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Response surface optimization of bioethanol production from third generation feedstock - *Eucheuma cottonii*

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ABSTRACT

Response Surface Methodology (RSM) based on Central Composite Design (CCD) was employed to optimize the conditions of enzymatic hydrolysis and fermentation of the seaweed *Eucheuma cottonii*. The significant influence of cellulose loading, enzyme loading and incubation time in enzymatic hydrolysis that has been screened by Plackett-Burman Design (PBD) was optimized using CCD. The optimum glucose concentration of 24.24 g/L (81% glucose yield) was obtained at 3% (v/v) of cellulose loading, 4% (v/v) of enzyme loading and 54 h incubation time. Subsequently, PBD analysis showed the significant effects of inoculum concentration, pH, temperature and time on fermentation process. Further optimization study by CCD revealed that 12% (v/v) of inoculum concentration, pH 5.2, 32 °C and 72 h of fermentation time enhanced the bioethanol production up to 9.77 g/L with the yield of 0.40 g/g and 78% conversion efficiency. Thus, the RSM based optimization of bioethanol from *Eucheuma cottonii* showed satisfactory results in this research. In short, it can be concluded that this optimization approach will serve as a good foundation for the realization of a consistent bioethanol production in the future.

1. Introduction

The great dependence on fossil fuels for global energy production has led to the tremendous increase of negative environmental impacts. Biofuels, specifically bioethanol have the immense prospective to be exploited as energy resources to substitute fossil-based fuels to some extent. According to [1], almost 80% of world biofuels production was contributed by bioethanol, which subsequently have highlighted it as one of the remarkable inventions in the 'biofuels world'. Bioethanol which can be produced from a wide range of renewable feedstock sources ensures its sustainability for a large scale production process. Despite of its renewability properties, bioethanol also gave a good impression in several aspects such as environment, trade, socio-economic and energy security [2,3].

Among different sources of bioethanol feedstock employed for research, seaweed has become one of the most trivial choices. This feedstock has many superior properties such as its abundance, high growth rate, easy to cultivate and its various components which can be extracted for many industrial purposes [4]. Specifically in Sabah, Malaysia, the seaweed cultivation activities have become one of the main income sources for the local people around the coastal areas. Seaweed has been widely cultivated for large scale production in four districts of Sabah including Semporna, Lahad Datu, Kudat and Kunak. The chemical composition of seaweed are varied which depends on the species and growth conditions. The high carbohydrate content of red seaweed makes it viable as one of the potential feedstock to be converted into bioethanol. Particularly for *Eucheuma cottonii*, it contains 26.49% of carbohydrate, 9.76% of protein, 1.10% of lipid, 5.91% of crude fibres, 10.55% of moisture, 18.25% of soluble fibres and 6.8% of insoluble fibres [5]. Cellulose is one of the examples of this seaweed composition which can be easily hydrolyzed into fermentable sugars (glucose). The low lignin composition of the seaweed enable for efficient disintegration of the cellulose during the hydrolysis process [6]. This is followed by fermentation as these sugars will be consumed by the microorganism for the conversion into bioethanol.

The research on production of bioethanol from third generation feedstock especially seaweed have been actively conducted for the past several years. In this case, optimization study is the most critical part as it determines the efficiency of the whole production processes and subsequently its commercial viability for market exposure [7]. Currently, the employment of optimization softwares

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such as Response Surface Methodology (RSM) has gained recognition as its competence in optimizing the process parameters by reducing the workload as well as the production cost [8]. Generally, RSM applies the statistical design which works by deducing the relationship between the parameters to be optimized to the main product of the research [9].

Up to now, there was no reported optimization research that employed RSM software to optimize the hydrolysis and fermentation processes from *E. cottonii* seaweeds for bioethanol production. Due to the lack of sufficient information in this field, it is very important to establish the optimum conditions for the conversion of the seaweed into bioethanol. Furthermore, the employment of RSM for optimization brought an obvious advantage as it offers a large amount of information from a few experimental runs, which subsequently is expected to be able to reduce the expensive cost of the analysis. Therefore, in this current study, the optimum conditions for enzymatic hydrolysis and fermentation of *E. cottonii* for bioethanol production was studied by employing the sequential statistical designs of factorial (Plackett-Burman Design) and response surface (Central Composite Design).

