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RESEARCH ARTICLE

Utilisation of raw palm oil mill effluent (POME) as a renewable substrate for fermentative H_2 production: Optimisation using statistical approach

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Abstract

Fermentative H_2 production was studied using raw POME as the substrate with heat-shock pre-treated POME anaerobic sludge acted as the inoculum. The effect of crucial operating variables (initial pH of medium, incubation temperature, and inoculum size) on H_2 production was studied using Box−Behnken Design. A second-order polynomial regression model was generated to evaluate H_2 production trend under conditions tested. The model analysis revealed the high significance of linear effects of initial pH, incubation temperature, and inoculum size ($P < 0.01$) towards H_2 production. Similar results indicated that the interaction effect between initial pH and inoculum size, and interaction effect between incubation temperature and inoculum size were highly significant ($P < 0.01$). The regression model suggested that the optimum conditions were set to a pH value, incubation temperature and inoculum size of 6.4, 58.0°C and 8.0% v/v, respectively. In order to validate the optimum conditions determined by the model, heat-shock pre-treated POME anaerobic sludge was incubated with raw POME under optimum conditions. Validation experiment showed that a cumulative H_2 volume of 239.0 mL was produced. Microbial community analysis of inoculum showed that mixed consortia between *Clostridium* sp. and other obligate anaerobic non-spore forming bacteria, mostly belonging to the *Firmicutes* and *Bacteroidetes* phyla were identified as the major H_2-producers and were hugely responsible towards fermentative H_2 production.

Keywords: Fermentative H_2 production, POME anaerobic sludge, optimisation, Box−Behnken Design, microbial community analysis

INTRODUCTION

The palm oil industry in Malaysia has grown rapidly over the years and unsurprisingly, Malaysia is well-known as the world's second largest producer of palm oil, accounted for 10.3% of the world's oils and fats production in the year of 2007 (Lam *et al*., 2009; Choong *et al*., 2018). Even though Malaysia is behind Indonesia as the world largest palm oil producer due to the rapid expansion of oil palm plantations, Malaysia still holds its position as the world's largest crude palm oil exporter (AIM, 2015; Awalludin *et al*., 2015). As for global distribution of palm oil supply annually, more than 80% is accounted between Malaysia, Indonesia and Thailand, combined altogether (Amiruddin *et al*., 2005; Awalludin *et al*., 2015). The industry serves as an important backbone to the country's economy and has significantly increased the standard of living of its populations (Yusoff and Hansen, 2007).

In spite of this, sustainability of palm oil production has always been doubted, particularly by non-governmental organisations (NGOs), especially the involvement of palm oil mills, which is palm oil mill effluent (POME), the most existing visible pollutant that is produced by the mills themselves (Poh and Chong, 2010). The extraction of crude palm oil (CPO) involves the boiling of fresh fruit bunch (FFB), which needs an obviously large amount of water during the extraction process. Each tonne of crude palm oil produced, it is estimated between 5.0 to 7.5 tonnes of water needed with more than 50% of that water used in the extraction process ends up as POME (Yacob *et al*., 2006; Wu *et al*., 2009).

Generally, raw POME is an acidic brownish colloidal suspension that consists of 95 – 96% water, 0.6 – 0.7% oil, and 4 – 5% total solids (Onyia *et al*., 2001; Ahmad *et al*., 2006), along with high polluting properties with an average level of chemical oxygen demand, COD (50 g/L), biochemical oxygen demand, BOD (25 g/L), total solids (41 g/L), suspended solids (18 g/L) and oil and grease (6 g/L) (Ahmad *et al*., 2006), which are produced from the mixture of steriliser condensate, separator sludge and hydrocyclone waster in a ratio of 9:15:1 (Wu *et al*., 2010). It also contains cellulosic material, fat, oil and grease (Rupani *et al*., 2010; Iskandar *et al*., 2018). Even though the effluent is non-toxic since no chemical is being added during the extraction process of CPO from FFB, it produces polluted wastewater which not only affects the aquatic niche, but also contributes odour problems to local community (Borja *et al*., 1996; Loh *et al*., 2013). Furthermore, due to intense colour of POME, it can directly prevent sunlight from penetrating into the water streams, hence compromising photosynthetic organisms that live in the water (Azreen *et al*., 2017). This concerning

situation leads to a growing necessity by the palm oil mill industry for a sustainable treatment of POME to reduce the pollution occurred. Along with more than 440 mills functioning throughout Malaysia which produce POME in huge scale every day (Cheng *et al*., 2010; Gobi and Vadivelu, 2013), the discharge of POME into the water bodies such as rivers poses a possible threat to the environment and its surrounding.

Hydrogen gas (H_2) can be classified as an ideal, green, and sustainable energy carrier. It contains the biggest gravimetric energy density compared to any hydrocarbon fuel. It can be viewed as an alternative source of renewable energy to fossil fuel since the combustion of H_2 produces no greenhouse gases (GHG) emission (Chong *et al*., 2009; Azwar *et al*., 2014; Rezania *et al*., 2017). H_2 has been proposed as the ultimate transport fuel for vehicles and vessels because of its non-polluting characteristics, and it also enables the use of highly efficient fuel cells to convert chemical energy into electricity (Forsberg, 2007). This energy is high in demand for various applications of numerous industries, such as the transportation sector, as coolant in electrical generators at power stations and rocket propulsion (Goyal *et al*., 2006).

