RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Suspended Growth Kinetic Analysis on Biogas Generation from Newly Isolated Anaerobic Bacterial Communities for Palm Oil Mill Effluent at Mesophilic Temperature.

Yee-Shian Wong[1, 2], Tjoon Tow Teng[1*], Soon-An Ong[2] , Norhashimah Morad[1] and Mohd Rafatullah[1]

[1]School of Industrial Technology, Universiti Sains Malaysia,
11600 Gelugor, Pulau Pinang Malaysia.

[2]School of Environmental Engineering, Universiti Malaysia Perlis,
Kompleks Pusat Pengajian Jejawi 3, 02600 Arau, Perlis, Malaysia

Corresponding author, E-mail : ttteng@usm.my (Prof. Tjoon-Tow Teng)

Tel.: +604-653-2215, Fax: +604-657-3678

Abstract

The anaerobic degradation of palm oil mill effluent (POME) was carried out under mesophilic temperature in anaerobic suspended growth closed bioreactor (ASGCB). Monod model was applied to describe the kinetic analysis of POME at different organic loading rates (OLR) in the range of 2.75 - 8.2 g TCOD/L day. The hydraulic retention time (HRT) was ranged between 8 and 24 days. The TCOD removal efficiency was achieved between 89.66% and 79.83%. The evaluated kinetic coefficients were: growth yield, Y_G (0.357 gVSS/ g TCOD), specific biomass decay rate, b (0.07/day), maximum specific biomass growth rate, μ_{max} (0.27/day), saturation constant for substrate, K_s (25.03 g TCOD/L) , critical retention time, Θ_c (3.72 day) and methane yield, Y_{CH4} (0.34 L CH_4/ $TCOD_{removed}$), respectively. Besides, new fermentative anaerobic bacteria isolated from POME were identified as *Escherichia fergusonii, Enterobacter asburiae, Enterobacter cloacae, Desulfovibrio*

aerotolerans, Desulfobulbus propionicus, Fusobacterium nucleatum, Paenibacillus pabuli, Bacillus subtilis, Methanobacterium sp., Methanosaeta concilii, Methanofollis tationis, Methanosarcina mazei and *Methanosarcina acetivorans* using 16S rDNA.

Keywords: Anaerobic Processes; Biodegradation; Biogas; Kinetics; DNA; Mesophilic.

Nomenclature

b	Specific biomass decay (1/day)
r_X	Specific substrate utilization rate (g TCOD/g VSS day)
r_v	Substrate utilization rate per volume (g TCOD/ day)
$r_{x,max}$	Maximum specific substrate utilization (g TCOD/g VSS day)
t	Time (day)
ASGCB	Anaerobic Suspended Growth Closed Biorector
Alk	Total Alkalinity
BOD	Biochemical Oxygen Demand (mg/L)
CO_2	Carbon Dioxide Gas
CH_4	Methane Gas
D	Dilution rate, 1/HRT (1/day)
DNA	Deoxyribonucleic Acid
HRT	Hydraulic Retention Time (day)
H_2	Hydrogen Gas
H_2S	Hydrogen Sulfide Gas
K_s	Saturation constant for substrate (g TCOD/L)
L	Liter
NH_3-N	Ammonia Nitrogen (mg/L)
O & G	Oil and Grease (mg/L)

51	OLR	Organic Loading Rate (g TCOD / L day)
52	PCR	Polymerase Chain Reaction
53	POME	Palm Oil Mill Effluent
54	Q	Volumetric Flow rate (L/day)
55	S	Substrate concentration in the reactor (mg/L)
56	S_1	Influent substrate concentration (mg/L)
57	S_2	Effluent substrate concentration, (mg/L)
58	SCOD	Soluble Chemical Oxygen Demand (mg/L)
59	SRT	Solid Retention Time
60	SS	Suspended Solid (mg/L)
61	TCOD	Total Chemical Oxygen Demand (mg/L)
62	TN	Total Nitrogen (mg/L)
63	TS	Total Solid (mg/L)
64	TVS	Total Volatile Solid (mg/L)
65	V	Reactor volume (L)
66	V_{CH4}	Methane Gas Volume (L)
67	VFA	Volatile Fatty Acid (mg/L)
68	VSS	Volatile Suspended Solid (mg/L)
69	X	Biomass concentration in the reactor, (mg/L)
70	Y_G	Growth yield (g VSS/g $TCOD_{removed}$)
71	Y_{CH4}	Methane yield (L CH_4/$TCOD_{removed}$)
72	**Greek letter**	
73	μ_{max}	Maximum specific biomass growth rate (1/day)
74	Θ_c	Critical retention time (day)
75	μ	Specific biomass growth rate (1/day)

1. Introduction

Palm oil industry produces high strength organic wastewater known as Palm Oil Mill Effluent (POME). POME is a viscous, brownish liquid containing about 95-96% water, 0.6-0.7% oil and 4-5% total solids (including 2-4% SS, mainly debris from fruit). It is acidic (pH 4-5), hot (80-90°C) with average Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) values of 50,000 mg/L and 25,000 mg/L, respectively [1].

Anaerobic digestion has often been used for high strength organic wastewater, since it produces less sludge compared to conventional aerobic treatment. An advantage of anaerobic treatment is operational cost effectiveness with simultaneous production of methane or hydrogen gas as energy resource. Anaerobic treatment is a complex process which involves decomposition of organic compounds and production of methane, hydrogen and carbon dioxide gases in the absence of molecular oxygen. The degradation process takes place by different types of anaerobic bacteria. The degradation mechanisms involve hydrolysis, acidogenesis (including acetogenesis) and methanogenesis [2]. Hydrolysis is the conversion reaction in which complex molecules such as carbohydrate, lipids and protein are converted into sugar, organic acids and etc. Acidogenic bacteria are responsible for breaking down the sugar, fatty acids and amino acids in acidogenesis process. Methanogenesis process occurs in which hydrogenotropic methanogens utilize the hydrogen and carbon dioxide gases produced by acetoclastic methanogens from short chain fatty acids to produce beneficial end product of methane gas [3].

For better control of degradation process with high operational efficiency, identification of microbial community of anaerobic digestion process is an essential requirement [4]. Currently only few anaerobic bacteria such as *Clostridium ssp.*, *Streptococcus cohnii ssp.*, *Lactobacillus* and *Thermoanaerobacterium spp.* have been reported with hydrogen producing ability from palm oil wastewater [5,6,7]. However,

hitherto no work regarding anaerobic bacteria with methane producing ability from palm oil wastewater has been reported. Kinetic study is required for a better understanding of the underlying phenomena in the anaerobic digestion process. Among many important applications, it can predict the compounds produced or consumed on their corresponding rates. The process kinetics for the anaerobic digestion are composed of several aspects. Firstly, there are the mass balance models involving input and output streams of the anaerobic system. Secondly, the selected model of flow or hydraulic must be formed for the anaerobic process. Finally, the selection of kinetic model for the reaction and stoichiometry can be conducted to determine the kinetic coefficients. Numerous kinetic models for anaerobic digestion such as Monod, First-order, Second-order, Modified Stover-Kinannon, Contois, Chen and Hashimoto have been proposed [8].