2. Materials and methods

2.1. Materials and chemicals

Eucheuma cottonii was purchased from Seaweed Research Unit of Universiti Malaysia Sabah. Hot water treatment was done to obtain the residue of *E. cottonii* [10]. The fresh *E. cottonii* sample was washed under running tap water, drained, cut and soaked in distilled water for a period of 30 min. Then, the seaweed was blended and boiled at 90 °C for 1 h. Later, the carrageenan extracts were filtered, leaving behind the cellulosic residues. This residue was washed with water and dried in oven for 48–72 h at 40 °C until a constant weight was obtained. The dried residue was ground into coarse powder, sieved and stored at 4 °C in a clean air-tight container until further use.

The enzymes such as Celluclast® 1.5 L and Viscozyme® L and reagents including 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP) and dextrose were purchased from Sigma Aldrich. HPLC grade acetonitrile, chloroform and acetone were supplied by Fischer Scientific. Yeast extract was purchased from Merck and peptone from FLUKA.

2.2. Microorganism cultivation and fermentation medium

Saccharomyces cerevisiae ATCC® 200062 was maintained as pure culture on YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 20 g/L agar) medium which was incubated at 30 °C for 48 h. The streaked plates after 48 h of incubation were stored in 2–8 °C until further use. Fermentation medium, 50 mL of YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose) was prepared in a 125-mL Erlenmeyer flask. Prior to use, the medium was autoclaved for 20 min at 121 °C.

2.3. Optimization of enzymatic hydrolysis

Sequential statistical designs of Plackett-Burman Design and Central Composite Design of Response Surface Methodology were used to screen and optimize various factors or parameters affecting the enzymatic hydrolysis of *E. cottonii* for glucose production.

2.3.1. Plackett-Burman Design (PBD) for parameters screening

PBD was applied to screen the significant parameters which mainly influence the enzymatic hydrolysis of *E. cottonii*. Five independent variables were evaluated at two levels, namely a high level (+1) and low level (−1) including cellulose loading (0.5–5% w/v), enzyme loading of Celluclast® 1.5 L and Viscozyme® L (ratio 20:80) (1–5% v/v), temperature (30–60 °C), pH (3.8–5.8) and incubation time (12–72 h). A total of 12 experiments were carried out in triplicates according to the experimental design matrix.

The hydrolysis experiments were performed in a fixed volume (50 mL) of sodium acetate buffer in a 125-mL Erlenmeyer flask and placed in an orbital shaker at 150 rpm. The glucose content of the sample was analyzed using HPLC. The independent variables which were significant at 5% level ($p < 0.05$) from the regression analysis were considered to have a greater impact on enzymatic hydrolysis of *E. cottonii* and were further optimized using CCD.

2.3.2. Central Composite Design (CCD) for optimization

CCD was applied to determine the optimum conditions of the significant parameters screened from PBD. The effect of cellulose loading (1–3% w/v), enzyme loading of Celluclast® 1.5 L and Viscozyme® L (ratio 20:80) (2–4% v/v) and incubation time (24–72 h) on the production of glucose were studied at five experimental levels (−2 (α), −1, 0, +1, +2 (α)). All the 20 experiments were carried out in triplicates according to the experimental design matrix generated from the CCD of RSM software.

The hydrolysis experiments were performed in a fixed volume (50 mL) of sodium acetate buffer (pH 4.8), temperature 50 °C in a 125-mL Erlenmeyer flask and placed in an orbital shaker at 150 rpm. The hydrolysate formed from the enzymatic hydrolysis was analyzed using HPLC to determine the glucose content of the sample. Analysis of variance (ANOVA), regression analysis and contour plots as well as 3D surface were generated from the Design-Expert® Version 7.0.0 Stat-Ease, Inc., Minneapolis software. The data produced were analyzed to determine the optimum conditions for enzymatic hydrolysis of *E. cottonii*.

2.4. Optimization of fermentation for bioethanol production

Once again, sequential statistical designs of Plackett-Burman Design and Central Composite Design of Response Surface Methodology were used to screen and optimize various factors or parameters affecting the fermentation of glucose hydrolysate from *E. cottonii* for bioethanol production.

