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Research

Medium Optimization for Biobutanol Production From Palm Kernel Cake (PKC) Hydrolysate By *Clostridium saccharoperbutylacetonicum* N1-4

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ABSTRACT

The study aims to optimize the medium composition for biobutanol production using a Palm Kernel Cake (PKC) hydrolysate by *Clostridium saccharoperbutylacetonicum* N1-4. Various nutrient factors affecting biobutanol production were screened using the Plackett-Burman design. These factors included: NH_4NO_3, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, yeast extract, cysteine, PABA, biotin, and thiamin. The results were analyzed by an analysis of variance (ANOVA), which showed that cysteine (P=0.008), NH_4NO_3 (P=0.011) dan yeast extract (P=0.036) had significant effects on biobutanol production. The established model from the ANOVA analysis had a significant value of $P_{model}>F$ = 0.0299 with an F-value of 32.82 which explains that the factors can explain in detail the variation in the data about the average and the interpretation is true with an R^2 value of 0.993. The estimated maximum biobutanol production was 10.56 g/L, whereas the optimized medium produced 15.49 g/L of biobutanol. Process optimizations with optimum concentration of cysteine, NH_4NO_3, and yeast extract have produced 21.33 g/L biobutanol which is a 37.7% improvement from the non-optimized medium. The findings show that PKC hydrolysate with the addition of optimal concentrations of the three types of medium namely, cysteine (0.15 g/L), NH_4NO_3 (0.50 g/L), and yeast extract (1.5 g/L) during ABE fermentation, yielded a maximum biobutanol concentration of 21.33 g/L. Therefore, the results of this study provide good indications for promoting PKC hydrolysate as a new source of novel substrates with great potential in producing high biobutanol through ABE fermentation by *C. saccharoperbutylacetonicum* N1-4.

Key words: Biobutanol, hydrolysate, medium optimization, Plackett-Burman design, Palm Kernel Cake

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INTRODUCTION

In light of the anticipated depletion of fossil fuels (gasoline), the global demand for biofuel is rising steadily over time. According to the Sustainable Development Goals (SDG) of the United Nations, biofuel will replace fossil fuels by 2100. To reach the International Energy Agency's objectives, the industry must triple its present biofuel production by 2030. To achieve this goal, a sustainable and renewable alternative biofuel source must be identified (Bao *et al*., 2020). Biobutanol is regarded as an advanced biofuel due to its lower auto-ignition temperature, decreased corrosivity, lower evaporation rate, high energy release per mass, and ability to blend without separation with base fuel. Despite biobutanol's superior attributes to those of bioethanol and biodiesel, it was the least produced biofuel due to its production characteristics, which were mostly a result of its

toxicity (Peabody *et al*., 2016).

Apart from that, numerous researches have been published on the viability of sugarcane bagasse, corn kernel, oil palm frond, and other biomass as prospective feedstocks for biobutanol production. The need to seek other viable options is necessary to ensure the continuous development of biofuel production (Kushwaha *et al*., 2019). Therefore, this study will provide the premise for the utilization of palm kernel cake (PKC) with an emphasis on medium optimization for biobutanol production. The palm oil industry is a driving force industry in Malaysia one of the largest producers of palm oil in the world. Therefore, this leads to various types of palm oil-based waste generated such as empty fruit bunch, palm oil mill effluent, palm kernel cake (PKC), fronds, mesocarp fiber, trunk, and shell. PKC is a type of waste produced mainly from the oil palm industry which is known as a by-product of palm kernel oil extraction and is found in large quantities in Asian countries like Malaysia and Indonesia (Shukor *et al*., 2016a)

Furthermore, waste from PKC accumulates over time, with the Malaysian Palm Oil Board (MPOB) reporting that more than two million metric tonnes of PKC were generated in 2021. Not only that, but improper waste management is environmentally detrimental and increases greenhouse gas emissions (GHG). As a result, critical actions are required to ensure an appropriate framework for waste management and to preserve our energy security. To mitigate this issue, this abundant renewable biomass can be utilized as it provides a great potential carbon source, that could be converted into biofuels and value-added products, giving rise to a new potential industry in Malaysia. Currently, PKC is widely exported and used as a protein, fat, and energy source in dairy cattle feed. In the same context, rather than focusing just on PKC for poultry use, this waste can be used to produce biofuels, particularly biobutanol, owing to its composition of cellulose (11.6%) and hemicellulose (35.2% mannan, 2.6% xylan) with 50% fermentable hexose sugars such as glucose and mannose (Shukor *et al*., 2016b)

In a similar vein, the focus of this study will be on optimizing the medium for biobutanol production from palm kernel cake (PKC) hydrolysate by *C. saccharoperbutylacetonicum* N1-4. In general, the development of fermentation medium for most *Clostridium* sp, such as tryptone, yeast extract, and ammonium acetate (TYA) medium, P2 medium, and Reinforced Clostridial Medium (RCM), necessitates the use of numerous chemicals, which is not only costly but also limits biobutanol production potential in terms of yield and productivity (Al-Shorgani *et al*., 2013). Additionally, medium optimization is required since it encourages the enhancement of biobutanol production, which can result in a lower production cost, resulting in a more cost-effective ABE fermentation, particularly when scaling up a butanol synthesis process. To further prove the efficacy of medium optimization, several successful applications have been proven for optimization for bioethanol production, pre-optimization alkaline protease production, and even for phenolic compounds extraction (Al-Shorgani *et al*., 2016; Sharma *et al*., 2017; de Moura *et al*., 2018). Generally, there are many types of strategies employed for medium optimization to improve the efficiency of the production medium such as central composite design (CCD), Box Behnken design, Plakett-burman design, Taguchi design, and even Response Surface Methodology (RSM) in which each design has its functional properties and advantages (Singh *et al*., 2017). The classical medium optimization method is One-Factor-at-a-Time (OFAT), which is preferred due to its convenience and simplicity, and the results may be analyzed using simple graphs. Nevertheless, the main disadvantage of this strategy is the difficulty in predicting the interaction when a large number of variables are involved in a scattershot sequence of experiments, which is time-intensive (Razali *et al*., 2018).