The literature study is abound with results of research on attached growth anaerobic treatment such as immobilised cell bioreactor [9], two stage up-flow anaerobic sludge blanket (UASB) [10], high rate up-flow anaerobic sludge fixed film [11], and combined high-rate anaerobic reactors [12] for the treatment of POME. Little research has been conducted on the application of anaerobic suspended growth process and also identification of anaerobe bacteria from POME for better control of the degradation process. Therefore, the aim of the present work was focused on the identification of anaerobic bacteria and kinetic coefficients for the anaerobic digestion of POME using suspended growth closed bioreactor.

2. Materials and Methods

2.1 Wastewater preparation

The raw POME was collected by onsite sampling from MALPOM Industries Bhd, in Nibong Tebal, Penang, Malaysia. The seeding required to acclimatize the Anaerobic Suspended Growth Closed Bioreactor (ASGCB) was taken from the anaerobic pond of

MALPOM Industries Bhd wastewater treatment plant. The composition and properties of the collected POME used in the present study are summarized in Table 1. The POME was stored in the refrigerator at 4°C until further experimental work was conducted. This storage observed no change of composition.

2.2 ASGCB set-up

The schematic configuration of the ASGCB set-up is shown in Figure 1. The ASGCB is 0.25 m in diameter and 0.36 m in height. The reactor consists of a cylindrical-shape flexi glass vessel with total and working volumes of 17.7 L and 14 L, respectively. It comprises an integrated on-line pH data recording system connected to a pH probe and an overhead stirrer. The operating temperature of ASGCB was maintained constant at 35 °C using thermal jacket. The ASGCB has a gas sampling valve (or clamp) which allows gas samples to be collected without interference with head space composition and is connected to a 50 L gas collection bag.

2.3 Bacteria Cultivation and Isolation

Cultivation of bacteria was conducted according to isolation techniques of microbiology [13]. The culture medium contained (in g/L): Heart Extract and Peptone 20; Yeast Extract 5.0; Sodium Chloride 5.0; Bacteriological Agar 15; Sodium Thioglycollate 2.0 and Sodium Formaldehyde Sulfoxylate 1.0. The pH of the medium was adjusted to pH 7.0 ±0.1 with 1 N Sodium Hydroxide (NaOH) or 0.1 N of Hydrochloric Acid (HCl) by using pH meter (Metrohm, 826 pH Mobile). The medium was sterilized at 121ºC for 15 minutes. Isolation of bacteria in the sample was done with serial dilution where one mL of the sample was diluted with 9 mL of sterile saline water. The dilution was repeated until the colonies on the agar plate was countable. Pure isolates were then stored on culture medium at 4 ^{0}C until

151 further analysis. The cultivation and isolation were done in the Anaerobic Chamber (Fisher
152 Scientific, Forma Anaerobic System) at 35°C.
153
154 2.4 Molecular identification of isolates
155 2.4.1 DNA extraction and PCR amplification
156 The bacteria genomic DNA was extracted by using I-genomic DNA extraction kit (QIAGEN)
157 according to the manufacturer's instruction from the cultivated medium. Then, PCR
158 amplification of 16S rDNA was conducted using the amplification kit (PROMEGA)
159 according to the manufacturer's operation procedure. The amplification reaction was done in
160 a 50 µL tube with mixtures of 10 µL of 5X colorless GoTaq buffer, 1mM of $MgCl_2$, 0.1 mM
161 of dNTP, 0.5 µM forward primer, 0.5 µM reverse primer, 0.02 units of Taq DNA polymerase
162 and nuclease-free water. Two types of primer pair were applied by using universal primer of
163 27F and 1492R (27F: AGA GTT TGA TCM TGG CTC AG ; 1492R: CGG TTA CCT TGT
164 TAC GAC TT) and archaea primer of ARC344F and ARC915R (ARC344F: ACG GGG
165 YGC AGC AGG GGC GA; ARC915R: GTG CTC CCC CGC CAA TTC CT). The PCR
166 amplification was 2 min at 95 ^{0}C followed by 34 cycles of denaturation at 95 ^{0}C for 50s,
167 annealing at 58 ^{0}C for 45s, elongation at 72 ^{0}C at 45s and the final elongation at 72 ^{0}C for 8
168 minutes. The PCR amplification procedures for archaea were set similar to that of bacteria
169 with only difference in the annealing temperature at 54 ^{0}C for 1 min. The PCR products were
170 then analyzed with 1% agarose gel electrophoresis (AGE).
171
172 2.4.2 Bacteria Identification based on 16S rDNA sequences
173 The PCR products were sent to the Genomics BioScience and Technology Co., Ltd. in
174 Malaysia for 16S rDNA sequence analysis. The clone libraries were finally built based on the
175 obtained sequences. The obtained sequences were analyzed through the gene bank database

of National Center for Biotechnology Information (NCBI) for highest similarity identification of bacteria. The maximum bacteria identity was determined through the Basic Local Alignment Search Tool (BLAST) between the similarity identification of aligned sequence nucleotides and nucleotides stored in the gene bank database.

2.5 ASGCB experimental procedure

The seeding required for starting the ASGCB was taken from the anaerobic pond of MALPOM Industries Bhd Penang, Malaysia wastewater treatment plant. About 14 liters of the anaerobic digested POME were used to acclimatize the laboratory ASGCB. The start-up of the ASGCB involved step increases of the influent organic volumetric loading rates from 2.72 g COD/L/day to 6.55 g COD/L/day. This acclimatization phase was important to allow the microorganisms present in the mixed liquor perfectly acclimatize to the new environment and reach a steady state condition before proceeding to the next phase of research studies. The pH in the ASGCB was adjusted to 7.50 ±0.05 using 1 N Sodium Hydroxide (NaOH).

Once the start-up phase has been completed, a series of continuous experiments using feed flow-rates of 0.58, 0.7, 0.88, 1.17 and 1.75 liters of raw POME per day, corresponding to Hydraulic Retention Time (HRT) of 24, 20, 16, 12 and 8 days was followed. The rate of influent substrate concentration of TCOD was controlled in the range of 62500-65500 mg/L. Biogas and samples from ASGCB were collected for analysis after 24 hours of input raw POME. For each batch of HRT, the ASGCB was continuously operated until steady state condition was achieved. The steady-state condition was reached once the values of TCOD removal efficiency, VFA, biogas production rate and biogas composition remained unchanged for five consecutive days. The variation of the actual results from the mean values was <3 %.

