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Physicochemical characteristics of attached biofilm on granular activated carbon for thermophilic biohydrogen production

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Abstract

In this study, thermophillic biohydrogen production by mixed culture obtained from a continuous acidogenic reactor treating palm oil mill effluent were improved by using granular activated carbon (GAC) as support material. Batch experiments were carried out at 60 °C by feeding the anaerobic sludge bacteria with a sucrose-containing synthetic medium at an initial pH of 5.5 under anoxic conditions. The physico-chemical characteristics of attached biofilm were evaluated after extraction of the extracellular polymeric substances (EPS) of the biofilm by formaldehyde- NaOH method. The main component of the biofilm was protein (60%); while carbohydrate content accounted for 40% of the EPS. Two major absorption bands at

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approximately 3400 cm^{-1} and 1650 cm^{-1}, characteristics of stretching vibrations of hydroxyl and amino groups, respectively, were identified in the FT-IR spectra, confirming the composition of the EPS. Observations using scanning electron microscopy (SEM) illustrated the attachment of rod-shaped bacterial cells on GAC at 60°C. The maximum hydrogen production rate of 4.3 mmol/L/h and hydrogen yield of 5.6 mol H_2/mol sucrose were obtained from this attached biofilm system. The major soluble metabolites of fermentation were acetic acid and butyric acid. The results showed that the granular activated carbon enhanced the biohydrogen production by stabilizing the pH and microbial metabolites and therefore could be used as support material for fermentative hydrogen production under thermophilic conditions in large scale.

***Keywords*:** Anaerobic sludge, Attached biofilm, Biohydrogen production, Granular activated carbon, Thermophilic anaerobic cultivation

Introduction

Hydrogen is a clean renewable energy with a great potential to substitute the depletion of limited fossil fuels. There are various ways to produce hydrogen. For example, anaerobic or fermentative hydrogen production is one of the most promising ways to stabilise waste organics and convert biomass into hydrogen using specific anaerobic bacteria. This process can fulfil a sustainable development as it only requires little energy input, utilises waste organics as substrates, and produces hydrogen, which a noncarbonaceous energy carrier.

Various microorganisms have been exploited to produce hydrogen, either in suspended or attached growth systems. Much have been focused on suspended culture systems to examine the performance of anaerobic biohydrogen production using either single/pure strain and/or mixed culture [1,2]. However, in case of mixed culture, several drawbacks have been reported with respect to the low hydraulic retention time (HRT), such as the washout of the biomass and unstable hydrogen-producing bacterial populations [3]. Therefore, stability of the microbial population has become one of the most crucial elements in biohydrogen production.

Some alternative strategies have been proposed e.g., by introducing attached growth system via immobilisation technique in an anaerobic fluidised bed (AFBR) [4], fixed bed [5], or upflow anaerobic sludge blanket (UASB) reactor [6] in order to enhance biomass retention time. Many studies have demonstrated that the yield and productivity in thermophilic fermentative hydrogen production is higher than in mesophilic hydrogen fermentation [7]. However, lower cell density has been a drawback of fermentation at thermophilic temperatures [8]. Therefore, attached biofilm instead of suspended growth seems to be very practical in keeping the culture density and at the same time increasing hydrogen performance [9].

Generally, immobilisation involves in the formation of a biofilm or granulation of cells during the fermentation process. This technology has relatively good potential due to the high cell density and adherence to the support material which avoids cell washout at a low HRT and, subsequently improves performance in two-phase separation [9]. There exists only a few reports on the biohydrogen production using attached growth systems under thermophilic conditions [9-10] as most investigators have focused on mesophilic conditions [11-12]. A long period of time is required for the granulation process, as reported by Yu and Mu [13]. Zhang et al. [14], on the other hand, demonstrated rapid formation of biofilms under anaerobic conditions in a fluidised bed reactor (FBR).

Biofilm formation depends on several factors such as (i) physical surface and chemical composition of support carrier, (ii) surrounding environment such as nutrient availability, pH, and temperature, and (iii) composition of microbial consortia [15]. Biofilm-based systems have been extensively used as immobilised cell systems as they enhanced the reaction rates and population dynamics [16]. However, these studies have been limited to the development of mixed microflora biofilms, and the physical conditions have not been well-characterised [17]. Further studies on the rapid development of biofilm and the characterisation of such films can minimise the mass transfer resistance and stabilise the hydrogen-producing bacteria on biofilm for a good hydrogen performance.

In this study, thermophilic fermentative hydrogen production was carried out by immobilization of the anaerobic sludge obtained from palm oil mill treatment plant on granular activated carbon (GAC) in batch mode. GAC has been known as an inert, hydrophobic sorbent favourable for cell attachment. The effect of pH on biohydrogen

production by thermophilic biofilm was compared with suspended cells (without support carrier of GAC) using sucrose-containing medium at 60°C. The characteristics and chemical composition of the GAC-attached biofilm developed under optimum pH condition was also examined. Finally, the biohydrogen production with this thermophilic biofilm using complex substrate like palm oil mill effluent (wastewater) as carbon source was also examined. This knowledge is important for the assessment of reactor performance with real wastewater in future studies.

1. Materials and methods

2.1 Mixed culture, carrier supports, and fermentation medium

Mixed microflora used in this study was obtained from a recent study [18].The anaerobic sludge underwent heat shock (heating at 80 °C for 60 min) and then acclimatized in a 500ml anaerobic reactor at 60°C with HRT of 48-12h by feeding palm oil mill effluent in a continuous mode [18]. The reactor effluent was collected (5L) and stored in laboratory cold room at 4^{o}C for further use.

The carrier used to attach the hydrogen-producing bacteria was granular activated carbon (GAC) grade VISORB with a mesh size of 10 × 16 VS 45 (Carbochem Inc., USA). The GAC was sieved using a sieve shaker (Model EFL 2000/2, Endecotts, London) to obtain the required particle size of 2–3 mm.

The medium used for biohydrogen production contained 10 g/L sucrose as the sole carbon and energy source and supplements (in g/L unless indicated otherwise) as follows: NH_4Cl, 1; NaCl, 2; $MgCl_2.6H_2O$, 0.5; $CaCl_2.2H_2O$, 0.05; $K_2HPO_4.3H_2O$, 1.5; KH_2PO_4, 0.75; $NaHCO_3$, 2.6; cysteine hydrochloride, 0.5; yeast extract, 2; resazurin, 0.5 mg; and trace

elements, 1 mL (R&M Chemical, UK) [19].