2.4.1. Plackett-Burman Design (PBD) for parameters screening

Five main independent variables which plays an important role in bioethanol fermentation employing *S. cerevisiae* was evaluated at two levels, namely a high level (+1) and low level (−1). These five variables were inoculum concentration (5–20% v/v), pH (4–7), temperature (20–40 °C), fermentation time (24–72 h) and agitation speed (100–200 rpm). A total of 12 experiments were carried out in triplicates according to the experimental design matrix generated from the PBD of RSM software.

The fermentation experiments were performed in a fixed volume (50 mL) of YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) seaweeds residue hydrolysate) in a 125-mL Erlenmeyer flask and placed in an orbital shaker. The fermentation broths were analyzed using GCMS to determine the bioethanol content of the sample. The independent variables which were significant at 5% level ($p < 0.05$) from the regression analysis were considered to have a greater impact on fermentation of glucose by *S. cerevisiae* and were further optimized using CCD of RSM.

2.4.2. Central Composite Design (CCD) for optimization

The effect of significant parameters from PBD analysis including inoculum concentration (5–15% v/v), pH (5–6), temperature (30–40 °C) and fermentation time (48–72 h) for bioethanol production was studied at five experimental levels (−2 (α), −1,

0, +1, +2 (α)). All these 30 experiments were carried out in triplicates according to the experimental design matrix generated from the CCD of RSM software.

The fermentation experiments were performed in a fixed volume (50 mL) of YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) seaweeds residue hydrolysate) in a 125-mL Erlenmeyer flask and placed in an orbital shaker at 150 rpm. The fermentation broths were analyzed using GCMS to determine the bioethanol content of the sample. Analysis of variance (ANOVA), regression analysis and contour plots as well as 3D surface were generated from the software. The data produced were analyzed to determine the optimum conditions for fermentation of *E. cottonii* for bioethanol production.

2.5. Analytical methods

2.5.1. Glucose determination

Derivatization of sample using PMP was done prior to HPLC analysis using the method from Ref. [11]. Shimadzu (Semi-Prep) HPLC machine was employed for the glucose determination analysis. The HPLC system consisted of a LC-6AD liquid chromatograph pump, DGU-20A_{3R} degassing unit, SIL-10AP auto-sampler, CTO-20A column oven, SPD-M20A diode array detector (DAD) and Waters Symmetry C18 column.

The sample was analyzed under the following conditions: injection volume (20 μl), mobile phase of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (v/v, %), flow rate 1 mL/min, run time 20 min, detection at 245 nm. The glucose compound of the sample was determined based on the retention time of the standard glucose. The glucose concentration was quantified based on the values of its peak area to a calibrated standard glucose curve and the glucose yield was calculated using the following formula [10]:

$$\text{Glucose yield}(\%) = \frac{\text{Concentration of sugar at time of t}(g/L) \times 100}{\text{Initial concentration of substrate}(g/L)}$$

2.5.2. Bioethanol determination

The bioethanol content of the sample was analyzed by using Gas Chromatography-Mass Spectrometry (Agilent Model 6890N) by modifying the method of [5]. The chromatographic separation of the compound of interest was accomplished with a HP-5MS column (0.25 mm × 30 mm × 0.25 μm ID) and detected with an HP 5970 mass spectrometer detector. The sample (1 μL) was injected into the GCMS in split mode with split ratio of 200:1. The initial oven temperature was programmed to 40 °C, followed by an increase at rate of 10 °C/min up to 100 °C. Helium gas was used as the carrier with a flow rate of 0.7 mL/min with the run time of 6 min.

The bioethanol content of the sample was determined based on the retention time and the mass fragmentation of the standard ethanol. The bioethanol concentration was quantified based on the values of its abundance to a calibrated standard ethanol curve while the bioethanol yield and conversion efficiency were calculated based on the following formula:

$$\text{Bioethanol yield}(g/g) = \frac{\text{Bioethanol concentration } (g/L)}{\text{Initial glucose concentration } (g/L)}$$

$$\text{Conversion efficiency}(\%) = \frac{\text{Bioethanol yield } (g/g) \times 100}{0.51}$$

where 0.51 is the maximum bioethanol yield per unit of hexose sugar from glycolytic fermentation (g/g) [10].