2.6 Analytical method

The TCOD of a sample was measured using reactor digestion method (HACH, range: 20 - 1500 mg/L) for two hours at 150 ^{o}C. The MLSS, MLVSS, O & G, NH_3-N, VFA and Alk were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Daily biogas production was recorded using Binder COMBIMASS gas analyzer consisted of five gas channels: CO_2, CH_4, O_2, H_2S and H_2. The volatile fatty acids such as acetic, propanoic, butanoic and iso-butanoic were extracted from the sample using 10% phosphoric acid (H_3PO_4) and methanol. The extracted samples were analyzed by Shimadzu GCMS QP 2010 Plus equipped with SGE BP21 capillary column (25 m length, 0.22 mm internal diameter and 0.25 um thickness). Helium was used as the carrier gas. The injection temperature was 220 °C and the column temperature was initially held at 120 °C for 6 minutes then was raised progressively at 15 °C/minute until 220 °C. The carbon, hydrogen, nitrogen, sulfur and oxygen contents of POME wastewater were measured by elemental analyzer (PerkinElmer 2400 Series II CHNS/O).

2.7 Kinetic model development

2.7.1 Substrate balance model

Determination of kinetic coefficients can be done on the specific parameters of the system performance. Different kinetic models were used for different biological systems to evaluate kinetic coefficients. For this purpose, various kinetic models such as Monod, Stover-Kincannon, Michaelis-Menten, first order and second order have been applied. In the present study, the mass balance, reaction rate equation and Monod models were used. The equations describing the performance of the system are the mass balance equations of both the substrate and biomass.

2.7.2 Mass balance equation

The substrate balance can be expressed as (1):

$$\frac{dS}{dt} = \frac{Q(S_1 - S_2)}{V} - r_v \quad (1)$$

where, S = substrate concentration in the reactor, Q = volumetric flow rate, S_1 = influent substrate concentration, S_2 = effluent substrate concentration (S_2 = S), V = working volume, r_v = substrate utilization rate per volume, and t = time.

Once the reactor was completely mixed and continuously fed without recycle, $\frac{dS}{dt} = 0$. Equation (1) then becomes:

$$r_v = (S_1 - S_2)\, D \quad (2)$$

where, D = dilution rate = $\frac{Q}{V} = \frac{1}{HRT}$

The specific substrate utilization rate, r_X is defined as $r_X = \frac{r_v}{X}$, where X = biomass concentration in the reactor:

$$r_X = \frac{(S_1 - S_2)D}{X} \quad (3)$$

On the other hand, the biomass balance gives:

$$\frac{dX}{dt} = \frac{-QX}{V} + \mu X \quad (4)$$

where, μ = specific biomass growth rate. At steady state, $\frac{dX}{dt} = 0$. Hence, Equation (4) yields

$$\mu = D = \frac{1}{HRT} \quad (5)$$

The growth of microorganism is proportional to the substrate utilization. Biomass decay or endogenous metabolism should also be taken into account to describe the specific growth rate [11]:

$$\mu = Y_G r_X - b \quad (6)$$

where, Y_G = growth yield coefficient and

b = specific biomass decay coefficient.

Combining Equations (5) and (6) gives:

$$D = Y_G r_X - b \quad (7)$$

or

$$r_X = \frac{D}{Y_G} + \frac{b}{Y_G} \quad (8)$$

2.7.3 Reaction Rate equation

Several types of reaction rate equations have been proposed to describe the relationship between the growth rate and the substrate utilization. Monod equation is the simplest and well known in biological treatment system [10]. The Monod equation is:

$$r_X = \frac{S}{K_S + S} r_{X,\max} \quad (9)$$

where, $r_{X,max}$= maximum specific substrate utilization rate, K_S = saturation constant for substrate, and S = substrate concentration in the reactor. r_X is half of $r_{X,max}$ when the substrate concentration is K_S. Equation (9) can be rearranged to the following three types of linear equation to obtain $r_{X,max}$ and K_S from experimental data of r_X and S.

$$\frac{1}{r_X} = \left(\frac{K_S}{r_{X,\max}}\right)\left(\frac{1}{S}\right) + \frac{1}{r_{X,\max}} \quad \text{Lineweaver-Burk plot} \quad (10)$$

$$r_X = r_{X,\max} - K_S\left(\frac{r_X}{S}\right) \quad \text{Eadie plot} \quad (11)$$

$$\frac{S}{r_X} = \frac{S}{r_{X,\max}} + \frac{K_S}{r_{X,\max}} \quad \text{Hofstee plot} \qquad (12)$$

The maximum specific biomass growth rate, μ_{max}, is obtained by substituting $r_{X,max}$ for r_X in Equation (6):

$$\mu_{max} = Y_G r_{X,max} - b \qquad (13)$$

Critical retention time Θ_C is a safety factor for anaerobic degradation process where substrate utilization does not happen but bacteria washout could happen if the reactor is loaded below the critical retention time [10]. Θ_C can be expressed as:

$$\Theta_C = \frac{1}{\mu_{max}} \qquad (14)$$

2.7.4 Methane yield

Methane yield is a vital economic factor for the anaerobic digestion process. In most cases, the methane yield coefficient, Y_{CH4} is determined from experimental data. If the volume of methane gas produced, V_{CH4} is assumed to be proportional to the amount of substrate consumed, then Equation (15) is formed:

$$V_{CH4} = Y_{CH_4} Q(S_1 - S_2) \qquad (15)$$

A plot of V_{CH4} versus $Q(S_1 - S_2)$ should yield a straight line passing through the origin with a slope equal to Y_{CH4}.

3. RESULTS AND DISCUSSION

3.1 Bacteria Identification

Bacteria cultivation and isolation processes were conducted from the ASGCB mix sludge samples at the end of each batch operating HRT. Table 2 summarizes the microbial identification of ASGCB mix sludge sample by using 16S rDNA. Eight types of bacteria and five types of archaea were obtained. The 16S rDNA data of ASGCB sample illustrates that

290 the microorganisms consisted of *Phylum Proteobacteria* (38.5%), *Firmicutes* (15.4%),
291 *Fusobacteria* (7.6%) and *Euryarchaeota* (38.5%).

292 *Escherichia fergusonii*, *Enterobacter asburiae* and *Enterobacter cloacae* which
293 belong to the family of *Enterobacteriaceae* were identified in the ASGCB. These types of
294 bacteria are known to produce hydrogen gas from carbohydrates and fatty acids [14,15].
295 Sulfur-reducing anaerobe bacteria such as *Desulfovibrio aerotolerans*, *Desulfobulbus*
296 *propionicus*, *Fusobacterium nucleatum*, *Paenibacillus pabuli* and *Bacillus subtilis* were also
297 obtained. These types of bacteria survived with sulfur content in POME wastewater in the
298 ASGCB (Table 1). To our knowledge this is the first report that *Fusobacterium nucleatum,*
299 *Paenibacillus pabuli* and *Bacillus subtilis* as sulfur-reducing bacteria are present in the
300 anaerobic process of POME wastewater. The sulfur-reducing bacteria consumed of sulfur as
301 the electron acceptor to form hydrogen sulfide gas (H_2S) from the hydrogen gas (H_2) as the
302 electron donor [16,17,18,19]. Therefore, high concentration of H_2S gas was obtained from the
303 anaerobic degradation process (Table 3). Low H_2 gas production was detected as most of the
304 hydrogen ion had been used to form H_2S gas (Table 3).