2.2 Batch fermentation

In this study, the effect of initial pH on thermophilic fermentative hydrogen production by anaerobic sludge was studied and compared using cells in suspended form as well as attached on granular activated carbon (GAC). The initial pH of the medium was varied from 5.0 to 7.5 with 0.5 increments and was adjusted using either 1 M HCl or 1 M NaOH. Batch cultivation was performed in a 100 ml serum bottle with working volume of 55 ml and 10% heat treated POME sludge as inoculum. Pre-grown, 24 hr old culture (5 ml) was added to 50 ml of aforementioned medium and the serum bottles were purged with nitrogen gas to create anaerobic condition. The immobilization of POME sludge were carried out by adding GAC in a ratio of 1:1 of heat treated POME sludge volume (ml) to GAC weight (g) in the serum bottle while fermentation without GAC was conducted in parallel. The serum bottles were incubated in a shaking water bath (Model SW22, Julabo, Germany) at 150 rpm, 60°C for 24 hours. The repeated batch cultivation process was continued for maximum five successive batches to compare the productivity and stability of hydrogen production at different initial pH. These experiments were done in triplicates.

The optimal initial pH obtained in repeated batch cultivation for biohydrogen production was used to conduct another batch study for profiling cumulative hydrogen productivity and to examine the biofilm formation. The experiment was conducted in a 50 ml serum bottle with a working volume of 25 ml medium. The bacteria grown and attached to the GAC during the final batch of cultivation (initial pH 5.5) was collected and added to 25 ml of the fresh medium adjusted to pH 5.5. The volume of medium (ml) to weight (g) of GAC ratio is

10:1. The serum bottles were purged with nitrogen gas to create an anaerobic condition and the serum bottles were incubated in a shaking water bath (Model SW22, Julabo, Germany) at 150 rpm, 60 °C. Samples were analyzed at every 3 hours interval for 48 hours. The biogas produced was sampled using disposable syringe for further analysis on the gas composition. The supernatant were analysed for soluble volatile fatty acids, total carbohydrates and zeta potential analysis. Biofilm characteristics were determined by compositional analysis of extracellular polymer substances (EPS) by chemical extraction and Fourier-transform infrared (FTIR) spectra. Scanning electron microscopy (SEM) was employed for visualization of biofilm on GAC. The experiment was conducted in triplicates.

2.3 Calculation of hydrogen production

The cumulative hydrogen production in the batch experiment was determined according to a modified Gompertz equation using Sigma Plot Software 10.0 (Systat Software Inc., USA). Theoretically, the modified Gompertz equation is as follows [20]:

$$H_t = H_m . exp\left\{-exp\left[\frac{R_m . e}{H_m}(\lambda - t) + 1\right]\right\} \qquad (1)$$

where H_t is the cumulative hydrogen production (mL), H_m is the maximum hydrogen production (mL), R_m is the maximum hydrogen production rate (mL.h^{-1}), e is Euler's number, e = 2.73, λ is the lag phase time (h), and t is the incubation time (h).

2.4 Monitoring and analysis of hydrogen gas, VFAs and sugar

Hydrogen gas production was calculated from headspace measurements of gas composition and the total volume of biogas produced at each time interval was determined using equation 2:

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \qquad (2)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and previous intervals, and V_H the total volume of headspace in the bottle [21]. The volume of biogas produced was measured using a gas-tight syringe (0.5 mL injection volume) and a gas chromatograph (GC, model SRI 8600C, USA) equipped with the helium ionization detector (HID) and the thermal conductivity detector (TCD). Helium (MOX 99.99%) gas was utilized as carrier gas for the GC and run at the flow rate of 25 mL/min. The initial oven temperature was set at 40^{o}C with the pressure of 2.7 psi. The temperature at 40^{o}C was maintained for 5 min, followed by a ramping of 30^{o}C per minute until the temperature achieved 220^{o}C before it was held for another 10 min.

The fermentation liquid was filtered through a 0.22 μm syringe filter before analysis. Soluble volatile fatty acids (VFAs) were analysed by HPLC analysis using an Agilent 1200 HPLC system (California, USA) with a REZEX ROA column (Phenomenex, USA) equipped with a refractive index detector (RID). The flow rate of 5 mM H_2SO_4 as the mobile phase was fixed at a constant 0.6 mL/min with isocratic elution at room temperature. The HPLC sample injection volume was 20 μL, and the standard curves were generated using different concentrations of mixed organic acids. Sucrose was analysed using phenol-sulphuric acid method.

2.5 Biofilm extraction, characterization and imaging

The biofilm attached to the carrier (GAC) was mashed with a pestle to detach the extracellular polymeric substance (EPS), placed in a centrifuge tube, and stored at −20 °C for

168 further analysis. The attached EPS was extracted according to the formaldehyde-NaOH
169 method as described by Liu and Fang [22]. Prior to extraction, EPS suspension was prepared by
170 adding 1.5 g (dry weight) of suspended biofilm in a 10 mL of ultrapure water (Sartorius,
171 Malaysia). Extraction was performed by treating the sample with 0.06 mL of 37% (w/w)
172 formaldehyde at 4 °C for 1 h. Following the exposure to formaldehyde, the suspension was
173 then treated with 5 mL of 1 M NaOH at 4 °C for about 3 h. Finally, the mixtures were
174 centrifuged at 10,000 g for 15 min to obtain the soluble EPS.

175 Once the EPS was extracted, the soluble EPS was evaluated for the carbohydrates
176 content using the phenol-sulphuric acid method. Proteins were determined based on the
177 Bradford method using bovine serum albumin (BSA) as the standard. The total of
178 carbohydrates and proteins were based on the quantity of volatile suspended solids (VSS) of
179 the biofilm and measured according to the American Public Health Association (APHA)
180 standard method.

181 The chemical composition of extracted EPS were also verified using FTIR spectrometry
182 (Model Nicolet 6700, Thermo Scientific, USA) via the attenuated total reflectance (ATR)
183 method. The FTIR spectra were acquired in the 4000–400 cm^{-1} region with a resolution of 2
184 cm^{-1} using transmission mode.