As a result, the OFAT approach was not chosen as a medium optimization tool as the central concept of this study centers on the application of a two-level factorial design known as the Plackett-Burman design (PBD). This statistical design approach is mostly utilized when investigating the impact of a large number of nutritional parameters. Furthermore, previous researchers have reported the efficiency of PBD in a variety of circumstances, such as for optimizing the medium of fermented tremella polysaccharide, medium trace nutrients for glycolipopeptide biosurfactant production, and laccase production from *Pleurotus ostreatus* (Soumya *et al*., 2016; Ekpenyong *et al*., 2020; Ma *et al*., 2020). In this study, 11 potential medium components namely NH_4NO_3, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, yeast extract, cysteine, PABA, biotin and thiamine in biobutanol synthesis using PKC hydrolysate as main carbon source will be investigated. The basis behind the chosen 11 components are mostly the ingredients found in TYA medium and P2 medium which are the main medium components needed for the growth of *Clostridium* sp. All the components from both mediums in a certain amount of concentration which further necessitates the need of conducting medium optimisation were proven to boost and enhance biobutanol production. Their effect as additional components in PKC hydrolysate for optimum production of biobutanol will reveal the importance of this component. Based on our knowledge, no study reveals the best medium composition in biobutanol production using PKC

hydrolysate as the most economical carbon source to replace conventional sugar sources.

MATERIALS AND METHODS

Inoculum and medium preparation

A stock culture of *C. saccharoperbutylacetonicum* N1-4 was acquired from the Biotechnology Lab of the Chemical and Process Engineering Department at Universiti Kebangsaan Malaysia. After activating the microbe with a heat shock in boiling water and a cold shock in freezing water which is a way to enhance the ability of the bacterial cells to take up foreign genetic material, 1 mL of stock culture was added to 9 mL of TYA medium in a test tube (Shukor *et al*., 2016a). The microbe was then incubated for one to two days in which the optical density (OD) must be more than 1.0 and within 24-48 hr at 30 °C under anaerobic conditions. The anaerobic conditions were achieved when oxygen-free nitrogen was purged into the test tube and sealed with parafilm before being kept in the incubator (Al-Shorgani, 2016). TYA medium composition was as follows (g/L): glucose, 20; tryptone, 6; yeast extract, 2; ammonium acetate, 3; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.3 & $FeSO_4.7H_2O$, 0.01.

Batch ABE fermentation

ABE fermentation for the production of biobutanol was carried out in a 100 mL serum container with a 50 mL working volume. PKC hydrolysate, which was released from the best treatment through hot water treatment (LHW) and enzymes step was employed as a substrate by *C. saccharoperbutylacetonicum* N1-4 to produce biobutanol. The hydrolysis yields a total amount of sugar of 97.81 g/L. In terms of composition, 22.01% of lignin, 22.95% of hemicellulose, and 17.83% of cellulose were found in PKC (Shukor *et al*., 2016b). The 1 L of respective medium components were added to 1 L of PKC hydrolysate to create the ABE fermentation medium. The culture medium was then autoclaved at 121 °C for 15 min (Shukor *et al*., 2016b). The anaerobic ABE fermentation conditions were provided by sparging oxygen-free nitrogen in the sterile fermentation medium. Then, 10% (v/v) of the fresh inoculum of *C. saccharoperbutylacetonicum* was inoculated in PKC hydrolysate and was tested one by one with various concentrations of 11 medium components and incubated at the temperature of 30 °C for 24 h. The initial pH of the ABE fermentation medium was adjusted to 6.5 using a solution of 6 M NaOH.

Plankett- Burman Design (PBD) for Screening and Medium Optimisation

Screening Medium Components Affecting Biobutanol Production

The Plackett-Burman design (PBD) was used to evaluate and screen various significant medium components in the production of biobutanol by *C. saccharoperbutylacetonicum* N1-4 using PKC hydrolysate treated with hot water treatment (LHW) and enzymes. The basis of this pre-treatment process helps break down complex polysaccharides and create conditions conducive to microbial growth and butanol production (Shukor *et al*., 2016a). 11 medium components need to be studied for their effect on biobutanol production and the PBD can analyzed in this large number of components by giving K+1 experiment with K as the total factor. All medium ingredients for ABE fermentation are coded with the alphabet A to K with two levels, namely the minimum level (-1) and the maximum level (+). The determination of medium material and the range of medium material are determined by following previous studies by using P2 and TYA medium as the basis (Al-Shorgani *et al*., 2013; Ranjan *et al*., 2013). The PBD is based on a polynomial model (Equation 1).

$$Y = B_0 + \Sigma B_1 X_1 \qquad \text{Equation 1}$$

Where Y is the activity, B_0 is the crossover model B_1 is the linear constant and X_1 is the variable being studied. A total of 12 experimental treatments were developed using the Design Expert software for the PBD to screen the influential medium material from the 11 components (Table 1).

Optimization of Medium Materials Affecting Biobutanol Production using Response Surface Reaction Method (RSM)

Three components have a significant effect on the production of biobutanol, namely medium H (cysteine), which is the medium that shows the greatest influence on the production of biobutanol followed by medium A (NH_4NO_3) and medium G (yeast extract) have been selected for the optimization process using the RSM method. These three factors are tested in 3 levels (low, medium, high) with codes (-1,0, +1) as shown in Table 2. For the 3 factors studied, 20 sets of experiments were given as shown in Table 3.