305 High contents of fatty acids were characterized from POME wastewater (Table 1).
306 Therefore, five species of methane producing bacteria such as *Methanofollis ationis*,
307 *Methanobacterium sp., Methanosaeta concilii, Methanosarcina mazei,* and *Methanosarcina*
308 *acetivorans* have been obtained in ASGCB. The methanogenic archaea bacteria converted
309 acetates into methane gas (CH_4) and carbon dioxide gas (CO_2) [20,21]. Perhaps, the presence
310 of the identified microbial community in the ASGCB played an important role in degrading
311 POME in the complex anaerobic degradation processes.
312
313
314

315 *3.2 Operational parameters and biodegradability of POME wastewater in ASGCB*

316 Table 3 summarizes the results obtained under various HRT. An analysis of variance
317 (ANOVA) was conducted to test the significance of experimental data at different HRT in
318 Table 3. Table 4 states the outcome of ANOVA test as F-value and p-value for each
319 operational variables under various HRT. High F-value with low p-value (< 0.05) was
320 obtained through ANOVA significant test. Therefore, the operational results are statistically
321 proven to be significant in the present study [22,23].

322 Throughout the study, the organic loading rates were varied from 2.75 g TCOD/L/d to
323 8.2 g TCOD/L/d. At each HRT, the operational parameters were operated until steady state
324 was reached as indicated by a constant effluent TCOD, biogas production, total VFA,
325 alkalinity, and MLVSS level. The effluent TCOD of the reactor increased as the HRT
326 reduced. This is due to the increase of input organic loading rates as the volumetric flow rate
327 of POME was increased. On the other hand, the anaerobic degradation process of POME is
328 operated without acidification risk in the ASGCB with the level of VFA/Alk between 0.05
329 and 0.50. The VFA/Alk levels fall between the recommended condition of 0.3 - 0.5 whereby
330 this process is considered to be operating favorably in anaerobic condition [24,25]. The level
331 of alkalinity reduced from 11400 mg $CaCO_3$/L to 7650 mg $CaCO_3$/L as the HRT reduced.
332 The high level of bicarbonate alkalinity was reached in the reactor due to the reaction of
333 ammonia with carbon dioxide and water in forming ammonium bicarbonate [26]. The mixed
334 liquor volatile suspended solid (MLVSS) inside the ASGCB was analyzed as the microbial
335 growth or biomass concentration. The MLVSS varied from 7610 mg MLVSS/L to 11570 mg
336 MLVSS/L. This result proves that the anaerobic degradation of POME could be well
337 performed as the increase of microbial growth with increasing organic loading rate (OLR).

338 The daily biogas production increased from 12.90 L/d to 30.81 L/d with increasing
339 OLR. This is because the POME was characterized with high concentration of palmitic fatty

acids as stated in Table1. Palmitic acids containing 16 carbons long-chain fatty acid decomposed faster to shorter chain fatty acids such as acetic; propanoic; butanoic; iso-butanoic and etc. The identified anaerobic bacteria *Escherichia fergusonii*, *Enterobacter asburiae* , *Enterobacter cloacae*, *Desulfovibrio aerotolerans, Desulfobulbus propionicus , Fusobacterium nucleatum, Paenibacillus pabuli, Bacillus subtilis, Methanobacterium sp., Methanosaeta concilii, Methanofollis tationis, Methanosarcina mazei* and *Methanosarcina acetivorans* further decomposed the fatty acids to produce methane (CH_4), carbon dioxde (CO_2), hydrogen (H_2) and hydrogen sulfide (H_2S) gasses through acidogenesis, acetogenesis and methanogenesis of anaerobic degradation pathway. The anaerobic degradation reactions are:

$$3C_{16}H_{32}O_2 + 42H_2O \rightarrow 7CH_3COOH + 4CH_3CH_2COOH + 3CH_3CH_2CH_2COOH + 10CO_2 + 52H_2 \quad \text{(Acidogenesis)} \quad (16)$$

$$2CH_3CH_2COOH + 2H_2O \rightarrow 3CH_3COOH + 2H_2 \quad \text{(Acetogenesis)} \quad (17)$$

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + H_2 \quad \text{(Acetogenesis)} \quad (18)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(Methanogenesis)} \quad (19)$$

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- \quad \text{(Sulphidogenesis)} \quad (20)$$

$$H^+ + HS^- \leftrightarrow H_2S \quad (21)$$

The contents of the CH_4, CO_2, H_2 and H_2S produced during the steady-state period at different HRTs are shown in Table 3. The CH_4 content decreased from 72.5 % to 65.9 % with decrease of HRT. A decrease in CH_4 content is caused by an increase of the VFA level and poor performance of effluent TCOD. This is because high OLR was characterized by high concentration of VFA at the low HRT. These results are in agreement with the finding by Hikmet [27] who reported that the CH_4 content decreased with TCOD removal efficiency and decrease in HRT.

365 *3.3 Evaluation of kinetic coefficients*

366 *3.3.1 Kinetic coefficients Y_G and b*

367 The experimental data under steady state condition (Table 3) were analyzed and
368 biological kinetic models were tested. The growth yield (Y_G) and specific biomass decay (b)
369 were obtained using Equation (8) by plotting r_x versus D. As illustrated in Figure 2, the
370 points fitted well on a straight line with a regression coefficient of 0.99. The values of Y_G and
371 b were calculated to be 0.357 gVSS/g $TCOD_{removed}$ and 0.07/ day, respectively from the slope
372 and intercept. The evaluated growth yield, Y_G was in the range of 0.181 gVSS/g $TCOD_{removed}$
373 and 0.99 gVSS/g $TCOD_{removed}$ as reported in the literature: 0.69 gVSS/g $TCOD_{removed}$ [28];
374 0.174 gVSS/g $TCOD_{removed}$ [11]; 0.99 gVSS/g $TCOD_{removed}$ [29] and 0.181 gVSS/g
375 $TCOD_{removed}$ [30]. Hitherto, this is due to the similar substrate of POME wastewater was
376 treated in the anaerobic degradation process.