3. Results and discussion

3.1. Significant parameters of enzymatic hydrolysis

Five parameters that possibly influenced the enzymatic hydrolysis of *E. cottonii* were initially screened for its significant effects. The design matrix of all the independent variables and the corresponding dependent variable are given in Table 1. The competency of the model was evaluated based on the Fischer's test for analysis of variance (ANOVA).

Natural log transformation was done due to the wide variations in the range of experimental data. From the ANOVA analysis, a p-value of 0.0040 indicated that the model term was significant. The linear regression coefficient (R^2) of this model was 0.9125. The Predicted R^2 of 0.6500 was in reasonable agreement with the Adjusted R^2 of 0.8396. The model also had a low standard deviation (0.34), C.V [%] (18.71) and PRESS (2.79). The Adequate Precision of this model was greater than 4 which indicates an adequate signal to be used to navigate the design space.

Independent variables which were significant at 5% level ($p < 0.05$) were considered to have an important effect on the response and were selected for further optimization studies. From the five independent variables, only cellulose loading ($p = 0.0013$), enzyme loading ($p = 0.0152$) and incubation time ($p = 0.0087$) were found to significantly influence the enzymatic hydrolysis of *E. cottonii* sample. The following equation was found to explain the production of glucose by enzymatic hydrolysis of *E. cottonii* in terms of coded factors.

Final equation in terms of coded factors:

$$\ln(\text{Glucose concentration}) = 1.82 + 0.56A + 0.33B - 0.19C + 0.090D + 0.38E$$

where A (cellulose loading), B (enzyme loading), C (temperature), D (pH) and E (incubation time).

Based on the results of PBD analysis, cellulose loading exerted the major effects in enzymatic hydrolysis of *E. cottonii* sample. Theoretically, high cellulose loading is important in the production process as it subsequently results in higher glucose yield. However, the selection range of cellulose loading is also important as some previous studies have reported an inverse relationship between cellulose loading to the conversion efficiency [12–15]. This could happen due to the accumulation of inhibitors and end products such as furfural and hydroxymethylfurfural (HMF) [16]. Furthermore, difficulties in mixing during the hydrolysis usually occur as the viscosity of the hydrolysate increases with an increase in cellulose loading [17].

Incubation time is the second significant variable that have major effects in enzymatic hydrolysis. According to [18], as enzymatic hydrolysis is a slow process, longer incubation period is needed to achieve higher yield of reducing sugar. On the other hand, the significant effect of enzyme loading also plays a major role in the enzymatic hydrolysis. Usually, higher enzyme loading is preferred as it increases the efficiency for the complete conversion of the substrate complex structure into its respective reducing sugars [19]. However, the optimum enzyme loading varies according to each type of feedstock due to the dissimilarity of the structures. The application of a combination of enzymes also may work synergically to achieve maximum substrate conversion [20]. Divergently, temperature and pH were found to have insignificant effects on enzymatic hydrolysis in this study.

Table 1
Experimental design matrix (actual and coded) and results of Plackett-Burman Design for enzymatic hydrolysis.

Run	Independent variables					Dependent variable
	Cellulose loading % (w/v)	Enzyme loading % (v/v)	Temperature (°C)	pH	Incubation time (h)	Glucose concentration (g/L)
1	5.00 (+1)	1.00 (−1)	60.00 (+1)	3.80 (−1)	12.00 (−1)	3.05
2	5.00 (+1)	5.00 (+1)	30.00 (−1)	5.80 (+1)	72.00 (+1)	18.98
3	0.50 (−1)	5.00 (+1)	60.00 (+1)	5.80 (+1)	12.00 (−1)	3.33
4	5.00 (+1)	5.00 (+1)	30.00 (−1)	5.80 (+1)	12.00 (−1)	20.03
5	0.50 (−1)	1.00 (−1)	30.00 (−1)	5.80 (+1)	72.00 (+1)	4.35
6	0.50 (−1)	5.00 (+1)	30.00 (−1)	3.80 (−1)	12.00 (−1)	3.31
7	5.00 (+1)	1.00 (−1)	60.00 (+1)	5.80 (+1)	12.00 (−1)	4.07
8	0.50 (−1)	1.00 (−1)	60.00 (+1)	5.80 (+1)	72.00 (+1)	4.32
9	5.00 (+1)	1.00 (−1)	30.00 (−1)	3.80 (−1)	72.00 (+1)	15.46
10	0.50 (−1)	1.00 (−1)	30.00 (−1)	3.80 (−1)	12.00 (−1)	2.15
11	5.00 (+1)	5.00 (+1)	60.00 (+1)	3.80 (−1)	72.00 (+1)	21.73
12	0.50 (−1)	5.00 (+1)	60.00 (+1)	3.80 (-1)	72.00 (+1)	4.50