185 The zeta potential was measured using Zetasizer Nano Particle Analyser (Nano ZS, Model
186 ZEN 3600, Malvern Instrument Ltd., UK) and analysed using Dispersion Technology
187 Software (DTS) version 5.02. The supernatant used for zeta potential analysis was maintained
188 at about 0.02% on a wet-weight basis with ultrapure water (Sartorius Malaysia Sdn. Bhd.,
189 Malaysia).

Biofilm developed on GAC was visualised using field emission scanning electron microscopy (FESEM) (Model Supra 55VP, Carl Zeiss AG, Germany). The GAC-immobilised cells were fixed with 2% (w/w) glutaraldehyde and left overnight at 4 °C. The fixed samples were washed with 0.1 M phosphate buffer solution for three times and left for 10 min each. Dehydration was done by successive passages through 30, 50, 70, 80, 90, and 100% (w/w) alcohol. The dehydrated particles were then transferred to a Critical Point Dryer (Model Leica EM CPD 300, Leica Microsystems, Germany) for 1 h and 30 min. The dried samples were sputter-coated with platinum and finally analysed using FESEM.

2.6 *Validation of GAC-attached thermophilic biofilm using POME as substrate*

In order to validate the efficiency of GAC-attached thermophilic biofilm to produce hydrogen from palm oil mill effluent (POME), batch experiments were carried out in 50 ml serum bottles with 25 mL of working volume. For the purpose, synthetic medium as mentioned in Section 2.1 was amended by adding raw POME (10% v/v) and replacing 10g/L sucrose with glucose and xylose(3:1) mimicking sugars available in raw POME. Hydrogen production performance was also compared using synthetic medium without addition of POME but with mixed sugars (Glucose and xylose) in the same ratio. Medium pH was adjusted to 5.5 and was purged with nitrogen gas to create an anaerobic condition. Serum bottles were incubated in a shaking water bath (Model SW22, Julabo, Germany) at 150 rpm, 60°C. Analysis of biogas, soluble volatile fatty acids, and total carbohydrates were carried out as described in section 2.4.

3. Results and discussion

3.1 Batch cultivation

The effect of the initial pH of the mixed culture from heat treated POME sludge with GAC and without GAC (control) was studied in the pH range of 5.0 – 7.5. The effect of

initial pH in hydrogen fermentation is a crucial aspect because inappropriate pH could inhibit hydrogenase activity [23]. The results shown in Fig. 1 are the steady-state data of hydrogen performance of the last batch at different initial pH.

In suspended cells, the biohydrogen production rate gradually increases from 2.82 to 4.28mmol/H_2/L with the initial pH increasing from 5.0 to 6.5. However, further increment of initial pH to 7.0 reduced the biohydrogen production rate two-folds compared to that in pH 5.0. The maximum H_2 content of 47.8% was obtained at pH 5.5. Generally, pH values between 5.0 and 6.5 are reported to be favorable for better microbial activity and hence better hydrogen production [24].

In GAC-attached biofilm, both H_2 production rate and H_2 content increased with increasing initial pH from 5.0 to 5.5. With a further pH increase, the performance declined. Highest H_2 production rate and H_2 content of 5.32 mmol H_2/L/h and 51.2%, respectively, were obtained at initial pH 5.5 with GAC. In this study, biohydrogen production rate could be enhanced up to 51% by using GAC as support material at initial pH of 5.5. The optimal cultivation pH of 5.5 for GAC is in agreement with reports from other researchers on thermophilic hydrogen fermentation at 60^{o}C [8].

Furthermore, the maximum hydrogen production rate at initial pH 7.0 and 7.5 for control experiment shows the lowest value among other initial pH with approximately 1.0 mmol H_2/L/h. This observation is similar to Chong et al (2013), who also reported that the growth of bacteria as well as hydrogen production is restricted at pH above 7.0 in suspended culture [25]. However, it is interesting to note that, higher value for hydrogen production was recorded at initial pH 7.0 (4.3 mmol H_2/L/H) in presence of GAC in this study than those of initial pH

5.0 – 6.5 (in the range of 2.82 – 4.28 mmol H_2/L/h) in control experiment (Fig. 1). This is due to the mechanical stability of the biofilms formed on the GAC that have great binding capacity for organic matter, hence provides an environment that is rich in nutrients promoting microbial adhesion [26].

Therefore the experiment in presence of GAC even at a non-favorable pH condition exhibited better hydrogen production than the suspended cell in control experiments in all different initial pH studied. The immobilization system enabled cells to withstand considerable shear force and stay active towards a stressful environment [26].

From the microbiological point of view, the suitable pH at which hydrogen production would be optimum is subjected to the type of hydrogen producing bacteria (HPB) in the inoculum. In our recent study [18] focusing on the community analysis of the anaerobic sludge from palm oil mill treatment plant, it was shown that the reactor effluent harbours species of *Bacillus* as dominant culture. *Bacillus smithii* CMB-B1 and *Bacillus coagulans* M36 species have been identified to be responsible for the production of hydrogen.

The same sludge was employed in this present study. Generally, basilli thermophilic (thermophilic bacilli) is aerobic or facultative anaerobic bacteria that live in the temperature range of 45-70 °C. *Bacillus* species have already been reported as producing hydrogen other than *Enterobacter* and *Clostridium* species [27].

The temperature range and optimum pH for growth and hydrogen production of *Bacillus smithii* has been reported to be 25°C to 60°C and pH 5.7 by Nakamura et al. (1988) [28]. The potential of *Bacillus smithii* as hydrogen-producing bacteria was also studied by Grady et al. (1998) who used this species to convert waste biomass into hydrogen [29]. *Bacillus coagulans*

257 species has also been identified as a hydrogen producer in either mesophilic and thermophilic
258 conditions [30].

259 *3.2 Kinetic analysis of batch fermentation using GAC-immobilized cells*

260 Fig. 2 shows the kinetic analysis of biohydrogen and soluble metabolite production at
261 initial pH 5.5 using GAC-attached POME sludge. The comparison between the experimental
262 data and the predicted modified Gompertz model of the cumulative hydrogen production of
263 GAC-attached biofilm is shown in Fig 2(a). The kinetic parameters obtained were based on
264 the cumulative hydrogen production data fitted by the modified Gompertz model with H_m =
265 58.6 ml, R_m = 2.8 ml.h^{-1}, and λ = 0.003 h. From the results, the hydrogen production rate
266 (HPR) obtained was 4.3 mmol/L/h. An experimental H_2 yield of 5.6 mol H_2/mol sucrose,
267 which equals 2.8 mol H_2/mol hexose was obtained by using acclimatized GAC-attached
268 biofilm as inocula.