377

378 *3.3.2 $r_{x,max}$; k_s ;* μ_{max} and Θ_C *biokinetic coefficients*

379 The maximum specific substrate utilization rate ($r_{x,max}$) and saturation constant for
380 substrate (k_s) were obtained through Equation (10) by Lineweaver-Burk plot. By plotting $1/r_x$
381 and 1/S data pairs, a straight line with regression coefficient of 0.98 is obtained (Figure 3).
382 The values of $r_{x,max}$ and k_s are 0.957 g TCOD/g VSS day and 25.03 g TCOD/ L, respectively,
383 from the intercept and slope. The maximum specific biomass growth rate (μ_{max}) and the
384 critical retention time (Θ_C) are 0.27 day^{-1} and 3.72 day, respectively, obtained from
385 Equations (13) and (14). The value of μ_{max} for the substrate of TCOD is close to that of the
386 reported work of 0.207/day [11] and 0.304/day [31]. This is due to similar activity for
387 microbial growth in the treatment of POME. The critical retention time, Θ_C at which the
388 washout of microorganisms was 3.72 days for the substrate of TCOD. Thus, the ASGCB was

operated without risk to avoid low performance of anaerobic degradation process as the kinetic analysis experiments were conducted between 24 days and 8 days of retention time.

The small values of $r_{x,max}$, k_s and μ_{max} were achieved as the anaerobe bacteria could easily degrade the organic matters in the input of palm oil wastewater. Therefore, the ASGCB significantly performed well between 79.83 % and 89.66 % reduction of TCOD with increase of OLR (Table 3). This significant finding is reflected by the newly isolated anaerobe bacteria: *Escherichia fergusonii*, *Enterobacter asburiae* , *Enterobacter cloacae*, *Desulfovibrio aerotolerans, Desulfobulbus propionicus , Fusobacterium nucleatum, Paenibacillus pabuli, Bacillus subtilis, Methanobacterium sp., Methanosaeta concilii, Methanofollis tationis, Methanosarcina mazei* and *Methanosarcina acetivorans* that are able to degrade the fatty acid contents of POME wastewater.

3.3.3 Kinetics of methane production

The experiment data listed in Table 3 were used to determine the yield of methane, Y_{CH4}. By plotting V_{CH4} against $(S_1 - S_2)Q$ in Equation (15), a value of 0.34 L CH_4/ g $TCOD_{removed}$ was obtained from the slope (Figure 4). In Figure 4, the straight line passing through the origin with a correlation coefficient of 0.99 suggests the validation of Equation (15). The value of Y_{CH4} of 0.34 L CH_4/ g $TCOD_{removed}$ is reported so far to be the highest methane yield for palm oil wastewater compared to the previously reported values of 0.325 L CH_4/ g $TCOD_{removed}$ [9]; 0.325 L CH_4/ g $TCOD_{removed}$ [11] and recently 0.32 L CH_4/ g $TCOD_{removed}$ [30]. Therefore, the presence of *Methanobacterium sp., Methanosaeta concilii, Methanofollis tationis, Methanosarcina mazei* and *Methanosarcina acetivorans* anaerobes performs well in suspended growth anaerobic process.

4. CONCLUSION

New anaerobic bacteria such as *Escherichia fergusonii*, *Enterobacter asburiae* , *Enterobacter cloacae*, *Desulfovibrio aerotolerans, Desulfobulbus propionicus* , *Fusobacterium nucleatum, Paenibacillus pabuli, Bacillus subtilis, Methanobacterium sp., Methanosaeta concilii, Methanofollis tationis, Methanosarcina mazei* and *Methanosarcina acetivorans* were isolated from ASGCB. Their presence in the ASGCB produced the highest ever methane yield at 0.34 L CH_4/ $TCOD_{removed}$. Monod model obtained for the suspended growth anaerobic process produces the following biological kinetic coefficients: Y_G (0.357 gVSS/ g TCOD), b (0.07/day), μ_{max} (0.27/day), K_s (25.03 g TCOD/L) , Θ_c (3.72 day) and $Y_{CH}4$ (0.34 L CH_4/ $TCOD_{removed}$), respectively.

5. ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to University Sains Malaysia Research University (RU) grant: 1001/PTEKIND/814160 for financial support, School of Environmental Engineering of University Malaysia Perlis (UniMAP) and MALPOM Industries Sdn Bhd.

6. REFERENCES

[1] Najafpour, GD, Zinatizadeh, AAL., Mohamed, AR., Hasnain Isa, Nasrollahzadeh H. High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor. Process Biochem. 2006; 41: 370–379.

[2] Zhang Dong, Chen Yinguang, Zhao Yuxiao and Zhu Xiaoyu. New sludge pretreatment method to improve methane production in waste activated sludge digestion. Environ. Sci. Technol. 2010; 44(12): 4802-4808.

[3] Siddique Tariq, Pennen Tara, Semple Kathleen and Foght Julia M. Anaerobic Biodegradation of longer chain n-Akanes coupled to methane production in oil sands tailings. Environ. Sci. Technol. 2011; 45(13): 5892-5899.

[4] Tabatabaei M, Rahim Raha A, Abdullah N, Wright A, Shirai Y, Sakai K, Sulaiman A, Hassan MA. Important of methanogenic archea populations in anaerobic wastewater treatmets. Process Biochem. 2010; 45: 1214 - 1225.

[5] Badiei M, Jahim Jamaliah Md, Anuar Nurina, Abdullah Siti RS, Swee Su Lim., Kamaruzzaman MA. Microbial community analysis of mixed anaerobic microflora in suspended sludge of ABSR producing hydrogen from palm oil mill effluent. Int. J. Hydrogen Energy. 2012; 37: 3169-3176.

[6] Yee-Shian Wong, Soon-An Ong, Tjoon-Tow Teng, Nabilah Aminah Lutpi, Kasthuri Kumaran. Production of Bioflocculant by Staphylococcus cohnii ssp.from Palm Oil Mill Effluent (POME). Water Air Soil Pollut. 2012; 223: 3775–3781

[7] Singh L, Siddiqui MF, Ahmad Anwar, Rahim MH Ab, Sakinah M, Wahid ZA. Application of polyethylene glycol immobilized Clostridium sp. LS2 for continuous hydrogen production from palm oil effluent in upflow anaerobic sludge blanket reactor. Biochem Eng. J. 2013; 70: 158-165.

[8] Buyukkamaci Nurdan and Filibeli Ayse. Determination of kinetic constant of an anaerobic hybrid reactor. Process Biochem. 2002; 38: 73-79.