Optimisation has been studied and applied in other research areas, including the H_2 production when dealing with wastes as substrates. It is of great significance to produce H_2 from organic wastes by fermentative H_2 production, because it plays the dual role of energy production and waste reduction (Wang and Wan, 2009). Thus, this step is to ensure the system developed fits and increases into projection of production scale when it has been optimised. The optimisation studies of fermentative H_2 production have been widely reported (Wang and Wan, 2008; O-Thong *et al*., 2008; Wang and Wan, 2009; Rasdi *et al*., 2009; Nath and Das, 2011; Sreela-or *et al*., 2011a; Sreela-or *et al*., 2011b; Shanmugam *et al*., 2014; Azman *et al*., 2016a; Azman *et al*., 2016b; Azman *et al*., 2016c). This experimental strategy allows multivariate analysis by modification of several variables at one time, and moreover to optimise for the responses of interest. However, due to the nature of the source of microorganisms, substrates used, and system approach, there are wide variations to pinpoint the range level of certain factors.

Suitable microorganism which acts as inoculum is regarded as a significant factor that contributes toward fermentative H_2 production. Even though pure cultures have been studied broadly and in-depth, mixed cultures and naturally mixed consortia still have huge prospect when dealing with more complex substrates such as domestic and industrial wastewaters (Wong *et al*., 2014; Arimi *et al*., 2015). Thus, a pre-treatment procedure is necessary to be studied when naturally mixed consortia are used as inoculum in producing H_2. The pre-treatment works by enriching the H_2-producers and suppressing the H_2-consumers. Hence, the degree of H_2 production rate and yield can be bolstered when dealing with inoculum samples that are composed of various microorganisms (Wong *et al*., 2014). However, it should be noted that this pre-treatment procedure should be imposed with minimal cost which later imposes a huge impact towards scaling-up the production (Pendyala *et al*., 2012). Heat-shock, acidification, chemical and aeration are among the pre-treatment procedures that have been applied (Mohan *et al*., 2008; Wang and Wan, 2008; Mohammadi *et al*., 2011; Pendyala *et al*., 2012; Wong *et al*., 2014), in which the heat-shock pre-treatment is preferred over other options due to its easy implementation and cost-effective, with high production rate of H_2 (Wang and Wan, 2008; Mohammadi *et al*., 2011; Wong *et al*., 2014).

In this study, Box−Behnken Design (BBD) was chosen by combining 2^k factorials with incomplete block design, that is very efficient in terms of the number of required runs. It also does not contain any point at the vertices of the cubic region created by the upper and lower limits for each variable compared to Central Composite Design (CCD) (Anderson and Whitcomb, 2000; Montgomery, 2009), which was the main reason for BBD to be chosen in this study. Furthermore, there are limited knowledge and information nowadays in maximising H_2 production, especially raw POME as the substrate and using BBD as a tool of statistical approach for optimisation. Finally, the molecular analysis of microbial population from the sludge used in the study was performed to identify the H_2-producers which were responsible for H_2 production.

EXPERIMENTAL

POME sample collection and characterisation

POME samples were obtained from a local palm oil mill factory in Kulai, Johor, Malaysia. The samples were consisted of POME anaerobic sludge and raw POME, which later were stored in cold room facilities at 4°C to minimise the degradation of samples by indigenous microbial activities, thus preserving the quality of the samples. The characteristics of POME are summarised in Table 1.

Table 1 Characteristics of raw POME sample.

Parameter	Value (g/L)
COD	35.0 ± 4.5
BOD_5	23.0 ± 2.3
Colour intensity*	21.2 ± 3.3
Ammoniacal nitrogen	0.2 ± 0.2
pH*	4.6 ± 0.2
Total suspended solids (TSS)	27.0 ± 1.8
Mixed liquor suspended solid (MLSS)	23.1 ± 3.5
Mixed liquor volatile suspended solid (MLVSS)	11.4 ± 3.6

**All values in g/L except colour (ADMI) and pH*

Preparation of POME anaerobic sludge as source of inoculum

The bacterial inoculum used in this experiment was obtained from the POME anaerobic sludge. The sludge was initially passed through a mesh screen to remove fragments. The concentration of the volatile suspended solids (VSS) of the sludge was 11.4 ± 3.6 g/L. The sludge was pre-treated with heat-shock methods at 80°C for 30 min in order to suppress the H_2-consumers.

Fermentation of H_2 production in batch system

The experiment was conducted in 60 mL serum bottles with working volume of 40 mL. Raw POME was used as the substrate for fermentation without any additional nutrient. Heat-shock pre-treated sludge was inoculated with raw POME and incubated for 24 h to produce H_2. The system was flushed with N_2 for 5 min to ensure anaerobic condition throughout the experiment and was capped tightly with rubber septum. The initial pH, incubation temperature, and inoculum size of experiment were adjusted according to the values determined by BBD. As for pH adjustment, 1 M HCl and 1 M NaOH were used throughout of this experiment.

The experimental design of Box-Behnken Design

A response surface methodology using BBD was introduced to limit the sample size as the number of parameters grew and to determine the most suitable level for the selected variables to achieve optimal H_2 production from raw POME using heat-shock pre-treated POME anaerobic sludge as the inoculum. Three significant variables, initial pH (A), incubation temperature (B), and inoculum size (C) were studied in BBD. These were assessed at three coded values: low level (−1), centre point (0), and high level (+1), which resulted in 17 experiments including 5 centre points. Each of the experiment was conducted in triplicate and the average of H_2 production was defined as the experimental responses. The coded values of the independent variables were determined using the following equations:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad \text{− Eq. 1}$$

where;

x_i is the dimensionless coded value of variable;
X_i is the actual value of the ith variable;
X_0 is the actual value of X_i in the centre point;
ΔX_i is the increment value.

