to bioreactor 1. Substrates of skim milk and mineral medium of 4.5 litres were added with an inoculum of proteolytic bacteria of 450 ml and the mixture was fed to bioreactor 2. Substrates of CMC and mineral medium of 4.5 litres were added with an inoculum of cellulolytic bacteria of 450 ml, and the mixture was fed to bioreactor 3. The degradation process was conducted for 15 days for each bioreactor. Measurement of a number of bacterial cells, pH, the concentration of oil, protein, cellulose, and biogas was conducted every 3 days. A sample of 5 ml was taken from each bioreactor for measuring pH, bacterial cell and concentration of substrate. Biogas was stored in the spuit, and the biogas concentration was analysed using Gas Chromatography (GC) Perkynelmer with ASTM D 1945 and ASTM 3588 test method. Bacterial populations were calculated using haemocytometers in units of cells per ml. Then, it was observed under a microscope, and the number of bacteria was counted in the small cubicle. Analysis of oil and fat in reactor sample was done by SNI 6989 20: 2009 method, protein analysis was done by Titrimetric test method, and cellulose analysis was done by VIS spectrophotometer test method.
3. Result and discussion

3.1. The relation of degradation time in bacteria population growth (X)
The bacterial growth curve was analyzed to determine whether the bacteria would continue to grow as the time of the process continued to increase or decrease as well as whether the bacterial population had an optimum point at a given process time as time went on. The generation time of bacterial growth is the time required by the cell to divide, varying depending on the species and growth conditions, as well as the type of substrate corresponding to the type of degrading bacteria. Bacterial growth is the process of increasing the size or substance or the mass of an organism, for example, we macro creatures are said to grow as it grows taller, bigger or heavier. In single-celled organisms, growth is more defined as the growth of colonies, namely the increase in the number of colonies, the size of the larger colonies or the substance or mass. Bacteria in the colony more and more, growth in Bacteria is defined as the increase in the number of bacterial cells themselves. Relation time degradation with the growth of bacterial population (X) can be seen in Table 1 below:

| t (day) | $X_L$ (cell/ml) | $X_P$ (cell/ml) | $X_S$ (cell/ml) |
|--------|-----------------|-----------------|-----------------|
| 0      | 1.00E+06        | 1.00E+06        | 1.00E+06        |
| 3      | 2.92E+06        | 4.02E+06        | 2.34E+07        |
| 6      | 4.06E+06        | 5.00E+06        | 2.61E+06        |
| 9      | 4.81E+06        | 6.20E+06        | 3.50E+06        |
| 12     | 6.03E+06        | 7.84E+06        | 3.88E+06        |
| 15     | 6.03E+06        | 8.94E+06        | 4.41E+06        |

As shown in Table 1, it can be seen that as time goes by the process, the increasing bacterial population as long as the oil, protein and cellulose contents are still present in the bioreactor. Bacterial population increased linearly on the 3rd day to 12th day, this phase is called exponential phase. Where bacteria divide by constant velocity. On the 12th day until the 15th day is called the declining phase, where the bacterial population begins to look slowing down.

Population growth of cellulolytic bacteria ($X_S$) to exponential phase only reached $3.88 \times 10^6$ mg/ml compared with growth of lipolytic bacteria population ($X_L$) $6.03 \times 10^6$ mg/ml and growth of the population of proteolytic bacteria ($X_P$) equal to $7.84 \times 10^6$ mg/ml. This is due to the relation between the amount of substrate required by 1 cell of bacterial biomass to develop is not the same compared to subsect type although biochemically the rate of substrate resuffle is also highly dependent on the ease of degradation of the substrate in terms of the strength of carbon atoms, hydrogen, phosphorus and others on various types of substrate.

3.2. The relation of degradation time and substrate concentration
The substrate used to view the performance of cellulotic, lipolytic and proteolytic products resulting from the isolation of palm oil wastes is cellulose substrate, fat and oil substrate and protein substrate. The rate of substrate degradation rate by cellulolytic, lipolytic and proteolytic bacteria can be seen from the relation time degradation to the amount of substrate remaining in the fermentation reactor during the time interval of the process.

Relation time of degradation in substrate amount showed in table 2 below:
Table 2. The relation time degradation of substrate concentration.

| t  | $S_L$ | $S_P$ | $S_S$ |
|----|------|------|------|
| 0  | 99.00| 99.00| 99.00|
| 3  | 125.00| 25.60| 36.60|
| 6  | 71.00| 21.10| 26.40|
| 9  | 70.00| 21.20| 14.30|
| 12 | 102.00| 22.20| 49.80|
| 15 | 38.00| 22.60| 18.20|

As shown in Table 2, changes in the number of subscripts in the analysis begin on the third day to 15th day. This is because the amount of substrate at the beginning of the process or zero days and after the third day of change is quite large. Thus, to understand the behavior of the growth relationship with the change in the number of substances performed on a 3rd day to 15th day. Of the three bacteria involved showed a decrease in the amount of substrate that more prevalent on the substrate proteolytic that substrate decreased as much as 76.40 mg/ml and reduced 80.80 mg/ml of cellulose substrate versus a reduced fat substrate of only 61.00 mg/ml by the 15th day. This is because the numbers obtained for fatty substrates are well above the substrate digits of protein and cellulose.