3.2. Optimum conditions of enzymatic hydrolysis

Based on the previous results of PBD, the significant parameters were further analyzed using CCD to locate the optimum conditions of enzymatic hydrolysis of *E. cottonii*. The design matrix of all the independent variables and the results of 20 runs in triplicates of experimental and predicted glucose concentration are exhibited in Table 2. A good correlation between experimental and predicted glucose concentration from various conditions were observed and this indicates high accuracy of a response surface model constructed in this study.

Quadratic model was suggested for this optimization as the model p-value was statistically significant (<0.0001). The R^2 value at 0.9863 indicated a high accuracy of this model. The Adjusted R^2 of 0.9726 was in agreement with the Predicted R^2 of 0.9246. The Adequate Precision ratio of this model also indicates an adequate signal to navigate the design space. On the other hand, the values of the coefficient of variation (C.V.% = 6.74), standard deviation (SD = 1.04) and predicted residual sum of squares (PRESS = 53.78) were relatively low, which demonstrated that the model had a good precision and the experiments were reliable.

Considering the ANOVA analysis, six model terms, A, B, C, AC, A^2 and C^2 with $p < 0.05$ were found highly significant affecting the enzymatic hydrolysis. All the studied variables have been proven to be significantly affecting the enzymatic hydrolysis of *E. cottonii*. The following second order polynomial equation was found to explain the production of glucose by enzymatic hydrolysis of *E. cottonii* in coded factors.

Final equation in terms of coded factors:

$$\text{Glucose concentration} = 18.56 + 5.90A + 0.87B + 1.16C + 0.70AB + 1.29AC + 0.20BC - 1.52A^2 + 0.023B^2 - 3.30C^2$$

where A (cellulose loading), B (enzyme loading) and C (incubation time).

From the optimization analysis of the experimental data, the suggested optimum levels of all the variables determined by the quadratic model of CCD in this study were 3% (w/v) cellulose loading, 4% (v/v) enzyme loading and 54 h of incubation. Under these optimum conditions, the predicted glucose concentration reached up to 25.01 g/L. Fig. 1 shows the respective response surface plots (3-D) of the CCD model. These plots illustrate the significant interaction of all the three independent variables and were used to determine the optimum conditions for enzymatic

Table 2
Experimental design matrix (actual and coded) and results of Central Composite Design for optimization of enzymatic hydrolysis.

Run	Independent variables			Dependent variable	
	Cellulose loading % (w/v)	Enzyme loading % (v/v)	Incubation time (h)	Glucose concentration (g/L)	
				Experimental	Predicted
1	3.00 (+1)	2.00 (−1)	72.00 (+1)	20.97	21.15
2	3.00 (+1)	4.00 (+1)	72.00 (+1)	24.71	24.68
3	3.00 (+1)	2.00 (−1)	24.00 (−1)	17.46	16.64
4	2.00 (0)	3.00 (0)	48.00 (0)	18.53	19.36
5	1.00 (−1)	2.00 (−1)	72.00 (+1)	8.36	8.17
6	2.00 (0)	3.00 (0)	48.00 (0)	18.94	19.36
7	3.00 (+1)	4.00 (+1)	24.00 (−1)	19.77	19.39
8	1.00 (−1)	2.00 (−1)	24.00 (−1)	9.36	8.80
9	2.00 (0)	3.00 (0)	48.00 (0)	18.70	19.36
10	1.00 (−1)	4.00 (+1)	72.00 (+1)	8.67	8.91
11	2.00 (0)	3.00 (0)	48.00 (0)	18.95	19.36
12	1.00 (−1)	4.00 (+1)	24.00 (−1)	9.52	8.76
13	2.00 (0)	1.32 (−1.682)	48.00 (0)	15.82	16.37
14	2.00 (0)	3.00 (0)	48.00 (0)	19.71	17.77
15	3.68 (+1.682)	3.00 (0)	48.00 (0)	23.04	23.39
16	0.32 (−1.682)	3.00 (0)	48.00 (0)	3.05	3.53
17	2.00 (0)	3.00 (0)	48.00 (0)	18.30	17.77
18	2.00 (0)	4.68 (+1.682)	48.00 (0)	19.02	19.30
19	2.00 (0)	3.00 (0)	7.64 (−1.682)	5.24	6.46
20	2.00 (0)	3.00 (0)	88.36 (+1.682)	10.77	10.38