Table 1. The level of mediums selected for screening using the Plackett-Burman design (PBD)

Code	Medium	Low level (-1) g/L	High level (+1) g/L
A	NH_4NO_3	1	4
B	KH_2PO_4	0.1	1
C	K_2HPO_4	0.1	1
D	$MgSO_4.7H_2O$	0.1	1
E	$MnSO_4.7H_2O$	0.001	0.1
F	$FeSO_4.7H_2O$	0.001	0.1
G	Yeast extract	1	4
H	Cysteine	0.1	1
I	PABA	0.01	0.2
J	Biotin	0.01	0.1
K	Thiamine	0.1	1

Table 2. The tested factors and their stages in the optimization process

Factors	Unit	Symbol	Level		
			-1	0	+1
Cysteine	g/L	X_1	0.05	0.1	0.15
NH_4NO_3	g/L	X_2	0.5	1	1.5
Yeast extract	g/L	X_3	0.5	1	1.5

Table 3. Experimental design of process medium optimization

STD	Run Order	Block 1	Cysteine (g/L)	NH_4NO_3 (g/L)	Yeast extract (g/L)
1	5	Block 1	0.05	0.5	0.5
2	19	Block 1	0.15	0.5	0.5
3	16	Block 1	0.05	1.5	0.5
4	14	Block 1	0.15	1.5	0.5
5	7	Block 1	0.05	0.5	1.5
6	12	Block 1	0.15	0.5	1.5
7	9	Block 1	0.05	1.5	1.5
8	6	Block 1	0.15	1.5	1.5
9	11	Block 1	0.02	1	1
10	18	Block 1	0.18	1	1
11	15	Block 1	0.1	0.16	1
12	2	Block 1	0.1	1.84	1
13	20	Block 1	0.1	1	0.16
14	17	Block 1	0.1	1	1.84
15	8	Block 1	0.1	1	1
16	3	Block 1	0.1	1	1
17	1	Block 1	0.1	1	1
18	4	Block 1	0.1	1	1
19	13	Block 1	0.1	1	1
20	10	Block 1	0.1	1	1

Biobutanol analysis

Sugar concentrations were determined using high-performance liquid chromatography (HPLC) with the model type of 12000 Series, Agilent Technologies, Palo Alto, CA, USA with a SUPELCOGEL C-611 HPLC column (300 7.8 mm ID) and a refractive index detector (RID) at 60 °C and a flow rate of 0.5 mL/min with 10^{-4} M sodium hydroxide as the mobile phase. The concentrations of ABE were determined using a gas chromatograph (7890A GC-System; Agilent Technologies, Palo Alto, CA, USA) with a flame ionization detector and a 30-capillary column (Equity 1; 30 m0.32 mm1.0 m film thickness; Supelco, Bellefonte, PA, USA). The oven temperature was designed to climb by 8 °C every min from 40 °C to 130 °C. The temperatures of the injector and detector were adjusted at 250 °C and 280 °C, respectively. The flow rate of the carrier gas, helium, was fixed at 1.5mL/min (Al-Shorgani *et al*., 2016).

RESULTS AND DISCUSSION

The development of a PKC-based medium for the production of biobutanol by *C. saccharoperbutylacetonicum* N1-4 was studied using the best treatment and hydrolysis by Hafiza *et al*.

(2016). Therefore, the ideal circumstances for the production of biobutanol via this treatment are utilized in the research into the development of medium materials based on PKC as the primary carbon source. To boost the production of biobutanol by *C. saccharoperbutlacetonicum* N1-4 via ABE fermentation, additional medium components, such as vitamins, are also introduced.

In a similar vein, the initial stage in the development of this PKC-based medium is to screen substances that must be included and have a beneficial impact on biobutanol production. This screening procedure is also viewed as a vital stage, particularly when there are numerous parameters or types of materials whose amount of use is less understood, which leads to ideal outcomes. In experimental design, determining the dosage or, more precisely, the concentration required for a chemical is extremely challenging. Only experimental experience and prior research may supply such knowledge. PBD is a statistical strategy that has been extensively recognized as the most effective method for completing medium screening work without the need for significant experimental treatment, which leads to saving costs, energy, and time and decreasing the usage of largely expensive chemicals (Ma *et al*., 2020). In this work, the PBD was used to screen materials that can improve product output and further optimize the medium material that has a significant impact on biobutanol synthesis. In addition, a total of 12 experimental treatments were developed using the Design Expert software for the PBD to screen medium materials influencing the production of biobutanol from 11 components using different combinations of medium material concentration values to be added to the PKC hydrolysate. The selection of studied medium ingredients is based on the components of the original medium for Clostridium, such as the materials found in the TYA medium and the P2 medium, namely NH_4NO_3, KH_2PO_4, KH_2HPO_8, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, yeast extract, cysteine, PABA, biotin, and thiamine (Al-Sorgani *et al*., 2013; Ranjan *et al*., 2013).

Table 4 displays 12 experimental results for 11 types of materials for the screening process utilizing the PBD of the medium optimization model employing PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4. Treatment 12 produced the most biobutanol production (10.52 g/L) with a yield of 0.74 g/g, followed by other treatments production and yield namely treatments 7 (10.15 g/L, 1.56 g/g), treatments 3 (9.77 g/L, 0.61 g/g), treatments 4 (8.14 g/L, 1.08 g/g), and treatments 8 (6.60 g/L, 1.35 g/g). The treatments with the least biobutanol synthesis were treatments 6 (2.20 g/L, 1.12 g/g) and treatment 1 (2.32 g/L, 0.48 g/g). The data for the production of biobutanol is then entered into the "Design Expert" software using the PBD to generate a Semi-Normal Probability Plot (half-normal plot). This plot is used to determine the effect of a large medium on the production of biobutanol, which is evident when the plot points point toward the right side of the plot. Figure 1 depicts a semi-normal probability curve that identifies the effect of medium on biobutanol production by *C. saccharoperbutlacetonicum* N1-4. The effect of the medium's weak influence on biobutanol production is shown by the plot point that approaches the left side of the plot and the value of O. From Figure 1, it can be shown that medium H (cysteine) has the greatest (far right) effect on biobutanol production, followed by medium A (NH_4NO_3) and medium G. (yeast extract). However, this study has not yet been able to determine if these three mediums have a beneficial or detrimental influence on biobutanol production.