The experimental results were then fitted into the following second-order polynomial regression model to define the behaviour of the system:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{ii=1}^{k} \beta_{ii} X_i^2 + \sum_{ii=1}^{k} \beta_{ij} X_i X_j \quad \text{– Eq. 2}$$

where;

Y is the predicted response values (H_2 production);
k is the number of factor variables;
β_0 is the fixed value at centre point;
β_i is the linear coefficient;
X_i is the value of ith variable;
β_{ii} is the quadratic coefficient;
β_{ij} is the interaction coefficient.

Statistical regression analysis, analysis of variance (ANOVA) and 3D graphical plotting, Design-Expert® 7.0 software (Stat-Ease Inc., Minneapolis MN, USA) were used to determine the fitness of model, significance of the variables and their interactions as well as their effects towards the H_2 production. Table 2 shows the summary of the BBD experimental design for this study.

Table 2 Coded and actual values of experimental variables used in BBD.

Variable	Component	Level		
		–1	0	+1
A	Initial pH	6.25	6.50	6.75
B	Incubation temperature (°C)	57.5	60.0	62.5
C	Inoculum size (% v/v)	5.0	7.5	10.0

Verification of the model

In order to validate the optimum conditions predicted by the model, a set of batch system was performed under the optimum conditions. The results obtained in the verification experiment were then used to determine the accuracy of the model.

Analytical methods and analysis

The water displacement method was used to collect and measure the volume of biogas produced. The fraction of H_2 in the biogas was determined by a Gas Chromatograph-Thermal Conductivity Detector (Agilent 7890B Model, Agilent Technologies, Santa Clara CA, USA) fitted with HayeSep Q and MolSieve 5A columns. He and Ar were used as the carrying gases at the flow rate of 45 mL/min. The temperatures of the oven, column inlet and detector were 60°C, 190°C and 250°C, respectively. The biogas was manually injected and the injection volume was 1 mL. All wastewater parameters that conducted in this study were measured according to the Standard Methods (APHA, 2012).

The H_2 production was calculated from the serum bottle headspace measurements of gas composition and the total volume of biogas at each interval using the following equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \quad \text{– Eq. 3}$$

$V_{H,i}$ and $V_{H,i-1}$ are cumulative H_2 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 $C_{H,i-1}$ the fraction of H_2 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 (Chong *et al*., 2009; Azman *et al*., 2016c).

Microbial community analysis

Sampling, DNA extraction, and PCR

Sludge was retrieved from the bottom of the serum bottle and stored at 4°C. The total genomic community DNA was extracted using DNeasy PowerSoil Kit (Qiagen, USA). The procedure was performed in accordance with the manufacturer's protocol. The PCR mixture (50 µL total volume) contained GoTaq® Master Mixes (Promega Corp., Fitchburg WI, USA), nuclease free water, 100 ng/L of DNA template and 10 µM of each primer (Fd1 = 5' AGA GTT TGA TCC TGG CTC AG 3' and rD1 = 5' ACG GCT ACC TTG TTA CGA CTT 3'). The PCR was performed in an EppendorfTM MastercyclerTM Nexus Thermal Cycler (Thermo Fisher Scientific, Waltham MA, USA). The amplification was performed with thermal cycling including initial denaturation at 95°C for 2 min, 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min), and finally extension at 72°C for 10 min (Bay, 2014).

Cloning 16S rRNA

PCR products were purified from a 1% agarose gel using a gel extraction kit according to the manufacturer's instructions (Qiagen, Germany). The recovered PCR fragment was cloned into pGEM®-T vector (Promega Corp., Fitchburg WI, USA) and transformed into JM109 High-Efficiency Competent Cells (Promega Corp., Fitchburg WI, USA). White colonies were randomly selected from plates.

Sequences analysis

The recombinant plasmids were extracted using EasyPure® Plasmid MiniPrep Kit (TransBionovo Co. Ltd., China) according to the manufacturer's instructions. Restriction endonuclease EcoRI (TransBionovo Co. Ltd., China) was used on a 1% agarose gel to verify the insertion of fragment. Approximately 5 nucleotides were sent to Apical Scientific Sdn. Bhd., Malaysia for sequencing. Chromatograms were edited using ABI Sequence Scanner 2 and GeneDoc software, while DECIPHER (Wright *et al*., 2012) was used to scan for potential chimeric sequences. The sequences were compared to known 16S rRNA sequences in the GenBank™ database, using the basic logical alignment search tool (BLAST) to locate nearly exact matches in the GenBank database. DNA sequences were aligned using the program ClustalW (Thompson *et al*., 1994) and further edited manually. Phylogenetic analyses were performed using the neighbour-joining (NJ) method by the MEGA ver. 7.0 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Box-Behnken design

The introduction of BBD, which formed by combining 2^k factorials with incomplete block design and is very efficient in terms of the number of required runs. It also does not contain any points at the vertices of the cubic region created by the upper and lower limits for each variable compared to CCD. A strong advantage of BBD is when the points on the corners of the cube represent factor-level combinations that are prohibitively expensive or impossible to test due to the physical process constraints (illustrates in Fig. 1) (Anderson and Whitcomb, 2000; Montgomery, 2009).