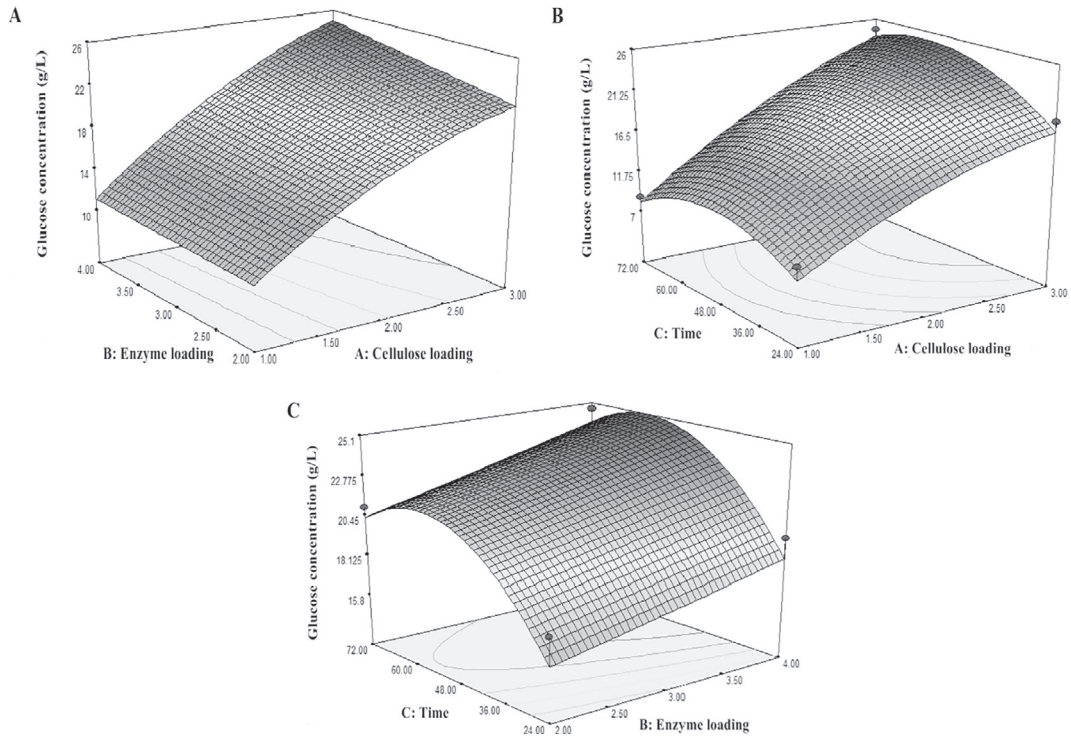

Fig. 1. The 3-D surface plot of interaction between the parameters on glucose production, **A)** cellulose loading and enzyme loading, **B)** cellulose loading and incubation time and **C)** incubation time and enzyme loading. (**Note:** The darker region represents the area of optimum conditions for enzymatic hydrolysis).

hydrolysis in this study.

3.2.1. Effect of cellulose loading

Fig. 1 (A and B) shows the response surface for the interaction of cellulose loading with enzyme loading and incubation time on glucose concentration. The results revealed that glucose concentration increases with an increase in cellulose. This indicates the significance of cellulose loading in influencing the production of glucose during enzymatic hydrolysis [21].