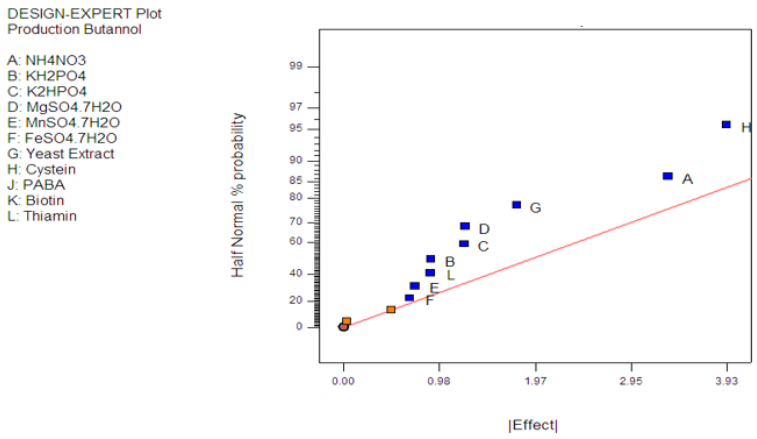

Fig. 1. Semi-Normal Probability Plot to identify the effect of medium influence by Plakett-Burman design for the medium screening process using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

Table 4. Screening process by Plackett-Burman design using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

Run Order	Block 1	A	B	C	D	E	F	G	H	J	K	L	Observed Biobutanol (g/L)	Estimated Biobutanol (g/L)
1	Block 1	4	0.1	1	0.1	0.001	0.001	4	1	0.2	0.01	1	2.32	2.09
2	Block 1	4	1	0.1	1	0.001	0.001	1	1	0.2	0.1	0.1	4.13	3.87
3	Block 1	1	1	1	0.1	0.1	0.001	1	0.1	0.2	0.1	1	9.77	9.51
4	Block 1	4	0.1	1	1	0.001	0.1	1	0.1	0.01	0.1	1	8.14	8.37
5	Block 1	4	1	0.1	1	0.1	0.001	4	0.1	0.01	0.01	1	4.15	4.41
6	Block 1	4	1	1	0.1	0.1	0.1	1	1	0.01	0.01	0.1	2.20	2.46
7	Block 1	1	1	1	1	0.001	0.1	4	0.1	0.2	0.01	0.1	10.15	9.92
8	Block 1	1	0.1	1	1	0.1	0.001	4	1	0.01	0.1	0.1	6.60	6.83
9	Block 1	1	0.1	0.1	1	0.1	0.1	1	1	0.2	0.01	1	6.03	5.80
10	Block 1	4	0.1	0.1	0.1	0.1	0.1	4	0.1	0.2	0.1	0.1	4.53	4.27
11	Block 1	1	1	0.1	0.1	0.001	0.1	4	1	0.01	0.1	1	2.39	2.62
12	Block 1	1	0.1	0.1	0.1	0.001	0.001	1	0.1	0.01	0.01	0.1	10.52	10.78

A: NH_4NO_3, B: KH_2PO_4, C: K_2HPO_4, D: $MgSO_4.7H_2O$, E: $MnSO_4.7H_2O$, F: $FeSO_4.7H_20$, G: yeast extract, H: cysteine, J: PABA, K: biotin, L: thiamine

Table 5 shows the results of the ANOVA analysis for the PBD in the screening of influential medium ingredients for the factorial model against 11 mediums for the production of biobutanol by *C. saccharoperbutylacetonicum* N1-4. This finding shows that only three medium ingredients have a significant effect on the production of biobutanol, namely medium H: cysteine (*P*=0.008), A: NH_4NO_3 (*P*=0.011) and G: yeast extract (*P*=0.036) while the rest of the ingredients medium B: KH_2PO_4, C: K_2HPO_4, D: $MgSO_4.7H_2O$, E: $MnSO_4.7H_2O$, F: FeSO4.7H_2O, J: PABA, K: biotin and L: thiamine was not significant. A small *P* value and its association with a large *F* value is due to the effect of the studied factor being larger than the standard error.

Apart from that, it can also be seen that the addition of some mineral salts and trace elements such as KH_2PO_4 and $MgSO_4.7H_2O$ in the PKC hydrolysate medium does not have a significant effect on the production of biobutanol by *C. saccharoperbutylacetonicum* N1-4. This may be because the PKC hydrolysate itself already contains mineral salts and trace elements such as zinc, copper, calcium, magnesium, and phosphorus, which not only promote the growth of Clostridium but also facilitate the formation of biobutanol (Marini *et al*. 2006). Therefore, the addition of minerals such as $FeSO_4.7H_2O$ and $MnSO_4.H_2O$ during ABE fermentation increased the iron and manganese mineral content already present in PKC, resulting in the opposite effect, a decrease in biobutanol synthesis.

Table 5 shows the ANOVA analysis by the PBD of the medium screening process using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4. Model interaction with a model probability value greater than *F*=0.0299 and an *F*-value of 32.82 suggests that the model's interpretation of the system response is credible. A high F value implies that the factor adequately explains the deviation of the data from the mean and that the interpretation is accurate. The R^2 score of this study is 0.99, indicating that this model can explain 99.3% of the variable content that positively contributes to the response, with less than 0.7% of the overall variance remaining unexplained. In contrast, the value of R is closer to 1 (0.993), indicating a strong correlation between the experimental value and the model's prediction.