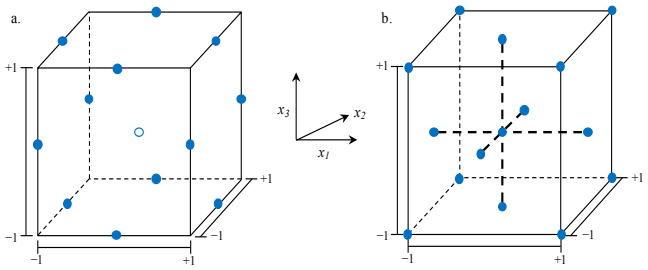

Fig. 1 A comparison between two designs; a) BBD for 3 factors, and b) face-centred CCD for k = 3.

A total of 17 experiments with different combinations among initial pH (A), incubation temperature (B), and inoculum size (C) were labelled as independent variables to determine their individual and combined effects on H_2 production (Y) through the development of a statistical correlation model using BBD. Central points consisted of five experiments were designated in this study and the remaining runs were evaluated in two different levels (lower level (−1) and higher level (+1)) as according to Table 3.

Table 3 The BBD experimental design.

Run	A	B	C	Cumulative H_2 production (mL)	
				Experimented	Predicted
1	6.25	57.5	7.5	231.3	232.5
2	6.75	57.5	7.5	195.4	196.8
3	6.25	62.5	7.5	209.7	208.3
4	6.75	62.5	7.5	171.0	169.8
5	6.25	60.0	5.0	167.2	169.3
6	6.75	60.0	5.0	103.9	105.7
7	6.25	60.0	10.0	194.0	192.2
8	6.75	60.0	10.0	183.8	181.7
9	6.50	57.5	5.0	172.1	168.9
10	6.50	62.5	5.0	127.6	127.0
11	6.50	57.5	10.0	201.4	202.0
12	6.50	62.5	10.0	189.5	192.7
13	6.5	60	7.5	227.0	222.4
14	6.5	60	7.5	223.0	222.4
15	6.5	60	7.5	226.0	222.4
16	6.5	60	7.5	218.0	222.4
17	6.5	60	7.5	218.0	222.4

Based on the 17 experimental runs by referring to Table 3, the data showed that the model could be best explained in quadratic model. Thus, the equation for this model was expressed as:

$$Y_{H_2} = 222.40 - 18.51A - 12.80B + 24.74C - 0.70AB + 13.28AC + 8.15BC - 15.49A^2 - 5.06B^2 - 44.69C^2 \quad \text{Eq. 4}$$

where Y is the predicted cumulative H_2 production while A, B and C represent for coded values of initial pH, incubation temperature, and inoculum size, respectively.

The determination of the fitness of the model was based on the *F*-value and *p*-value from the statistical characteristic of the quadratic polynomial model. The *F*-value demonstrates the significant effect of the variance towards the model by comparing the ratio of the mean square (MS) model to error mean square. By referring to Table 4, *F*-value of the model was 132.88 with the *p*-value of < 0.0001. This indicated that this model was significant based on the *p*-value obtained.

In addition, the *F*-value and *p*-value could also be used to determine the significance and interactive strength among each of the individual model term. According to this study, results showed that factor A, B, C, AC, BC, A^2, B^2 and C^2 had profoundly affected the H_2 production with *p*-value of < 0.05, excluding factor of AB. Besides, the *p*-value lack-of-fit test of the model was > 0.05 (*p*-value of 0.5567), which was illustrated as insignificant. Hence, with the insignificant of lack-of-fit test, it showed that the model had been fitted well with the data and would provide a solid foundation for predicting H_2 production under any variable combination.

Table 4 Analysis of variance (ANOVA) and results of regression line analysis of BBD.

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	19971.78	9	2219.09	132.88	< 0.0001
A	2741.70	1	2741.70	164.17	< 0.0001
B	1310.72	1	1310.72	78.48	< 0.0001
C	4895.55	1	4895.55	293.14	< 0.0001
AB	1.96	1	1.96	0.12	0.7420
AC	704.90	1	704.90	42.21	0.0003
BC	265.69	1	265.69	15.91	0.0053
A^2	1009.95	1	1009.95	60.47	0.0001
B^2	107.91	1	107.91	6.46	0.0386
C^2	8408.31	1	8408.31	503.48	< 0.0001
Residual	116.90	7	16.70		
Lack of Fit	43.70	3	14.57	0.80	0.5567
Pure Error	73.20	4	18.30		
Cor Total	20088.68	16			

Note: R^2 = 0.9942; adjusted R^2 = 0.9867; predicted R^2 = 0.9595; adequate precision = 40.444

The statistical significance of the model was further checked by other regression analyses based on Table 4. The R^2 coefficient value was 0.9942, which ~~is~~ suggested as a good correlation between experimental and predictor variables since 99.42% of the response variation could be explained using the model. The adjusted R^2 value (0.9867) was very close to predicted R^2 value (0.9595), which indicated that the model was highly accurate in predicting the response. The adequate precision value for this study was 40.444. This adequate precision value demonstrated that the model has strong adequate discrimination ability since the value was greater than 4. Cao *et al*. (2008) reported that the coefficient of variation (CV) can be used to represent the relative variability of the model, which is measured in terms of the distribution of residual data variations around the mean value, and the model can be classified as reasonably reproducible if the CV is below 10%. Hence, the CV for this study was reflected at 2.13%, indicating that the model was sufficient to navigate in high precision and was dependable.