According to [22], the employment of low to high substrate or cellulose loading can enhance the enzymatic hydrolysis rate. At some point, too high substrate loading resulted in the opposite trend in the efficiency. Cellulose loading up to 10% (w/v) was found to increase the glucose production as well as its yield and efficiency. However, any increase in cellulose loading higher than that did not significantly induce higher production of glucose [14].

3.2.2. Effect of enzyme loading

Enzyme loading is also one of the most critical parameters in enzymatic hydrolysis. For substrates like cellulose, high enzyme (cellulase) loading favoured the hydrolysis since more enzymes were available for breakdown of cellulose during longer incubation time (Fig. 1 [A and C]). On the other hand, at low enzyme loading, the conversion efficiency is expected to be low especially with high substrate loading.

In this case, it triggers for enzyme inactivation as well as loss in the reactivity of the substrate during the enzymatic hydrolysis reaction [23]. This occurs because at low enzyme loading, it is quite difficult for the enzyme to diffuse or adsorb onto the substrate due to its recalcitrance structure [21]. Since each feedstock has some distinction in their compositions and structures, the optimum loading of enzyme is also different.

3.2.3. Effect of incubation time

Interaction of incubation time with cellulose and enzyme loading on glucose production is shown in Fig. 1 (B and C). Incubation time enables the cellulose and enzyme to interact cooperatively to produce its respective reducing sugars after the hydrolysis process. According to [24], longer incubation time is needed to produce higher concentrations of glucose.

This is due to the fact that more time is needed to completely convert all the cellulose into glucose. However, at certain period of time, the glucose production and the yield may reduce. Most of the time, it is caused by the occurrence of end-product inhibition which is the accumulation of glucose and cellobiose from the interaction of cellulose substrate with cellulase [25].

3.3. Validation of the CCD model of enzymatic hydrolysis

The validation of the model was performed under the optimum conditions and 24.24 g/L of glucose (81% glucose yield) was obtained from *E. cottonii* which was in close agreement with the model prediction value of 25.01 g/L. The difference between the predicted and experimental value was only 3.11%. The differences of less than 10% are enough to justify the validity of the response model [26]. The hydrolysate then was employed as the glucose source for the optimization of fermentation for bioethanol production.

3.4. Significant parameters of fermentation for bioethanol production

Five parameters or independent variables that possibly influenced the fermentation were initially screened for its significant effects. The design matrix of all the independent variables and the corresponding dependent variable in terms of bioethanol concentration are given in Table 3. Fischer's test for analysis of variance

Table 3
Experimental design matrix (actual and coded) and results of Plackett-Burman Design for fermentation.

Run	Independent variables					Dependent variable
	Inoculum concentration % (w/v)	pH	Temperature (°C)	Fermentation time (h)	Agitation speed (rpm)	Bioethanol concentration (g/L)
1	5.00 (−1)	4.00 (−1)	20.00 (−1)	72.00 (+1)	100 (−1)	0.83
2	5.00 (−1)	7.00 (+1)	40.00 (+1)	72.00 (+1)	100 (−1)	1.06
3	20.00 (+1)	4.00 (−1)	40.00 (+1)	72.00 (+1)	100 (−1)	1.57
4	5.00 (−1)	4.00 (−1)	20.00 (−1)	24.00 (−1)	100 (−1)	0.76
5	20.00 (+1)	7.00 (+1)	20.00 (−1)	72.00 (+1)	200 (+1)	2.62
6	5.00 (−1)	4.00 (−1)	40.00 (+1)	24.00 (−1)	200 (+1)	0.40
7	20.00 (+1)	7.00 (+1)	40.00 (+1)	24.00 (-1)	100 (−1)	1.06
8	5.00 (−1)	7.00 (+1)	40.00 (+1)	24.00 (−1)	200 (+1)	0.54
9	20.00 (+1)	7.00 (+1)	20.00 (−1)	24.00 (−1)	100 (−1)	1.98
10	5.00 (−1)	7.00 (+1)	20.00 (−1)	72.00 (+1)	200 (+1)	2.06
11	20.00 (+1)	4.00 (−1)	40.00 (+1)	72.00 (+1)	200 (+1)	1.11
12	20.00 (+1)	4.00 (−1)	20.00 (−1)	24.00 (−1)	200 (+1)	0.73

(ANOVA) was used to evaluate the data and the competency of the model.