Table 5. ANOVA analysis for selected factorial model

Source	Sum of squares	DF	Mean square	*F*-value	Prob> *F*	
Model	106.09	9	11.79	32.82	0.03	Significant
A	33.3	1	33.3	92.70	0.01	
B	2.39	1	2.39	6.648	0.12	
C	4.6	1	4.60	12.81	0.07	
D	4.65	1	4.65	12.95	0.07	
E	1.59	1	1.59	4.43	0.17	
F	1.37	1	1.37	3.814	0.19	
G	9.45	1	9.45	26.31	0.04	
H	46.37	1	46.37	129.10	0.01	
L	2.37	1	2.37	6.59	0.12	
Residual	0.72	2	0.36			
Cor total	106.81	11				

R^2 = 0.993; Adj R^2 = 0.96; Std Dev. = 0.599; *P*< 0.05 is significant

Besides, a strong correlation between the regression model and the experiment can be obtained if the R^2 value is greater than 0.9 (Chen *et al*. 2009). In addition, because the R^2 for the model in this study is 0.993, it can be concluded that this model shows accuracy for changes and the R^2 value represents a very good accuracy between the observed experimental values and the predicted value of biobutanol production. The equation of the individual parameter interaction model (as a first-order equation) can be shown as shown in Equation 2:

$$\text{Biobutanol} = 12.86 - 1.11*NH_4NO_3 - 0.99*KH_2PO_4 + 1.38*K_2HPO_4 + 1.38*MgSO_4.7H_2O - 7.36*MnSO_4.7H_2O - 6.82 * FeSO_4.7H_2O - 0.59*\text{Yeast Extract} - 4.37 * \text{Cysteine} - 0.99*\text{Thiamine}$$

The effect of 11 mediums on the production of biobutanol in ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 is displayed in the Pareto plot (Figure 2). It is observed that the

most important medium that affects the production of biobutanol is cysteine which is 43.42%, followed by NH_4NO_3 (31.18%) and yeast extract (8.85%). Biotin is among the types of nutrients that have the least contribution to the production of biobutanol from PKC hydrolysate with a contribution percentage of 0.003%, PABA 0.67%, and $MnSO_4$, 1.49%.

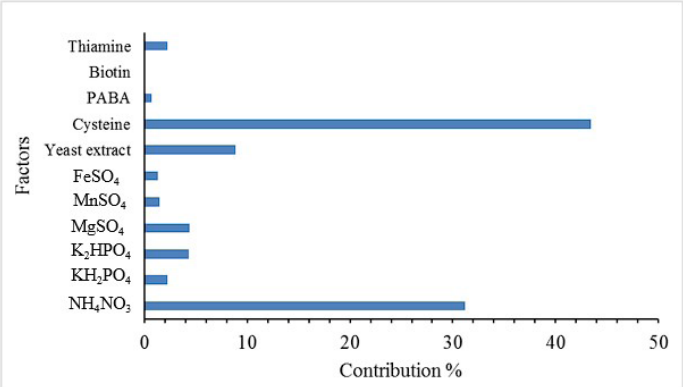

Fig. 2. Pareto plot of the Plackett-Burman design for parameter estimation using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

Figure 3 shows the estimated effect of 11 factors on the production of biobutanol by *C. saccharoperbutylacetonicum* N1-4. Nutrients such as K_2HPO_4, $MgSO_4$, PABA, and biotin are among the mediums that have a positive effect on the production of biobutanol if the concentration of the medium is increased. Besides, nutrients such as NH_4NO_3, KH_2PO_4, $MnSO_4$, $FeSO_4$, yeast extract, cysteine, and thiamine are among the mediums that hurt the production of biobutanol. A reduction in the concentration of this medium is seen to be able to improve the production of biobutanol.

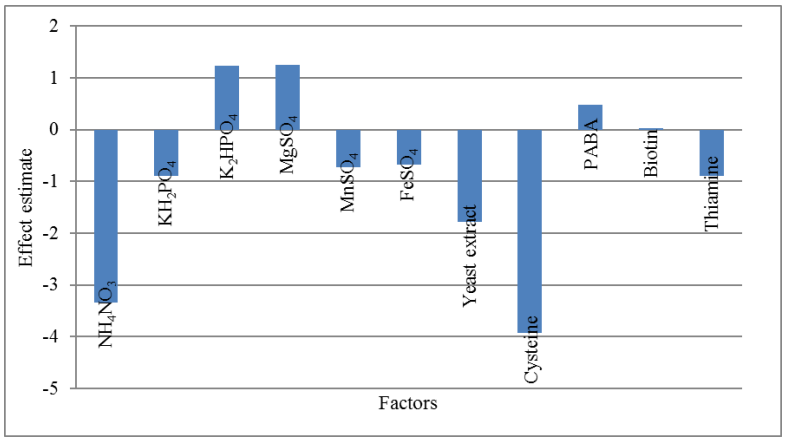

Fig. 3. Estimate of the effect of factors on biobutanol production using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4.

In addition, from the results of this PBD, three types of nutrients have the most significant effect on the production of biobutanol using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4 in this ABE fermentation. The factor or nutrient that has the most significant effect in the production of biobutanol is the concentration of cysteine, $C_3H_7NO_2S$ (P=0.0077) which is a type of amino acid that has reducing power (reducing agent) added to the medium for ABE fermentation to lower the redox potential of the system and thereby increasing the ability to form reducing products such as biobutanol (Shukor *et al*., 2016b). Therefore, the addition of cysteine is seen to have a large impact on the production of biobutanol, especially when PKC hydrolysate is used.

The addition of ammonium nitrate, NH_4NO_3 in ABE fermentation has the same function as yeast extract where these two substances are added as a source of nitrogen for cell growth. Nitrogen contributes 10-14% to the dry cell weight of a microorganism by entering the cell mass in the form of proteins and nucleic acids (Razak *et al*., 2013). Ammonium salts such as NH_4NO_3 are among the commonly used nitrogen sources compared to complex organic nitrogen sources such as yeast extract and peptone which are often used as a nitrogen source for most laboratory studies only considering

their expensive price compared to ammonium salts. However, the results of this study show that the presence of yeast extract in low concentration is still needed to increase the production of biobutanol. This is owing to the substantial vitamin, protein, amino acids, and mineral content of yeast extract, which functions as a cofactor capable of promoting the development of *Clostridium* during biobutanol synthesis (Hanh *et al*. 2011)

Yeast extract is one of the ingredients commonly added in most fermentation processes including ABE fermentation (Ouephanit *et al*., 2011; Li *et al*., 2012; Al-Sorgani *et al*., 2013; Gottumukkala *et al*., 2013b). Previous research has demonstrated that yeast extracts and minerals such as K_2HPO_4 and $MgSO_4$ significantly enhance Clostridium's biobutanol synthesis (Survase *et al*., 2012; Al-Shorgani *et al*., 2013). The addition of yeast extract from 0.4% to 1% in ABE fermentation using *Clostridium* sp. G117 is seen to increase the production of biobutanol from 8.52 g/L to 8.61 g/L (Chua *et al*., 2013). Al-Shorgani *et al*. (2016) in their study using the ratio of carbon to nitrogen (C/N) to optimize the medium for the production of biobutanol using *C. acetobutylicum* YM1 showed that a C/N ratio of 65 is the best to produce 13.87 g/L of biobutanol. ABE fermentation for the production of biobutanol from date waste shows that yeast extract has a strong effect in increasing the production of biobutanol (Abd-Alla & Elsadek El-Enany, 2012). Not only that, an increase in the use of yeast extract from 0.4% to 1% in ABE fermentation by *Clostridium* sp G117 proved that there was an increase in the production of biobutanol from 8.52 g/L to 8.61 g/L (Chua *et al*., 2013).