269 The concentration of soluble metabolite products (SMP) (as shown in Fig. 2b) increased with
270 an increase in the fermentation time, which was reflected in more hydrogen production, as
271 shown in Fig. 2(a). Primary SMP was acetic acid (HAc), comprising 40–55% of the SMP
272 followed by butyric acid (HBu) accounted for approximately 30–40% of the fermentation
273 liquor. In contrast, the production of ethanol (EtOH), which is considered an unfavourable
274 metabolite for hydrogen production, was less than 20% of SMP. The amount of acetic acid
275 produced at 48 h was 24.2 mM, followed by 22.5 mM butyric acid and 11 mM ethanol. The
276 prevalence of acetic acid and butyric acid in the culture supernatant suggested that the
277 acidogenesis pathway was favoured to produce higher biohydrogen [31]. Study by Kotay and
278 Das (2007) using *Bacillus coagulans* IIT-BT SI isolated from sewage sludge also produced

acetic acid, butyric acid and ethanol as the primary metabolite during fermentative biohydrogen production [30].

In this study, a higher yield of H_2 is obtained by using GAC-attached POME sludge in batch cultivation process under thermophilic condition, compared to Wu et al [31] using acclimatized, attached biofilm at mesophilic condition. The H_2 evolution was rapid with no lag time and consisted of 43.8 % hydrogen content of total biogas produced with almost threefold the working volume of fermentation. No methane was detected throughout the experiment.

Table 1 summarizes similar studies on biohydrogen production from mixed culture attached on activated carbon using sucrose as sole carbon source. Even though the carrier material and carbon source used are similar for all these studies, the current study seems to be unique in developing bioprocess for hydrogen production by acidogenic, thermophilic biofilm on GAC. It also revealed that employing repeated batch system, hydrogen yield even in smaller scale (serum bottle) can be improved up to 3-4 folds compared to Wu et al [31]. Further improvements on the fermentation performance can be achieved by using bioreactor systems where operational parameters such as pH and temperature can be controlled throughout the experiment.

The total volatile fatty acids (TVFAs) concentrations achieved at initial pH 5.5 after 48 h fermentation in presence of GAC were 46.7 mmol TVFAs (referring to the total amount of acetic and butyric acid) and hence contributed to higher hydrogen performance. Immobilization of hydrogen-producing bacteria on GAC gave protection from exposure to metabolite accumulation, low acid condition, and low substrate concentration during

fermentation.

3.3 Biofilm characterization and imaging

Biofilms are complex assemblages of microorganisms that are embedded in a matrix of extracellular polymeric substances (EPS). During repeated batch cultivation, mixed microbial cells grew in association with activated carbon surfaces resulted in the formation of biofilm. The characterization of biofilm was carried out by extracting EPS using chemical extraction followed by chemical content, zeta potential measurements and observation under FESEM.

3.3.1 Extraction and chemical composition of Extracellular polymeric substances (EPS)

EPS is a component of aggregation of hydrogen-producing bacteria (HPB) accumulating in the biofilm on GAC. EPS produced at the solid surface of the GAC promote microbial adhesion by altering the physicochemical characteristics of the colonized surface. They create scaffolds with suitable physical characteristics and interconnected GAC pore structure that promote cell attachment [26].

In this study, extraction was carried out using the formaldehyde-NaOH method as it is the most effective extraction method by Liu and Fang[22]. Composition of total carbohydrate (TC) and protein in extracellular polymeric substances (EPS) of GAC immobilised-cell biofilm at different interval time of fermentation are shown in Table 2. As can be seen in Table 2, the carbohydrate and protein levels in the extracted EPS constituents increased with an increase of the fermentation time, which may have been the result of enhanced adhesion between bacteria and GAC during biofilm formation. At the beginning of the fermentation (3-hour), both carbohydrate and protein contents were low. However, after 48 h of fermentation, carbohydrate and protein comprised of 9.2 mg/g VSS (40%) and 13.2 mg/g

VSS (60%) of the extracted EPS, respectively. The ratio of protein to carbohydrate (P/C) in the EPS for all contact times varied between 1.4 and 2.6. Similar results for protein/polysaccharide ratios of biofilm EPS between 1.8 and 5.4 was reported by Ras et al. [34].

Moreover, there seemed a direct relationship between increment of total EPS concentration (given in Table 2) with total soluble metabolites produced (as shown in Figure 2b) during hydrogen production. In this study, the total EPS concentration increased from 13.6 mg/g VSS after 3 h fermentation to 22.4 mg/g VSS after 48 h fermentation which can be correlated with the increment of total SMP (SMP = TVFA + EtOH) from 9.1 mM to 57.7 mM during 48 h of fermentation. EPS secreted by mixed microflora can be subdivided into bound EPS (eg: attached organic materials) and soluble EPS or sometimes referred as soluble microbial products (SMP) [35]. It is well established that the production of this SMP reflects the hydrogenase metabolic pathway and hence, the performance of hydrogen [23]. Thus, EPS concentration plays an important role in establishing the structural and functional integrity of microbial biofilms and exhibit a direct relationship with hydrogen production [36]. However, the types of SMPs and the degree of microbial adhesion on the immobilized carrier will vary depending on the microbial species and operational conditions such as temperature and pH.

3.3.2 FTIR spectra

Fig. 3 shows the FTIR spectra of EPS formed after 48 h of fermentation by the GAC-attached biofilm. As depicted in Fig. 3, three major absorption bands were found in the sample. The broadest absorption band at approximately 3400 cm^{-1} revealed the presence of –OH groups while absorption bands at approximately 1650 cm^{-1} indicated the presence of

346 COOH groups. The band near 700 cm−1 reflected the presence of unsaturated bonds in the
347 samples. These functional groups confirmed that the samples were exopolysaccharide.
348 Similar results have also been reported by Iyer et al (2005) and Kumar et al (2011) [37-38].