464 [9] Borja R. and Banks CJ. Anaerobic digestion of palm oil mill effluent using an up flow
465 anaerobic sludge blanket (UASB) reactor. Biomass Bioenerg. 1994; 6: 381-389.
466
467 [10] Borja R, Banks CJ, Sanchez E. Anaerobic treatment of palm oil mill effluent in a two-
468 stage upflow anaerobic sludge blanket (UASB) system. J. Biotechnol. 1996; 45: 125-135.
469
470 [11] Zinatizadeh, AAL, Mohamed AR, Najafpour GD. Kinetic evalution of palm oil mill
471 effluent digestion in a high rate up-flow anaerobic sludge fixed film bioreactor. Process
472 Biochem. 2006; 41: 1038-1046.
473
474 [12] Choi Won-Ho, Shin Chang-Ha, Son Sung-Min, Ghorpade A., Kim Jeong-Joo and Park
475 Joo-Yang. (2013) Anaerobic treatment of palm oil mill effluent using combined high rate
476 anaerobic reactors. Bioresour. Technol. 2013; 141: 138-144.
477
478 [13] Bacteriological Analytical Manual Website: http://cfsan.fda.bam.gov.
479
480 [14] Blairon L, Maza ML, Wybo I, Pierard D, Dediste A, Vandenberg O. Vitek 2 ANC card
481 vesus BBL Crystal Anaerobe and RapID ANA II for identification of clinical anaerobic
482 bacteria. Anaerobe 2010; 16: 355-361.
483
484 [15] Sriram MI, Gayathiri S, Gnanaselvi U, Jenifer PS, Raj SM, Gurunathan S. Novel
485 lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal tolerant
486 bacterium Escherichia fergusonii KLU01 as a potential tool for bioremediation. Bioresour.
487 Technol. 2001; 102: 9291-9295.
488

[16] Mogensen GL, Kjeldsen KU, Ingvorsen K. Desulfovibrio aerotolerans sp. nov., an oygen tolerant sulphate-reducing bacterium isolated from activated sludge. Anaerobe 2005; 11: 339-349.

[17] Paul SS, Deb SM, Singh D. Isolation and characterization of novel sulphate-reducing Fusobacterium sp. and their effects on in vitro methane emission and digestion of wheat straw by rumen fluid from indian riverine buffaloes Anim. Feed Sci. Technol. 2011; 166-167: 132-140.

[18] Zainudin MHM, Hassan MA, Tokura M, Shirai Y. Indigenous cellulolytic and hemicellulolytic bacteria enhanced rapid co-composting of lignocellulose oil palm empty fruit bunch with palm oil mill effluent anaerobic sludge. Bioresour. Technol. 2013*;* 147: 632-635.

[19] El-Midany AA, Abdel-Khalek MA. Reducing sulfur and ash from coal using Bacillus subtitis and paenibacillus polymyxa. Fuel 2014; 115: 589-595.

[20] Lins P, Reitschuler C, Iiimer P. Methanosarcina spp., the key to relieve the start-up of a thermophilic anaerobic digestion suffering from high acetic acid loads. Bioresour. Technol. 2014; 152: 347-354.

[21] Maus I, Wibberg D, Stantscheff R, Cibis K, Eikmeyer, Felix-Gregor, Konig H, Puhler A, Schluter A. Complete genome sequence of hydrogenotrophic Archaeon Methanobacterium sp. Mb1 isolated from a production scale biogas plant. J. Biotechnol. 2013; 168: 734-736.

514 [22] Chaganti SR, Kimb DH, Lalmanc JA, Shewac WA. Statistical optimization of factors
515 affecting biohydrogen production from xylose fermentation using inhibited mixed anaerobic
516 cultures. Int. J. Hydrogen Energy 2012; 37: 11710-11718.
517
518 [23] Wong Yee-Shian, Tjoon-Tow Teng, Soon-An Ong, Norhashimah M, Rafatullah M, Jing-
519 Yong Leong. Methane Gas Production from Palm oil wastewater- An Anaerobic
520 Methanogenic Degradation Process in Continuous Stirrer Suspended Closed Anaerobic
521 Reactor. J. Taiwan Inst. Chem. Eng. 2014; 45: 896-900.
522
523 [24] Borja R, Martin A, Sanchez E, Rincon B, Raposo F. Kinetic modeling of the hydrolysis,
524 acidogenic and methanogenic steps in the anaerobic digestion of two- phase olive pomace
525 (TOPO). Process Biochem. 2005; 40: 1841-1847.
526
527 [25] Borja, R, Sanchez E, Rincon B, Raposo F, Martin MA, Martin A. Study and
528 optimization of the anaerobic acidogenic fermentation of two phase olive pomace. Process
529 Biochem. 2005; 40: 281-291.
530
531 [26] Zhao HW and Viraraghavan T. Analysis of the performance of an anaerobic digestion
532 system at the Regina wastewater treatment plant. Bioresour. Technol. 2004; 95: 301-307.
533
534 [27] Hikmet T. Temperature and organic loading dependency of methane and carbon dioxide
535 emission rates of a full-scale anaerobic waste stabilization pond. Pergamon. 1994; 0043-
536 1354: 251-257.
537

538 [28] Yeoh BG. A kinetic based design for thermophilic anaerobic treatment of a high strenght
539 agro industrial wastewater. Env Technol. 1986; 7(1): 509-518.
540
541 [29] Wong Yee-Shian, Mohd Omar A.B. Kadir, Teng Tjoon-Tow. Biological kinetics
542 evaluation of anaerobic stabilization pond treatment of palm oil mill effluent. Bioresour.
543 Technol. 2009; 100: 4969–4975.
544
545 [30] Chan Y J, Chong MF, Law CL. An integrated anaerobic-aerobic bioreactor (IAAB) for
546 the treatment of palm oil mill effluent (POME): Start-up and steady state performance.
547 Process Biochem. 2012; 47: 485-495.
548
549 [31] Faisal M. and Unno Hajime. Kinetic analysis of palm oil mill wastewater treatment by a
550 modified anaerobic baffled reactor. Biochem. Eng. J. 2011; 9: 25-31.
551
552
553
554
555
556
557
558
559
560
561
562

Captions of Figures:

Fig 1 Schematic configuration of the Anaerobic Suspended Growth Closed Bioreactor (ASGCB)

Fig 2 Plot of specific substrate utilization rate, r_x versus dilution rate, D (Equation 8)

Fig 3 Determination of maximum specific substrate utilization, $r_{x,max}$ and saturation constant, k_s through LIneweaver-Burk plot (Equation 10)

Fig 4 Determination of methane yield, Y_{CH4} by Equation (15)

Table 1: Composition and properties of POME used

Parameter *	**Concentration range (mg/L)**	**Mean**
pH	4.3 – 4.75	4.5
COD	62500 – 65000	63750
SCOD	30500 – 34500	32500
BOD	31500 – 38500	35000
MLSS	35050 – 41090	38070
MLVSS	30230 - 34700	32465
O & G	15500 – 16600	16050
TN	725 – 835	780
NH_3-N	65 - 70	67.5
Palmitic Acid	7420 - 7500	7460
Acetic Acid	1900 - 2110	2005
Propanoic Acid	53.2 - 56	54.6
Butanoic Acid	28.6 - 33.4	31
Iso- Butanoic Acid	2.8 - 5.8	4.3
Carbon (%)	35.8 - 59.5	47.7
Hydrogen (%)	5.3 - 9.1	7.2
Nitrogen (%)	0.9 - 2.7	1.8
Sulfur (%)	0.5 - 1.3	0.9
Oxygen (%)	21 - 55	38

* Unit for all parameter is mg/L except pH, carbon, hydrogen, nitrogen, sulfur and oxygen.