Singh *et al*. (2013) reported that the normal plot of residual is a crucial tool to diagnose, validate and explain the systematic departures of residuals from the statistical assumptions. Fig. 2 illustrates the residuals were plotted against the normal distribution of the model. Results indicated that the residuals for H_2 production were normally distributed since all the points have been clustered around the diagonal line and were independent of each other. Hence, the normal distribution plot ~~had~~ obeyed the assumption of normality which specified that the deviation from the linear was very minor. Therefore, it could be concluded that all residuals came from a normal distributed population and the error of variances was homogenous (Anderson and Whitcomb, 2000; Montgomery, 2009).

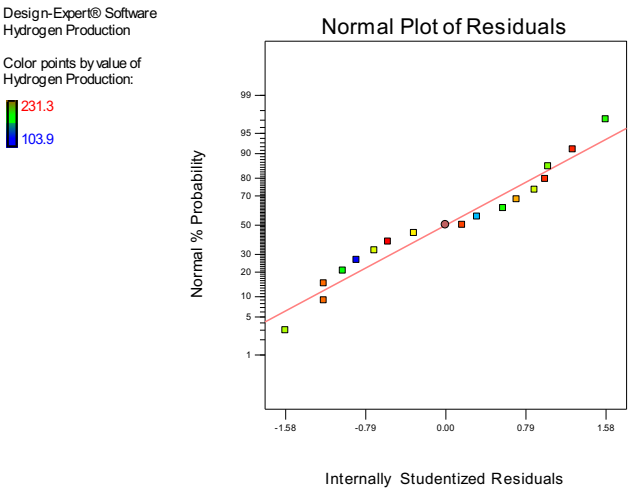

Fig. 2 Normal plot of residuals for H_2 production.

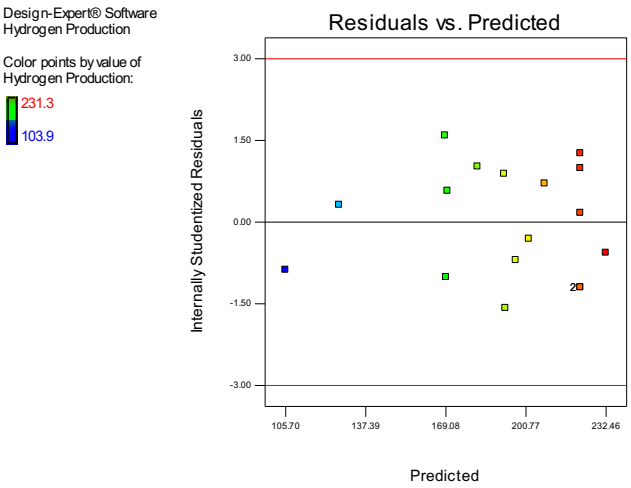

Fig. 3 Residuals against predicted plot for H_2 production.

Fig. 3 illustrates the residuals against predicted response plot which subjected for checking the appearance of non-constant variance in model by evaluating the pattern of the plotted points. In this study, the residual was randomly scattered without any specific pattern and trend such as a V-shaped pattern, which demonstrated that the residuals were from a common normal distribution with constant variance, but with no correlation between different residuals (Montgomery, 2009; Hoerl, 2012). Hence, the model was adequately fitted to the data, and was able to predict the response.

Localisation of H_2 production optimum conditions

Fig. 4 until Fig. 6 illustrate the three-dimensional (3D) response surface plots and corresponding contour graph for H_2 production. These were indicative of the infinite combined effects of any two independent variables towards maximum H_2 production with all other independent variables fixed at zero level. The optimum H_2 production value was at the central point of the smallest ellipse in the contour graph or at the maximum hump of the 3D graph.

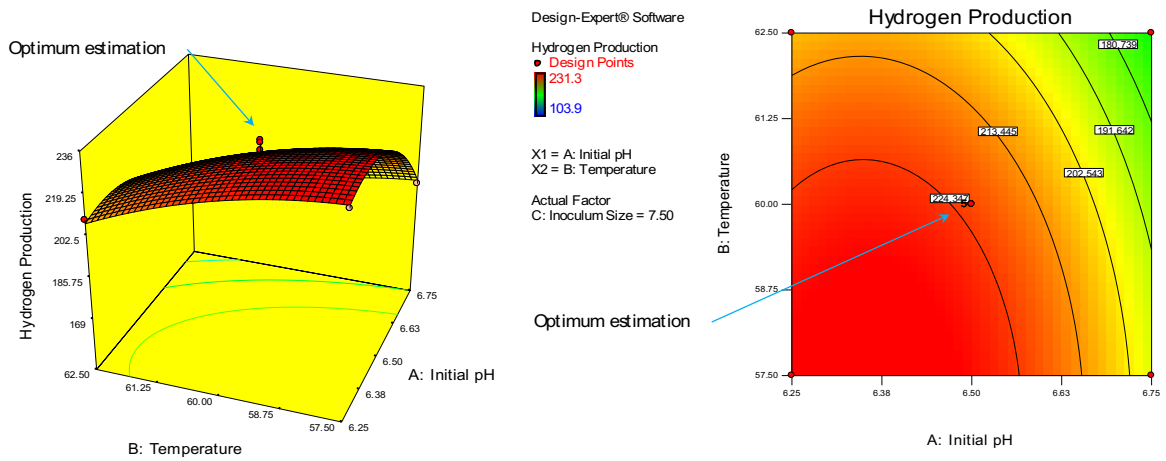

Fig. 4 3D response plots and 2D contour plots showing the effects of initial pH, incubation temperature and their mutual interaction on cumulative H_2 production with optimum level of inoculum size at 7.5% v/v