The protein substrate concentration ($S_P$) tends to fall at a constant rate after 3rd day to 15th day with the number of the substrate at optimum day (day 15) of 22.60 mg/ml. As for fat substrate concentration ($S_L$) and cellulose concentration ($S_S$) occurs a different phenomenon compared with changes in protein substrate mass, where the mass changes in fatty substrates and cellulose increase again on 12th day. Increase in the number of the substrate on the 12th day for fatty substrates and cellulose due to the reversible reaction of the few components of the degradation product to form the substrate molecule as a result of environmental factors in the fermentation system that can be caused by some pathogenic bacteria that start plants by the 12th day.

3.3. The relation of degradation time to the production of CH$_4$

The time-relation graph of production of methane gas production can be seen in Figure 1. below:

![Figure 1. Production of CH$_4$ along degradation process.](image-url)
bacteria in producing methane gas. While the flow charts for proteolytic bacteria more gentle than the groove graph of cellulolytic and lipolytic bacteria. This does not mean that proteolytic bacteria have a slower rate of protein substrate degradation to produce methane gas. However, the formation of a unified methane gas amount of time depends greatly on the number of moles of methane gas which can form for every 1 mole substrate degraded.

From Figure 1 the optimum time occurring for producing biomass from the three bacteria is the same ie at the time of 12 days process of producing methane gas 51.40 mole % by cellulolytic bacteria (a1), 47.04% mole by lipolytic bacteria (a2), and 19.67% mole by proteolytic bacteria (a3). This process takes place optimally under a 12th day for the research process that has been done. Methanogenesis is an important step in anaerobic processing as a whole since it is the slowest process of biochemical reactions.

3.4. The parameter of kinetics in the growth of lipolytic, proteolytic and cellulolytic bacteria

The isolated indigenous bacteria required tests related to their level of ability to degrade the liquid waste. Required values of indigenous bacteria kinetics parameters (lipolytic, cellulolytic and proteolytic) are specific growth rate values ($\mu$) [11]. In the balanced growth, the specific growth rate will be either determined by cell mass or cell number. The nutrient concentration in this phase is large, so the growth rate is not affected by nutrient concentration. The growth rate in the exponential phase follows the first-order differential equation [15]. Exponential growth occurs when all the needs for growth are met [14]. The relationship of degradation time and the specific growth rate is shown in Table 3. Below:

| $t$ (day) | $\mu_L$ (day$^{-1}$) | $\mu_P$ (day$^{-1}$) | $\mu_S$ (day$^{-1}$) |
|----------|---------------------|---------------------|---------------------|
| 0        | 0.00                | 0.00                | 0.00                |
| 3        | 4.82                | 4.97                | 4.70                |
| 6        | 4.65                | 4.60                | 4.16                |
| 9        | 4.67                | 4.67                | 4.57                |
| 12       | 4.67                | 4.77                | 4.28                |
| 15       | 4.17                | 4.64                | 4.40                |
| Average $\mu$ | 4.57                | 4.73                | 4.42                |

The specific growth rate ($\mu$) shows the same trend for cellulolytic, lipolytic and proteolytic bacteria, but the growth rate for proteolytic bacteria is above the other two types of bacteria. This indicates the growth of proteolytic bacteria has a better growth rate. The three growth rates of the visible bacteria tend to be slightly flat and slightly decreased, this is because the exponential phase of bacterial growth occurs under the 3rd day of processing time. From the graph, groove the specific growth rate of the bacteria has decreased up ahead of the degradation time. This is due to the slightly changed changing conditions at any time such as the temperature and the pH of the fermentation solution and the presence of the degraded substrate component undergoing repolarization or the formation of the substrate component by the various compounds of the reaction product own.

The kinetic parameter values of lipolytic bacteria specific growth rate ($\mu_L$) averaged 4.57 / day, proteolytic bacteria ($\mu_P$) of 4.73 / day and cellulolytic bacteria ($\mu_S$) of 4.42 / day. The value of a kinetic parameter of growth rate for the three types of bacteria has unequal value although the difference is not very significant. For proteolytic bacteria has the highest value compared to lipolytic and cellulolytic bacteria. The growth rate of proteolytic bacteria is greater than 7.01% of the growth rate of
cellulolytic bacteria and when it is compared with the rate of growth of lipolytic bacteria, then the value of the growth rate of proteolytic bacteria greater 3.50%. This indicates the growth rate of cellulolytic bacteria is smaller than lipolytic bacteria and proteolytic bacteria. Lower cellulolytic bacteria growth due to degradation of compounds containing long chain C chains by bacteria is more difficult than proteins. The average value of growth kinetic parameter (μ) for all three types of bacteria is 4.57/day.

4. Conclusion
The population of lipolytic, proteolytic and cellulolytic bacteria increases along the degradation time of substrates. The population growth of lipolytic, proteolytic and cellulolytic bacteria at 15 days achieves 6.03x10^6 cell/ml, 8.9x10^6 cell/ml and 4.41x10^6 cell/ml, respectively. The average of specific growth rates for lipolytic, proteolytic and cellulolytic bacteria achieves 4.56 day^{-1}, 4.73 day^{-1} and 4.42 day^{-1}, respectively. Production of methane increases along the degradation process, and it reaches 47.04 % by lipolytic bacteria, 19.67 % by proteolytic bacteria and 51.40 % by cellulolytic bacteria at 15 days of degradation time.

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