Table 2: Microbial community identification in ASGCB.

Microbial Group		Sequence similarity (%)	Accession no.	Phylogenetic affiliation		
				Phylum	Class	Family
Bacteria	Escherichia fergusonii	99	NR 074902	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae
	Enterobacter asburiae	99	NR 024640	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae
	Enterobacter cloacae	99	NR 044978	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae
	Desulfovibrio aerotolerans	99	NR 043163	Proteobacteria	Deltaproteobacteria	Desulfobulbaceae
	Desulfobulbus propionicus	99	NR 074930	Proteobacteria	Deltaproteobacteria	Desulfovibrionaceae
	Paenibacillus pabuli	99	NR 040853	Firmicutes	Bacilli	Paenibacillaceae
	Bacillus subtilis	99	NR 102783	Firmicutes	Bacilli	Bacillaceae
	Fusobacterium nucleatum	99	NR 074412	Fusobacteria	Fusobacteria	Fusobacteriaceae
Archaea	Methanobacterium sp.	99	NR 102889	Euryarchaeota	Methanobacteria	Methanobacteriaceae
	Methanofollis tationis	99	NR 041717	Euryarchaeota	Methanomicrobia	Methanomicrobiaceae
	Methanosarcina mazei	99	NR 118787	Euryarchaeota	Methanomicrobia	Methanosarcinaceae
	Methanosaeta concilii	99	NR 104707	Euryarchaeota	Methanomicrobia	Methanosaetaceae
	Methanosarcina acetivorans	99	NR 044724	Euryarchaeota	Methanomicrobia	Methanosarcinaceae

Table 3: The stable operation results obtained under various HRT in ASGCB.

Composition	**HRT (day)**				
	24	**20**	**16**	**12**	**8**
OLR (g TCOD/ L day)	2.75± 0.01	3.3± 0.06	4.1± 0.05	5.45± 0.06	8.2± 0.05
Volumetric feed flow rate (L POME/ day)	0.583± 0.00	0.7± 0.00	0.875± 0.00	1.167± 0.00	1.75± 0.00
TCOD removal efficiency (%)	89.66± 0.03	86.06± 0.10	85.15± 0.03	84.56± 0.29	79.83± 0.03
Effluent TCOD (mg/ L)	6810± 4.65	9130± 2.90	9720± 2.90	10130± 10.00	13200± 5.85
Inside TCOD (mg/ L)	12650± 5.00	14250± 14.45	16860± 44.80	21270± 36.00	32150± 11.55
Total Alkalinity in ASGCB (mg $CaCO_3$ /L)	11400± 13.30	10400± 28.90	9160± 5.00	8400± 5.75	7650± 2.90
Total VFA in ASGCB (mg CH_3COOH/ L)	514.29± 2.50	1017.14± 0.50	2028.57± 0.82	2497.14± 9.80	3860.57± 5.35
VFA:Alk	0.05± 0.01	0.10± 0.01	0.22± 0.01	0.30± 0.01	0.50± 0.01
MLVSS in ASGCB (mg/ L)	7610± 2.90	8220± 28.85	9290± 25.05	10780± 5.05	11570± 5.80
Total Volume Biogas Production (L /day)	17.79± 0.05	19.85± 0.03	23.82± 0.01	32± 0.06	46.76± 0.11
Daily Volume Methane Production, Q_{CH4} (L CH_4 /day)	12.90± 0.06	14.17± 0.06	16.41± 0.06	21.71± 0.05	30.81± 0.05

Methane Gas (CH_4) %	72.50± 0.03	71.40± 0.29	68.90± 0.15	67.90± 0.30	65.90± 0.26
Carbon Dioxide Gas (CO_2) %	27.30± 0.25	28.40± 0.11	31± 0.23	31.80± 0.06	33.90± 0.06
Hydrogen Sulfide Gas (H_2S) (mg /L)	1825± 3.00	3245± 5.05	5820± 28.80	6975± 15.25	12370± 5.25
Hydrogen Gas (H_2) (mg/ L)	214± 0.50	488± 0.55	717± 0.50	1998± 0.55	3841± 0.55

± Standard Deviation

Table 4: Analysis of variance (ANOVA) for the anaerobic degradation in ASGCB.

Factors	Statistics		
	F-value	p-value	Remark
TCOD removal efficiency	48.08	6.15×10^{-3}	Significant
Effluent TCOD	49.22	5.95×10^{-3}	Significant
Reactor TCOD	87.76	2.58×10^{-3}	Significant
Total Alkalinity in ASGCB	284.76	4.53×10^{-4}	Significant
Total VFA in ASGCB	97.22	2.22×10^{-3}	Significant
VFA:Alk	230.09	6.22×10^{-4}	Significant
MLVSS inside ASGCB	70.21	3.57×10^{-3}	Significant
Total Biogas Production	54.94	5.08×10^{-3}	Significant
Daily volume Methane Production	50.36	5.76×10^{-3}	Significant
Methane Gas	96.43	2.25×10^{-3}	Significant
Carbon dioxide Gas	27.37	4.24×10^{-2}	Significant
Hydrogen Sulfide Gas	27.37	1.36×10^{-2}	Significant
Hydrogen Gas	251.13	5.46×10^{-4}	Significant

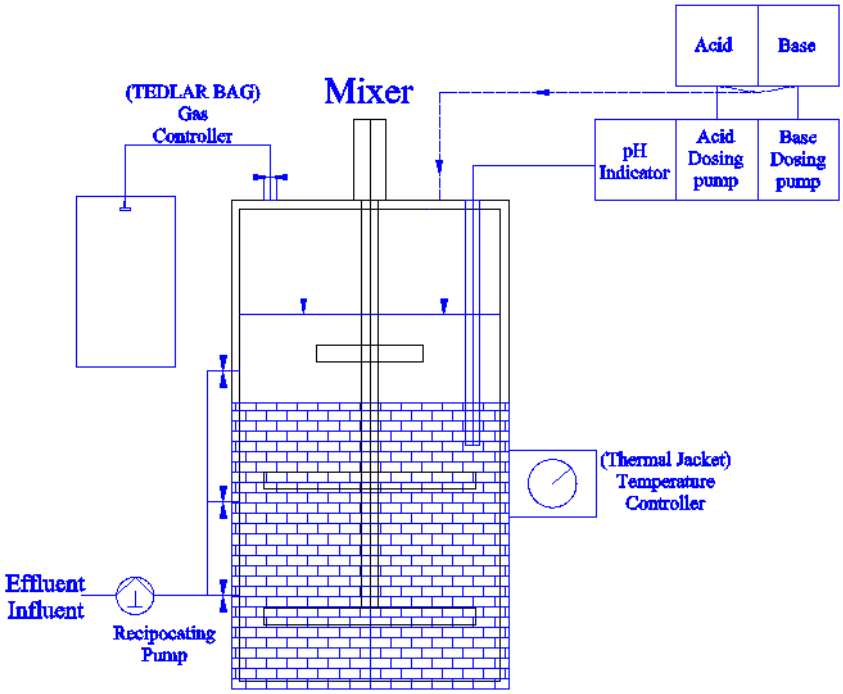

Figure 1: Schematic configuration of the Anaerobic Suspended Growth Closed Bioreactor (ASGCB)