349 *3.3.3 Zeta potential*

350 Zeta potential is an important index to characterize the surface charge of sludge, which
351 would tend to decrease gradually with the reduction of negative charge of sludge surfaces [39].
352 Fig. 4 shows changes in the zeta potential and pH of the culture supernatant during
353 biohydrogen production under thermophillic condition. Before immobilization and
354 cultivation, the zeta potential of raw sludge from POME at pH 5.5 was −2.04 mV.
355 After cultivation, the zeta potential or surface charge of the supernatant reduced to a negative
356 value with increasing fermentation time. Just after 3 h of fermentation, the zeta potential was
357 slightly reduced from -2.04 mV to −2.58 mV, and finally to −9.17 mV after 48 h, at the end of
358 batch fermentation. Culture pH was shown to have similar trend as zeta potential and the pH
359 value decreased from an initial pH of 5.5 to 4.74.

360 In contrast, Lin et al. [40], reported that the zeta potential of the pure culture increased
361 with decreasing culture pH. Similar phenomena was observed by Zhang et al.[41] during
362 biofilm formation under acid incubation. In their study, an increase in the zeta potential of
363 GAC from 2.74mV to 76.41 mV accompanied with reduction in pH from 5.5 to 2.0. However,
364 it is worthy to note that, in the present study, pH fluctuation was not abrupt as other studies
365 and hence both pH and zeta potential showed similar trend. In addition, the increase in EPS
366 content with increasing fermentation time (as shown in Table 2) suggested that the culture
367 zeta potential could be influenced by adsorbing EPS that carried more negative charges [14].

Recently, Su et al [39] have demonstrated that the decrease of zeta potential may speed up the granulation progress as a necessary condition. Bacterial adhesion is determined by an interplay between hydrophobic and electrostatic interactions. When bacteria approach the surface of the support material, they experience an electrostatic repulsion since both the bacteria and the GAC particle surface are negatively charged [14]. However, as the zeta potential decreased, microbial aggregation tends to strengthen because the low zeta potential decreased the repulsive electrostatic interactions. According to Gottenbos et al. [42], the positively charged surface adversely affects biofilm formation. Thus, a negative surface charge as in cultivated sludge POME is an advantage for biofilm formation.

3.3.4 Microbial observation

The GAC-attached biofilm at the end of fermentation (48 h) was selected for field emission scanning electron microscopy (FESEM) analysis. The surface morphology (crevices and micropores) of GAC before attachment are shown in Fig. 5(a) and 5(b) under different magnifications. The porous structure and irregular surface of GAC provided the space for cell growth. Fig. 5(c) and 5(d) illustrates the biofilm on the GAC carrier. As can be seen in Fig 5(c), the rod-shaped bacterial cells with a length of 1.0–5.0 μm and a width of 0.5–0.6 μm were dominated on the biofilm. A closer examination (10,000× magnification) in Fig 5(d) revealed the attachment of individual cells onto the surfaces as well as within the cavities of GAC. These SEM images indicated that a stable and successful immobilization was achieved at thermophillic conditions. The surface porosity of GAC eased the bacteria to attach to the surface with the aid of the conditioning layer and the EPS formation at the substratum. EPS at

the substratum minimised the mass transfer resistance and stabilised the hydrogen-producing bacteria on biofilm for a good hydrogen performance.

3.4 Validation of GAC-attached thermophilic biofilm using POME as substrate

Sucrose (10g/L) has been used throughout the study to establish whether the GAC-immobilized system developed herein is successful for producing biohydrogen at thermophilic conditions or not. We have also examined biohydrogen production with this thermophilic biofilm using diluted palm oil mill effluent (POME) as carbon source. The POME used in this study had total suspended solids about 47.9±0.4 g/L. Generally, attached growth system is not suitable for substrates with high solid content. Therefore, we attempted to use diluted POME to study the performance of GAC-attached thermophilic biofilm on biohydrogen production. Fig. 6 depicts the experimental data and predicted profile by modified Gompertz model of cumulative hydrogen production from POME with GAC attached cells at 60°C. The maximum hydrogen produced in 25 mL medium is H_m of 40.3 mL or equivalent to R_m of 4.8 $ml.h^{-1}$. This is slightly lower than control (H_m of 44.3 mL and R_m of 5.7 $ml.h^{-1}$) which consisted of 100 % synthetic medium and glucose and xylose mimicking sugar composition in raw POME.

In this study, hydrogen yield in presence of POME was 1.75 mol H_2/mol sugar consumed, whereas the maximum hydrogen production rate was 4.1 mmol H_2/L/h. The soluble metabolite products (SMP) produced was 33.3 mM, with acetate and butyrate comprised of 99 % of the total SMP and 1% ethanol. The dominance of acetate and butyrate formation indicated that the pathway of hydrogen is the acidogenic pathway. Lower hydrogen yield was obtained when using POME (1.75 mol H_2/mol sugar) compared to sucrose (2.8 mol H_2/ mol

411 hexose) (Section 3.2), suggesting that the GAC immobilized cells need some adaptation time
412 to a new combination of carbon source which consisted of hexose (glucose) and pentose
413 (xylose) [43]. Nevertheless, the hydrogen production rate of POME (4.1 mmol/L/h) was almost
414 similar when using sucrose (Section 3.2) with 4.3 mmol/L/h probably because the culture
415 used in this study has already been acclimatized in POME. Moreover, as fermentation was
416 performed under optimum pH and temperature, the enriched community remained same over
417 the cultivation period with consistent fermentation performance.

418 **4. Conclusions**

419 The biohydrogen production performance of suspended sludge and immobilized cell
420 systems with GAC were investigated in this study. The optimal initial pH for thermophilic
421 biohydrogen production was found to be 5.5. Adding GAC resulted in a prompt microbial
422 colonization and biofilm development with a H_2 yield of 5.6 mol H2/mol sucrose and a
423 hydrogen production rate of 4.3 mmol/L/h. This may be due to the protective effect of GAC
424 carrier of the attached growth biofilm against the acidic environment compared to the
425 suspended culture. Further improvements on the thermophilic biohydrogen production by
426 GAC-immobilized cells are currently being attempted in fluidised bed anaerobic bioreactor to
427 examine the microbial adhesion and tolerance at low hydraulic retention time (HRT).