PBD has proposed several sets of experiments regarding the combination of the medium in maximizing the production of biobutanol for the medium optimization process. Therefore, a set of experiments was selected with the highest desirability value of 1.0 which contains NH_4NO_3 (1.09 g/L), KH_2PO_4 (0.99 g/L), K_2HPO_4 (0.66 g/L), $MgSO_4.7H_2O$ (0.56 g/L), $MnSO_4.7H_2O$ (0.09 g/L), $FeSO_4.7H_2O$ (0.001 g/L), yeast extract (1.01 g/L), cysteine (0.10 g/L), PABA (0.04 g/L), biotin (0.09 g/L) and thiamine (0.10 g/L). The production of biobutanol using the proposed medium of PBD has succeeded in producing 15.49 g/L compared to 10.56 g/L estimated by this design, which is a value that is less than the estimated value. The outcomes of this study provide a strong indication for promoting PKC hydrolysate as a new source of novel substrate with high potential for biobutanol production by *C. saccharoperbutylacetonicum* N1-4 via ABE fermentation. Although it has been stated that the maximal production of biobutanol by wild-type Clostridium is only able to create 9 to 12 g/L of biobutanol for batch systems, however, this work indicates the contrary whereby 15.49 g/L of biobutanol can be produced in ABE fermentation. The findings of this study indicate that a moderate concentration of all nutritional components given to the ABE fermentation process utilizing PKC hydrolysate can boost biobutanol production by *C. saccharoperbutylacetonicum* N1-4 in a batch fermentation system.

Optimization of medium materials affecting biobutanol production by *C. saccharoperbutylacetonicum* N1-4

After the medium screening process is completed, the most potential and significant medium material in increasing the production of biobutanol in ABE fermentation is selected for the optimization process. Three types of medium namely cysteine, NH_4NO_3, and yeast extract were used as parameters to study their effect in maximizing the production of biobutanol using the reaction surface method (RSM) in Design-Expert version 8.0 software (DOE, Stat-Ease, USA). This RSM method is one of the most popular methods in performing statistical optimization processes for most chemical and biological processes (Vishwanatha *et al*., 2010; Ba-Abbad *et al*., 2013). Furthermore, this approach also saves time throughout the optimization process by recommending a small number of experiments and simplifying overall data processing (Bezerra *et al*. 2008). Table 6 displays the RSM outcomes for the medium optimization process using PKC hydrolysate.

To predict the optimal production of biobutanol, the model in this software was matched with experimental data. For the medium optimization process in ABE fermentation using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4, the following equation was produced (Equation 3):

$$\text{Biobutanol} = 24.73 - 74.16 * \text{Cysteine} - 5.52 * NH_4NO3 - 5.87 * \text{Yeast Extract} + 594.39 * \text{Cysteine}^2 + 6.42 * NH_4NO3^2 + 5.07 * \text{Yeast Extract}^2 - 28.6 * \text{Cysteine} * NH_4NO3 - 7.09 * \text{Cysteine} * \text{Yeast Extract} - 3.26 * NH_4NO3 * \text{Yeast Extract}$$

Apart from that, Table 7 displays the findings of the ANOVA for the medium optimization method used to produce biobutanol utilizing PKC hydrolysate. The accuracy of the model with the experimental

data was assessed with an R^2 value of 0.85 which indicates that this statistical model only explains 85% of the variability in biobutanol values. According to Chauhan *et al*. (2007), a model's strength and ability to predict outcomes properly are indicated by an R^2 value that is close to 1. These three forms of medium material, cysteine, NH_4NO_3, and yeast extract, showed a linear influence in the ANOVA analysis, and the quadratic term (cysteine)^2, $(NH_4NO_3)^2$, and (yeast extract)^2 was significant with a value of $P<0.05$, suggesting that this component affects the generation of biobutanol. This important ingredient is likewise regarded as a finite element of the medium, and a little change in concentration will affect both the rate of cell growth and the rate of product generation. Additionally, results from a linear function for cysteine, NH_4NO_3, and yeast extract suggest that these medium components have less of an impact on the production of biobutanol. Furthermore, contrary to what this model indicated, the interaction between AB (P=0.1494), AC (P=0.7068), and BC (P=0.1050) was not significant ($P>0.05$).

Table 6. Experimental design and results by response method surface (RSM) for the medium optimization using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

Run Order	Factors			Biobutanol (g/L)	
	Cysteine (g/L)	NH_4NO_3 (g/L)	Yeast extract (g/L)	Actual	Predicted
1	0.05	0.5	0.5	17.74	17.98
2	0.15	0.5	0.5	19.31	20.67
3	0.05	1.5	0.5	20.74	22.24
4	0.15	1.5	0.5	21.14	22.06
5	0.05	0.5	1.5	19.56	20.26
6	0.15	0.5	1.5	22.12	22.24
7	0.05	1.5	1.5	20.99	21.25
8	0.15	1.5	1.5	18.99	20.37
9	0.02	1	1	20.79	19.97
10	0.18	1	1	22.96	21.49
11	0.1	0.16	1	20.72	20.06
12	0.1	1.84	1	23.69	22.07
13	0.1	1	0.16	21.47	19.86
14	0.1	1	1.84	21.03	20.35
15	0.1	1	1	16.20	16.53
16	0.1	1	1	16.72	16.53
17	0.1	1	1	16.80	16.53
18	0.1	1	1	16.16	16.53
19	0.1	1	1	16.52	16.53
20	0.1	1	1	16.38	16.53