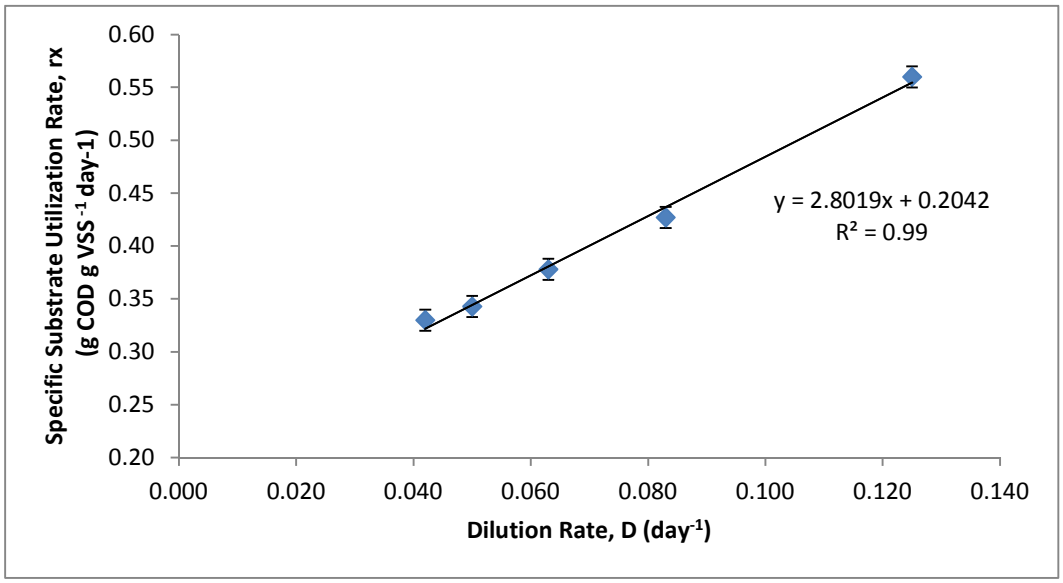

Figure 2: Plot of specific substrate utilization rate, r_x versus dilution rate, D (Equation 8).

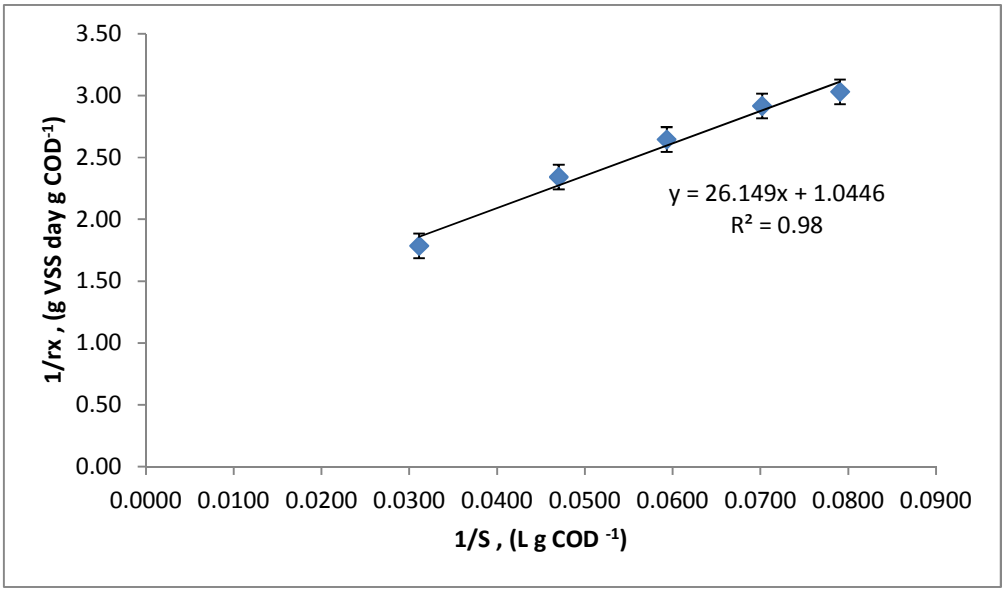

Figure 3: Determination of maximum specific substrate utilization, $r_{x,max}$ and saturation constant, k_s through Lineweaver-Burk plot (Equation 10)

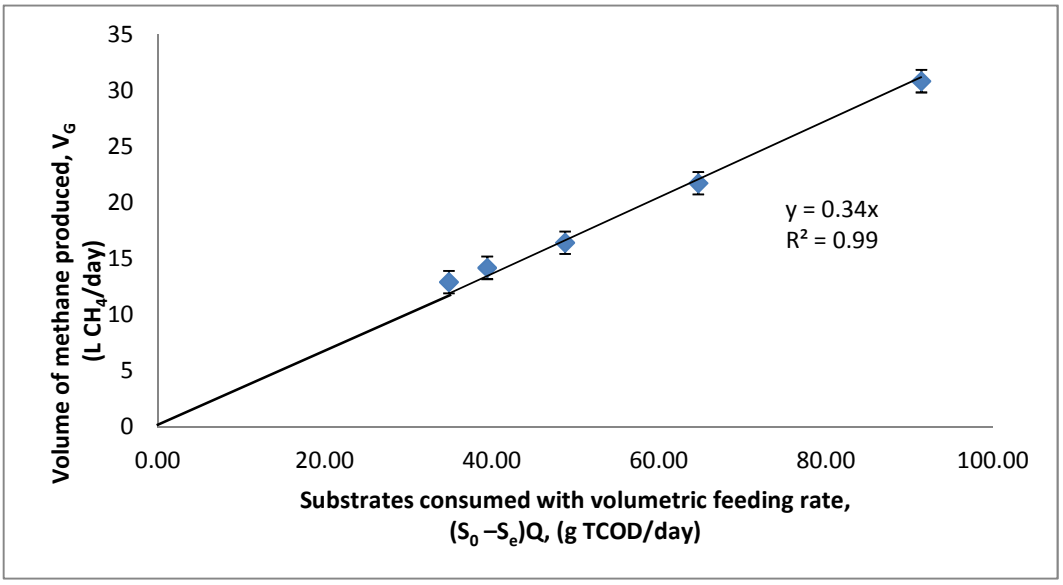

Figure 4: Determination of methane yield, Y_{CH4} by Equation (15).