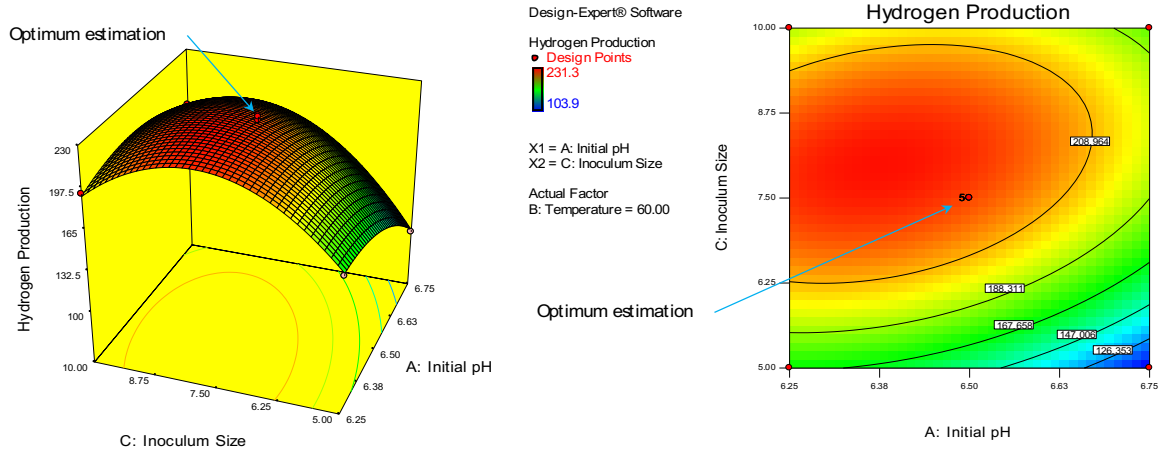

Fig. 5 3D response plots and 2D contour plots showing the effects of initial pH, inoculum size and their mutual interaction on cumulative H_2 production with optimum level of incubation temperature at 60°C

Initial pH of the substrate which acted as the medium for H_2-producers was considered as an important factor which affected fermentative H_2 production. It was directly involved with the metabolic activity of the H_2-producers as well as the metabolism pathway (Wang and Wan, 2009; Azman *et al*., 2016c; Wang and Yin, 2017). Based on Fig. 4 and Fig. 5, when the initial pH value of substrate was increased from 6.50 to 6.75, a drastic drop in H_2 production could be observed. Similar studies were reported previously by Lin and Cheng (2006) and Dareioti *et al*. (2014), in which their H_2 production was increased until pH values of 6.0 and 6.5 before being subsequently decreased when the pH values exceeded pH 7.5 and pH 9.0, respectively. It has been demonstrated that in an appropriate range, increasing the pH could improve the ability of H_2-producers to produce H_2 during fermentative H_2 production. However, pH value of the substrate that ~~is~~ was too high might ~~may~~ result in a decrease in the activity of hydrogenase enzyme of H_2-producers with the shifting metabolic pathway from the acidogenic phase to the solventogenic phase, which in turn could lead to a reduction in H_2 production (Gadhe *et al*., 2013; Azman *et al*., 2016c).

Incubation temperature was a crucial factor which would influence H_2 production by H_2-producers (Wang and Wan, 2009; Junghare *et al*., 2012; Azman *et al*., 2016c; Wang and Yin, 2017). As shown in Fig. 4 and Fig. 6, the production of H_2 was gradually decreased when the incubation temperature was elevated from 60.0°C to 62.5°C. This scenario was probably due to the switch from acidogenesis pathway of fermentative H_2 production to the solventogenesis phase in which acetic acid and butyric acid were further processed to produce solvents including acetone, butanol and ethanol (Liu *et al*., 2011; Khamaiseh *et al*., 2014; Azman *et al*., 2016c). Furthermore, high temperature imposed on H_2-producers might deteriorate the optimum physiological condition which involved its functional enzymes (Wang and Wan, 2009). Yokoyama *et al*. (2007), Lee *et al*. (2008) and Zhang *et al*. (2015) showed in their reports that beyond their optimal temperature, the H_2 production was tended to decline even though their inoculum were productive at thermophilic condition. Thus, an optimal condition of incubation temperature was ~~is~~ highly dependable on microbial biocatalysts which later were responsible towards higher H_2 production.