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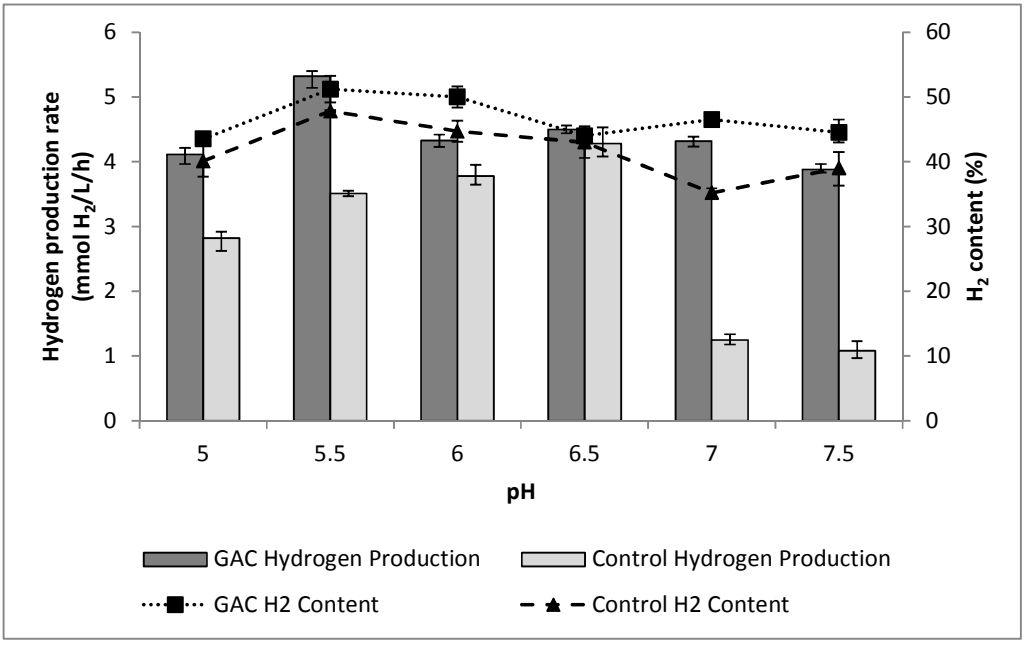
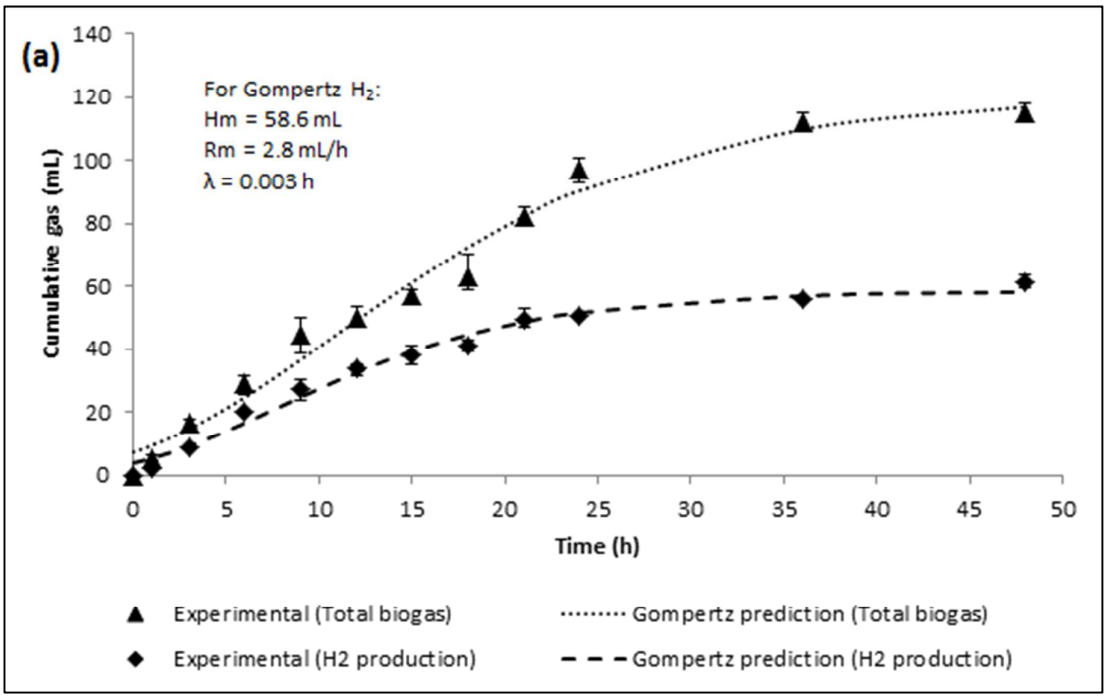


Fig 1 Hydrogen production rate and hydrogen content of final batch cultivation with GAC and without GAC (control) at various initial pH