Table 7. ANOVA analysis of the medium optimization process using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

Source	Sum of squares	F- value	P- value
Model	94.58	6.27	0.0042
Cysteine, A	2.78	1.66	0.2266
NH_4NO_3, B	4.84	2.89	0.1201
Yeast extract, C	0.29	0.17	0.6865
A^2	31.82	18.98	0.0014
B^2	37.08	22.11	0.0008
C^2	23.11	13.78	0.0040
AB	4.09	2.44	0.1494
AC	0.25	0.15	0.7068
BC	5.33	3.18	0.1050

*$P<0.05$ indicates the model is significant

Figure 4 shows a normal probability plot for the residuals to be normally distributed between straight lines. This circumstance further validates the statistical analysis is acceptable. In addition, Figure 5 shows the constant difference between the residual and the prediction where the even distribution of the residual data above and below the red line on the x-axis shows that this difference does not depend on the value of biobutanol produced and this supports the assumptions made by the model.

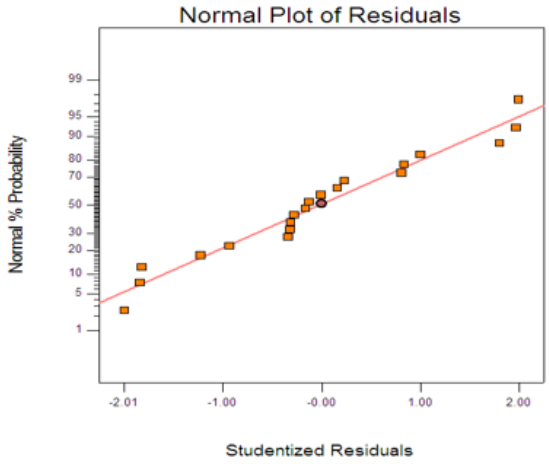

Fig. 4. Normal probability plot of medium optimization using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4.

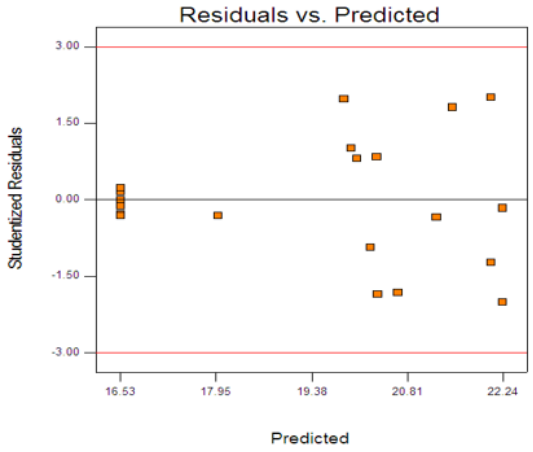

Fig. 5. Plot of residual against prediction for medium optimization using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

In addition, Figure 6 indicates a good correlation between the experimental data and the anticipated value created by the model utilizing Equation 3 of the medium optimization process employing PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4. Furthermore, many points scattered around the diagonal of the line indicate that the model provides optimal accuracy since the deviation between the estimated value and the experimental value is still minimal.

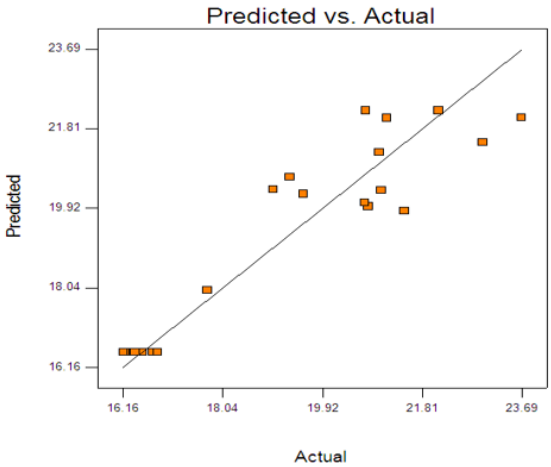

Fig. 6. Distribution equivalence plot of experimental data and predicted values of medium optimization model using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

The major goal of the RSM method optimization process is to find the ideal value by either maximizing or decreasing the response acquired from experimental data by defining the purpose of a study. The optimal medium for the medium optimization method employing PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4 can be obtained by maximizing the response (biobutanol production). Furthermore, the maximum predicted value refers to the surface bounded by the smallest ellipse in the contour diagram. A perfect interaction between the independent variables can be seen when an ellipse contour is produced.

The three-dimensional (3D) plot in the RSM produced using the model equation (Equation 3) is usually used to identify interactions between factors and also to validate the optimal concentration for each factor studied for maximum biobutanol production. From the 3D plot (Figure 7), the interaction between (a) CA: yeast-cysteine extract; (b) CB: yeast extract-NH_4NO_3, and (c) BA: NH_4NO_3-cystein for the medium optimisation model using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4 can be seen and the effect between two factors (different types of medium) in the production of biobutanol when one other factor is kept constant can be seen in this 3D plot. On the other hand, Figure 7 (a) shows the interaction between yeast extract and cysteine in the production of biobutanol when the concentration of NH_4NO_3 is constant. The highest biobutanol yield (19.76 g/L) was obtained by increasing the concentrations of these two substances namely yeast extract (1.5 g/L) and cysteine (0.15 g/L) to the maximum levels.