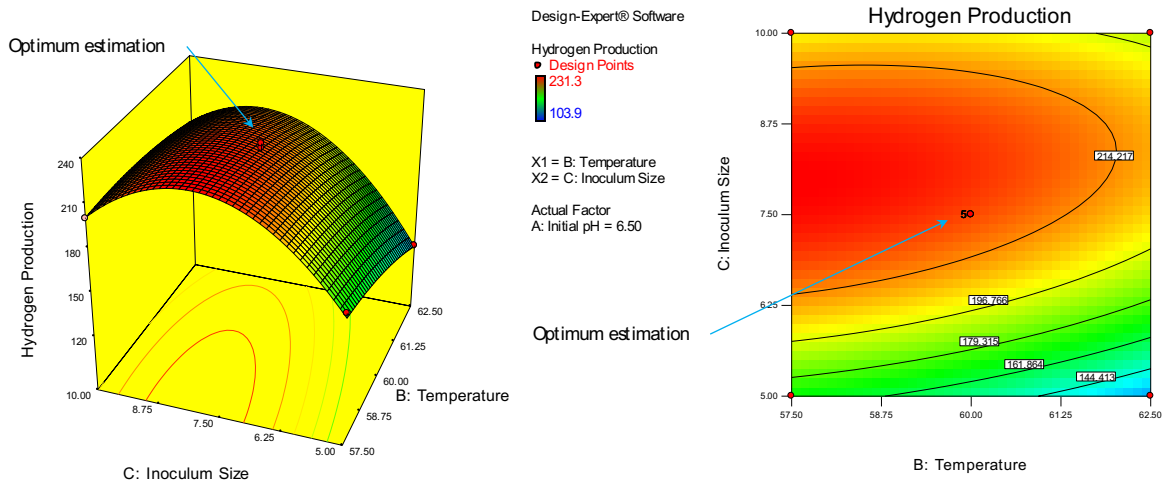

Fig. 6 3D response plots and 2D contour plots showing the effects of incubation temperature, inoculum size and their mutual interaction on cumulative H_2 production with optimum level of initial pH at 6.5

An appropriate inoculum size mixed with substrate might lead to higher H_2 production as demonstrated in Fig. 5 and Fig. 6. H_2 production was increased from 5.0% v/v until 7.5% v/v but started to decline when inoculum size exceeded 7.5% v/v. This scenario explained that when the inoculum size was increased, the substrate available for the H_2-producers was tended to be limited, leading to certain microorganisms starving, hence decreasing the survival rate of H_2-producers available for H_2 production. This scenario was also observed by Chittibabu *et al.* (2006) and Lazaro *et al.* (2015) in their studies which both concluded that up to a certain and suitable value, an increase in inoculum concentration could positively affect the H_2 production.

Based on Fig. 4 until Fig. 6, it was obvious that initial pH, incubation temperature, and inoculum size exhibited significant influence towards H_2 production. The value of initial pH would affect the electric charge on the cell membrane surface, which gave impacts on both microbial biocatalysts activity and nutrient adsorption. The optimal pH condition for fermentative H_2 production was ranged from pH 4.5 to pH 9.0, in which pH value lower than 4.5 could lead to the microorganisms' deterioration and would further suppress the activity of H_2 production (Ghimire *et al.*, 2015). Therefore, a low initial pH could simply prevent substrate from being further utilised for H_2 production. However, Khanal *et al.* (2003) reported that the neutral operational initial pH favoured the acetic acid pathways which significantly produced higher H_2 production compared to butyric acid pathways that preferred acidic pH condition. Incubation temperature influenced the microbial biocatalysts activity significantly, where low temperature might inhibit the crucial enzymes for H_2 production and lead to both low H_2 production rate and low substrate utilisation rate. Even though there was no concrete conclusion of optimal temperature for fermentative H_2 production, the best and effective H_2 production was between 35°C to 37°C and 55°C to 60°C for mesophilic and thermophilic temperature, respectively (Wang and Wan, 2009; Wang and Wan, 2011; Zhang *et al.*, 2015). Suitable inoculum size was crucial in producing high yield of H_2, hence both inoculum size and substrate available were dependent towards each other. Inoculum size that exceeded the limit in the system could lead to low substrate available which later resulting in low H_2 production and vice-versa due to survival of microorganisms' lack of nutrient (Chittibabu *et al.*, 2006; Lazaro *et al.*, 2015).

The 3D and contour plots from Fig. 4 until Fig. 6 were used to present the predicted response as a function of process factors (Anderson and Whitcomb, 2005). Yet, the effects of two factors could only be presented for each graph. Therefore, perturbation plots were used to demonstrate the effects by changing each factor towards the response and at the same time keeping all other factors constant (Anderson and Whitcomb, 2005). Fig. 7 illustrates the perturbation plot consisted of all factors studied in BBD. Based on the Fig. 7, it was clear that all factors had contributed towards H_2 production. Both initial pH (factor A) and incubation temperature (factor B) showed that increasing the level could cause H_2 production to decrease gradually after the centre point value while the inoculum size (factor C) depicted an increase in H_2 production up to the centre point value and started to decline beyond the point.

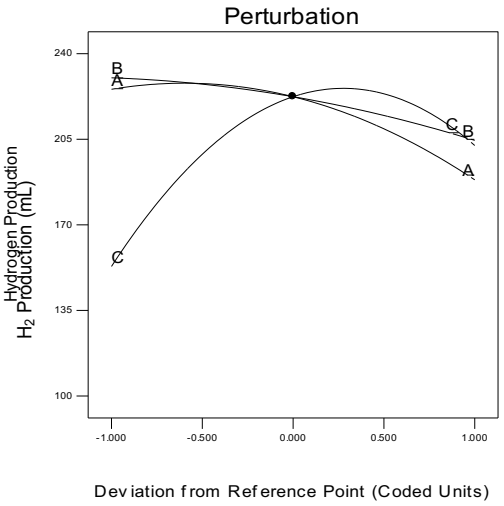

Fig. 7 Perturbation plot showing the effect of each factor towards H_2 production at their respective middle point. Legend: A – initial pH, B – incubation temperature, and C – inoculum size

Model validation

The optimum H_2 production using raw POME as the substrate with heat-shock pre-treated POME anaerobic sludge as the inoculum was predicted by the Design-Expert® software according to the desirability function which had assigned relative importance to the maximum H_2 production. Based on the solution with highest desirability as suggested by the program, the optimum parameters for maximum H_2 production were as followed: 6.4 of initial pH, 58.0°C of incubation temperature and 8.0% v/v of inoculum size. All validation experiments were carried out in 60 mL serum bottle, in triplicate ~~as~~ for the accuracy of the data.