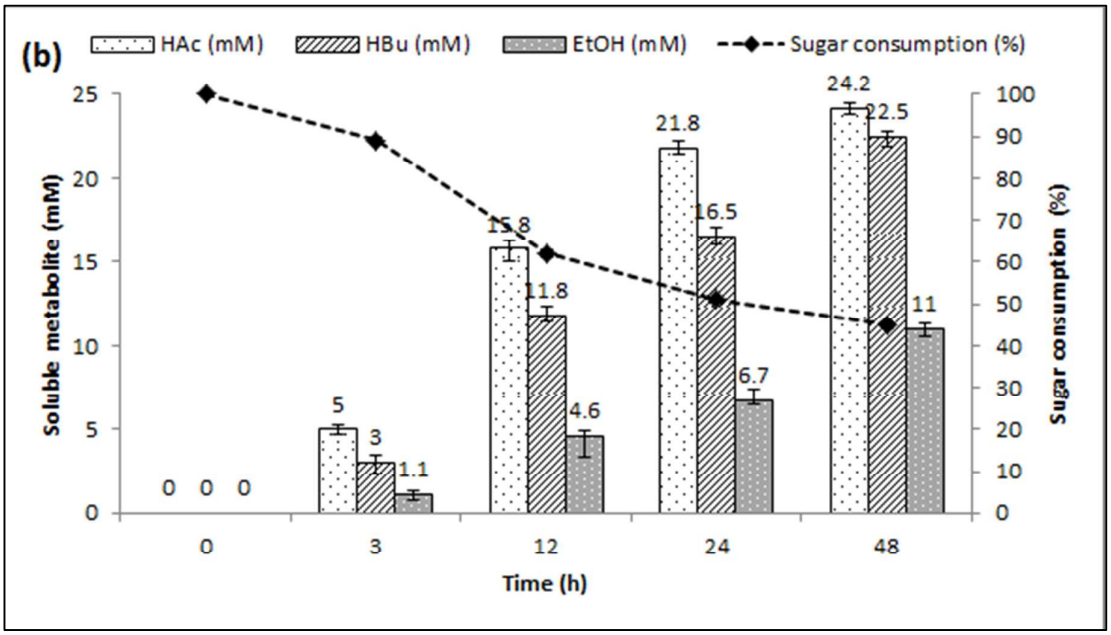


Fig. 2 –Batch kinetics of hydrogen production from sucrose with GAC attached biofilm at 60°C at initial pH of 5.5 (a) Gompertz curve fitting graph of cumulative gas production and (b) Composition of soluble metabolites (Acetic acid-HAc, Butyric acid-HBu and Ethanol-EtOH) and sugar consumption

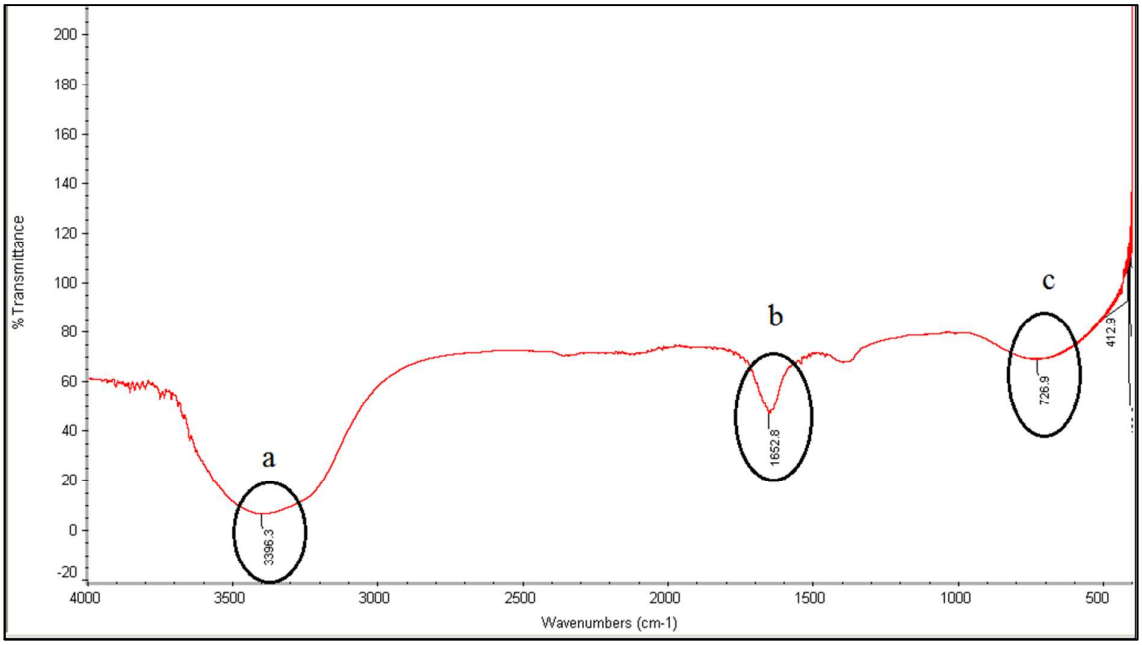


Fig. 3 FTIR spectra of GAC attached biofilm at 48 h interval time of fermentation with the stretching vibrations of (a) combination of hydroxyl and amino groups, (b) C(N)=O and C–N+N–H groups in protein, and (c) unsaturated bonds.

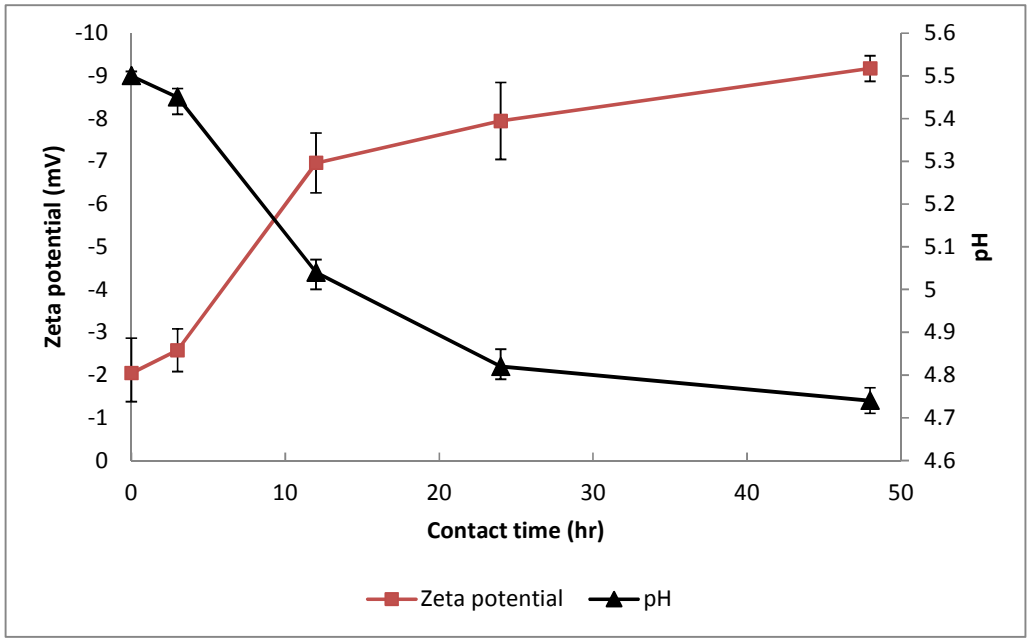


Fig. 4 Zeta potential and pH of supernatant at different interval time during biohydrogen production.