Moreover, when one of these two medium components is supplied at a low concentration or dose, the generation of biobutanol is reduced. Therefore, the results show that the optimum medium for yeast extract is 1.5 g/L while cysteine is at 0.15 g/L. For the interaction between yeast extract and NH_4NO_3 in the condition of constant cysteine concentration (Figure 7 (b)), at a concentration of 0.5 g/L yeast extract (minimum), the addition of 1.5 g/L NH_4NO_3 has increased the maximum production of biobutanol (20.66 g/ L). If these two substances are given at high concentrations, the opposite effect occurs in which the production of biobutanol will decrease. The same pattern can also be seen for the interaction between NH_4NO_3 and cysteine when the yeast extract is at a constant concentration (Figure 7 (c)). A high production of biobutanol will only be obtained if one of these mediums is at the maximum concentration. However, at 0.05 g/L cysteine and 1.5 g/L NH_4NO_3 gave the highest biobutanol yield of 20.48 g/L.

Apart from that, the experimental results show that the polynomial regression model has satisfactory adequacy to represent the medium optimization model using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4. The optimal concentration of the three types of medium material that is significant suggested by the model is 0.15 g/L cysteine, 0.50 g/L NH_4NO_3, and 1.5 g/L yeast extract. In the same context, biobutanol production of 22.24 g/L was predicted by the empirical model under the recommended optimum concentration of the medium. It can be seen that the total concentration of the proposed nitrogen-sourced medium is 2 g/L (0.50 g/L NH_4NO_3 and 1.5 g/L yeast extract), which is a sufficient amount for *C. saccharoperbutylacetonicum* N1-4 to grow and produce biobutanol.

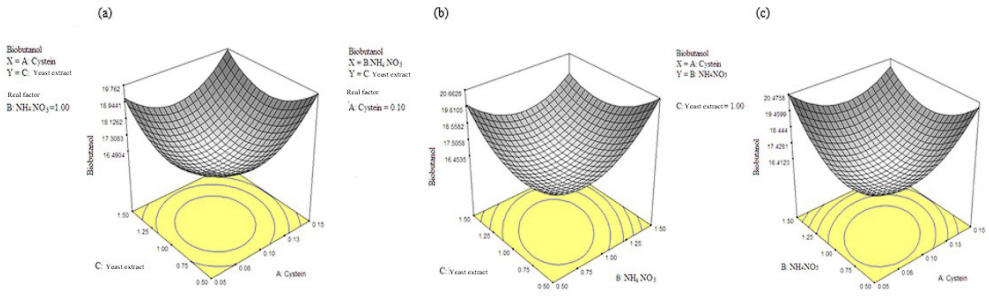

Fig. 7. 3D plot of the interaction between (a) CA; (b) CB and (c) BA of the medium optimisation model using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1-4

The amount of nitrogen source used in this study is seen to be low compared to the total concentration of nitrogen source used in the TYA medium (2 g/L yeast extract, 6 g/L tryptone, and 3 g/L ammonium acetate) (Oshiro *et al*., 2010; Zheng *et al*., 2013) and medium P2 (2 g/L NH_4NO_3 and 1 g/L yeast extract) (Al-Shorgani *et al*., 2012; Li *et al*., 2013) and this gives an advantage to the cost reduction of chemicals used in ABE fermentation using this PKC hydrolysate. The addition of sufficient yeast extract in this ABE fermentation medium can promote a phase shift from acidogenic to solventogenic by increasing gene

transcription 16-fold, and indirectly increase biobutanol synthesis by accelerating the accumulation of histidine and aspartic acid families (Li *et al*., 2011). In hydrogen production, Alvarado-Cuevas *et al*. (2013) also reported that the use of sufficient nitrogen resources is necessary to promote the use of carbon resources in the fermentation medium and subsequently to produce a high product. A total of 3 g/L of yeast extract and 4 mg/L of PABA was seen to help increase the production of biobutanol in ABE fermentation using rice straw hydrolysate by *C. acetobutylicum* MTCC 481 (Ranjan *et al*., 2013).

To confirm the optimal conditions, a set of ABE fermentation experiments was carried out using *C. saccharoperbutylacetonicum* N1-4 against PKC hydrolysate using optimal concentrations of cysteine, NH_4NO_3, and yeast extract. The experimental results showed that biobutanol with a concentration of 21.33 g/L was obtained These findings corroborate the empirical model's accuracy by offering highly similar values between the regression model and the experimental results. In addition, the use of this moderately concentrated medium (with a concentration range of only 0.15 – 1.5 g/L), suggests that good conditions may be obtained when an adequate supply of nutrients is used without any excess for cell growth and product production. Besides, biobutanol production of 15.49 g/L for the unoptimized medium is produced, representing a 37.7% increase when the medium optimization process is carried out. As a result, the findings of this study demonstrate the efficacy of the quadratic model in predicting and thus improving the medium for optimal biobutanol production using PKC hydrolysate by *C. saccharoperbutylacetonicum* N1- 4.

CONCLUSION

In conclusion, the optimization of medium composition for biobutanol production in batch culture by *C. saccharoperbutylacetonicum* N1-4 was successfully conducted through screening of significant nutrient factors using the PBD design. The results of the study revealed that among the 11 factors tested, three types of nutrients namely cysteine, NH_4NO_3, and yeast extract are the components that give the most impact in the production of biobutanol through ABE fermentation and these mediums have met the requirements in ABE fermentation using PKC hydrolysate as the main carbon source by *C. saccharoperbutylacetonicum* N1-4. The findings show that PKC hydrolysate with the addition of optimal concentrations of the three types of medium namely, cysteine (0.15 g/L), NH_4NO_3 (0.50 g/L), and yeast extract (1.5 g/L) during ABE fermentation, yielded a maximum biobutanol concentration of 21.33 g/L. In addition, this has improved biobutanol production and this further reveals that PKC hydrolysate with the addition of extra nutrients could enhance and boost biobutanol production. Apart from that, the information from the results of this study on the most suitable and optimal medium for the production of biobutanol using hydrolysate from PKC by *C. saccharoperbutylacetonicum* N1-4 not only promises to be cost-effective for its production as it can reduce the cost in terms of chemicals (medium materials) that need to be used to obtain a high yield of biobutanol but the outcome from this study are also seen to have contributed to serving the premise in overcoming the challenges of low biobutanol production for most biobutanol production processes from lignocellulosic materials.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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