The maximum predicted and average cumulative H_2 production observed after validation experiments were 234.8 mL and 239.0 mL, respectively. By comparing between predicted and actual experiment results, it showed that there was 1.79% increment of observed H_2 production value compared to predicted value. Therefore, the findings demonstrated the high effectiveness of the model in predicting the optimal level of factors, which indicated the optimisation process was successful.

Microbial community analysis

16S rRNA analysis was carried out based on the DNA extract from the heat-shock pre-treated sludge taken from the bottom of serum bottle at the end of the experiment. All had a DNA insert approximately 1350

bp from 22 screened clones. The phylum *Bacteroidetes* recorded the biggest clone portion at 59.1%, followed by phylum *Firmicutes* (31.8%) and phylum *Thermotogae* (9.1%) (Table S1, Supplementary Data). Partial DNA sequences were obtained from representative amplicons. Five clones were sequenced and were used to search the GenBank nucleotide database with the BLAST search tool. The sequences obtained in this study have been deposited in the GenBank database under accession numbers of MG808388 (ZF01), MG808387 (ZF02), MG825424 (ZF03), MG829860 (ZF04) and MG829859 (ZF05). Fig. 8 shows the phylogenetic tree between clones and other related microorganisms with *Saccharomyces cerevisiae* as the out-group.

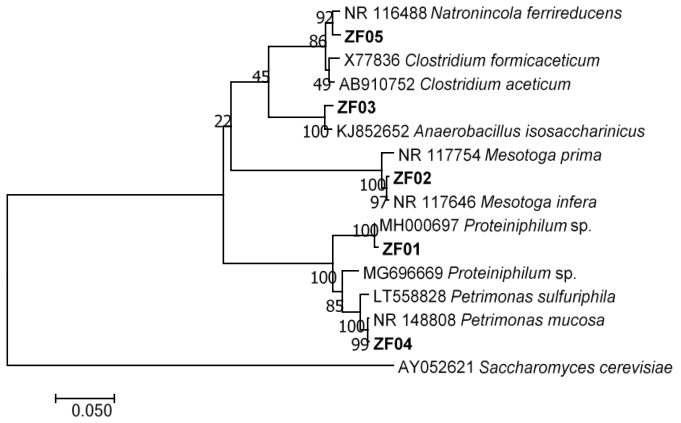

Fig. 8 Phylogenetic tree showing how clones ZF01 – ZF05 are related to other microorganisms

Based on Fig. 8, *Clostridium* sp., which are mainly the H_2-producers were presented. However, Cabrol *et al*. (2017) reported that *Clostridium* sp. might not be the most adequate H_2-producers and other obligate anaerobic non-spore forming bacteria, mostly belonging to the *Firmicutes* and *Bacteroidetes* phyla, have been identified as major H_2-producers, with specific metabolisms which were enable to maintain acceptable (even though suboptimal) H_2 performance when *Clostridium* were inactive. This scenario was supported by reports from Luo *et al*. (2008) and Liu *et al*. (2009) which stated that the non-spore formers were persistent and even dominant in the microbial community of mixed consortia even the operating conditions were favourable such as inoculum source, selection strategy, substrate, and operative parameters. As discovered by Hahnke *et al*. (2016), *Petrimonas* sp. and *Proteiniphilum* sp. were those contributed on H_2 production which belong to phylum *Bacteroidetes*.

CONCLUSION

In conclusion, H_2 production was successfully optimised through BBD based on these three factors; initial pH, incubation temperature, and inoculum with their assigned range. The maximum cumulative H_2 production was obtained at 239.0 mL under an optimised condition of initial pH of 6.4, incubation temperature at 58.0°C and 8.0% v/v of inoculum size. The POME anaerobic sludge used in this study was also studied for microbial community analysis. Findings showed that not only *Clostridium* sp. were contributed on H_2 production, but also other obligate anaerobic non-spore forming bacteria, mostly belonging to the *Firmicutes* and *Bacteroidetes* phyla, have been identified as major H_2-producers.

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Supplementary Data

Table S1 16S rRNA clone analysis of microbial community in sludge.

Clone types	No. of clones	Size (bp)	Phylogenetic linkage				Relative abundance (%)
			Phylum	Family	Closest relative	Similarity (%)	
ZF01	8	1384	Bacteroidetes	Dysgonamonadaceae	*Proteiniphilum* sp.	99	36.4
ZF02	2	1345	Thermotogae	Kosmotogaceae	*Mesotoga infera*	99	9.1
ZF03	2	1329	Firmicutes	Bacillaceae	*Anaerobacillus arseniciselenatis*	96	9.1
ZF04	5	1385	Bacteroidetes	Porphyromonadaceae	*Petrimonas mucosa*	99	22.7
ZF05	5	1314	Firmicutes	Clostridiaceae	*Clostridium aceticum*	97	22.7