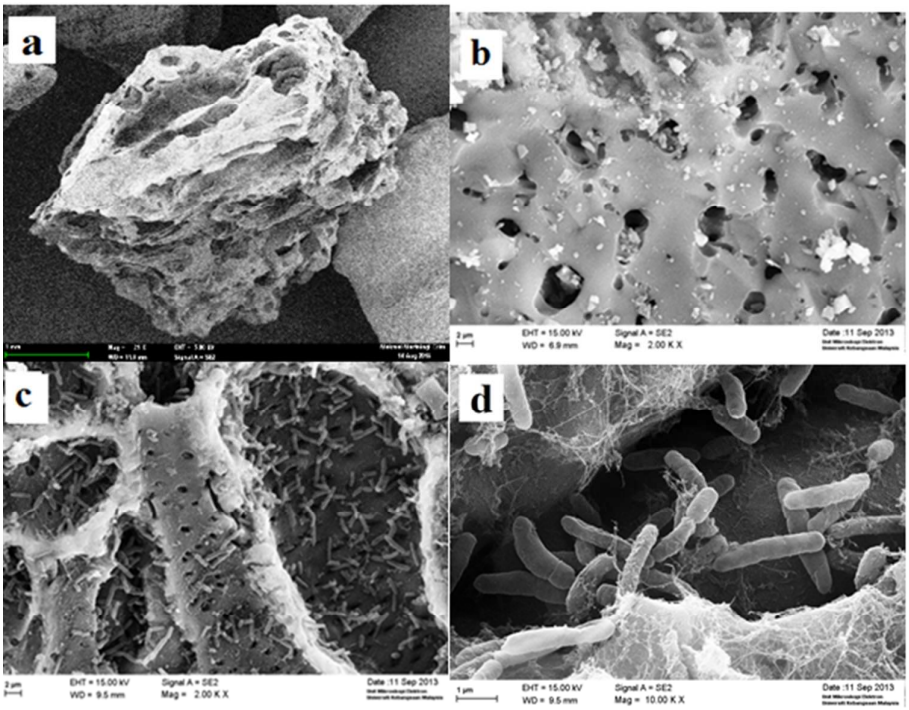


Fig. 5 SEM images of (a) single GAC (magnification 21×) particle and (b) porous surface structure of GAC (magnification 2,000×) before attachement, (c) bacterial colonization onto GAC after 48 h of fermentation (magnification 2,000×), and (d) close-up view of rod-shaped bacteria (magnification 10,000×) found as predominant species during biohydrogen production under thermophilic conditions (60 °C)

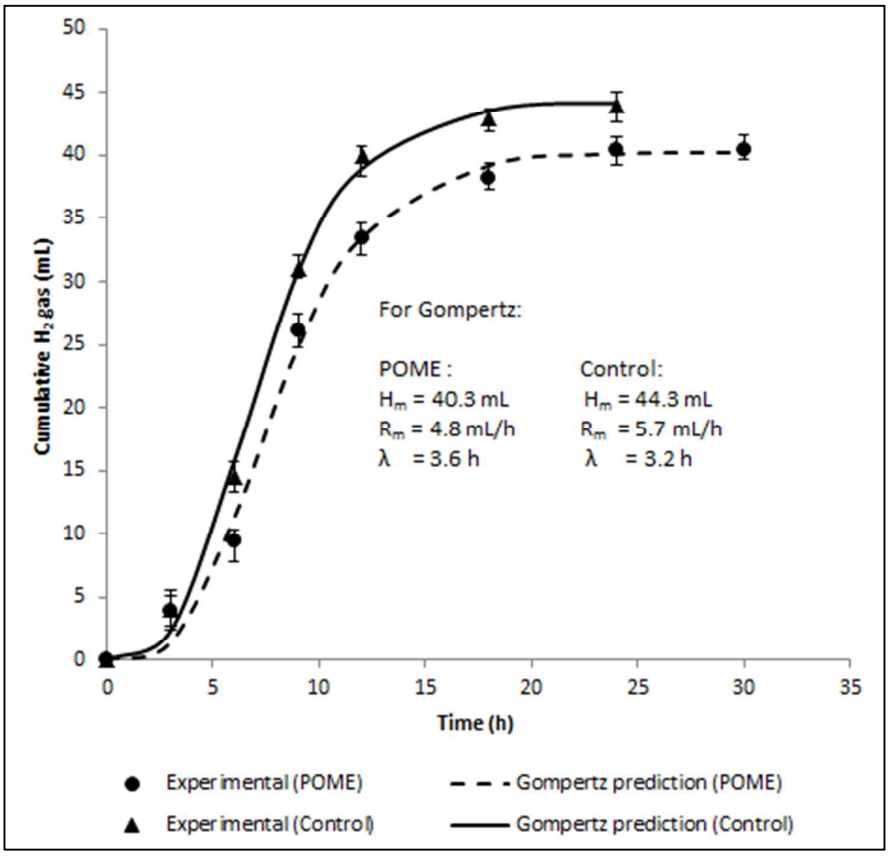


Fig. 6 - Batch kinetics of hydrogen production from palm oil mill effluent (POME) with GAC attached biofilm at 60°C

Table 1 Maximal hydrogen yield by mixed culture grown on activated carbon using sucrose as sole carbon source under different operational conditions

Microorganisms	Substrate	Immobilization carrier	Mode/ Process	Initial pH	Optimal Operation Temperature (°C)	Max. H_2 Yield (mol H_2 / mol hexose)	Reference
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Continuous/ Fixed-bed	6.7	35	0.59	[5]
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Continuous/ Packed-bed	6.7	35	1.45	[32]
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Continuous/ CIGSB	6.7	35	1.5	[33]
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Continuous/ CSABR	6.6 ± 0.2	40	1.93	[7]
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Batch/ Serum vial	6.7	40	0.87	[31]
Sewage sludge/ Mixed culture	Sucrose	Activated carbon	Continuous/ FBR	6.4	40	1.88	[11]
POME sludge/ Mixed culture	Sucrose	Granular activated carbon	Batch/ Serum bottle	5.5	60	2.80	This study

CIGSB - carrier-induced granular sludge bed; CSABR - continuously stirred anaerobic bioreactor; FBR – Fluidized-bed reactor

565 **Table 2** Composition of total carbohydrate and protein in EPS at different interval time of fermentation

Time (h)	Total EPS composition (mg/g VSS)		Ratio
	Carbohydrate	Protein	Protein/Carbohydrate
3	3.8 ± 0.4	9.8 ± 0.8	2.6
12	7.3 ± 0.3	10.6 ± 0.9	1.5
24	7.7 ± 0.2	12.3 ± 0.5	1.6
48	9.2 ± 0.3	13.2 ± 0.4	1.4

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