Transformation using inverse square root was done to increase the value of Predicted R-squared and Adjusted R-squared. From the ANOVA analysis, p-value of 0.0036 indicated that the model term was significant. In addition, the linear regression coefficient (R^2) of this model was 0.9157. The Predicted R^2 of 0.6630 was in reasonable agreement with the Adjusted R^2 of 0.8455. The model also had a low standard deviation (0.11), C.V [%] (11.13) and PRESS (0.30). The Adequate Precision of this model also showed an adequate signal to navigate the design space.

From the five independent variables studied, only four were found to be significance with respect to fermentation. These four variables were inoculum concentration ($p = 0.0056$), pH ($p = 0.0109$), temperature ($p = 0.0219$) and fermentation time ($p = 0.0035$). The following equation was found to explain the production of bioethanol in terms of coded factors.

Final equation in terms of coded factors:

$$0/\text{Sqrt}\,(\text{Bioethanol concentration}) = 1.01 - 0.14A - 0.12B + 0.099C - 0.15D + 0.057E$$

where A (inoculum concentration), B (pH), C (temperature), D (fermentation time) and E (agitation speed).

ANOVA analysis from the PBD model has revealed that fermentation time exerted a significant effect in bioethanol fermentation of glucose by *S. cerevisiae* ATCC® 200062. The design favoured longer fermentation time for this study. However, the employment of longer fermentation time also has its own side effects such as formation of toxic materials caused by the increase of bioethanol concentration [27]. Sometimes, the occurrences of these limitations depend on the mode of fermentation conducted in which the conditions inside the fermentation broth must be monitored frequently.

Inoculum concentration was proved to be the second most significant fermentation parameter in this study. It was suggested from the PBD that employment of higher concentration of inoculum led to the production of higher bioethanol concentration and yield as well as better conversion efficiency. This result was found to be in agreement with [28], where high concentration of inoculum increased the bioethanol yield to certain extent.

Another factor was found to affect the fermentation process was pH. It was found out that the optimum pH for *S. cerevisiae* were in the range of 4–6 [29]. The type of feedstock and fermentative microorganism employed usually will influence the optimum pH for fermentation [30]. Temperature was found to be the last factor which has significant impact on the production of bioethanol. Increase in temperature significantly increases the bioethanol concentration [31]. Sometimes, in certain cases, high temperature can be the stress factor that can limit the growth of the microorganism as they produce shock proteins that can inactivate their ribosomes [27]. Usually, at temperature of 50 °C, reduction in the bioethanol concentration can be observed [32]. In this study, lower temperature was found to be favourable for *S. cerevisiae* ATCC® 200062 to produce higher yield of bioethanol.

Among all the studied parameters, only agitation speed was found to be not significant for fermentation of glucose from *E. cottonii*. Varying the agitation speed from 100 to 200 rpm did not show any significant effect, hence the speed employed during the reaction can be fixed at any value within this range. For fermentation utilizing *S. cerevisiae* cells, the agitation speed between 150 and 200 rpm is the most employed one during the production. Increase in agitation speed above this range can restrain the metabolic activities of the cells [33].

3.5. Optimum conditions of fermentation for bioethanol production

Four parameters or independent variables, including inoculum concentration, pH, temperature and fermentation time which were found to have a significant influence on fermentation for bioethanol production were further optimized using CCD of RSM. The design matrix of all the independent variables and the results of 30 runs in triplicates of experimental and predicted bioethanol concentration are shown in Table 4. It can be observed that there was a good correlation between experimental and predicted bioethanol concentration from various conditions. This clearly shows the high accuracy of response surface model constructed in this study.

Quadratic model was suggested as the model p-value was statistically significant (<0.0001). The R^2 value at 0.9831 indicated a high accuracy of this model. The Adjusted R^2 of 0.9663 was in agreement with the Predicted R^2 of 0.8975. The Adequate Precision ratio of this model also indicates a satisfactory signal to navigate the design space. On the other hand, the values of coefficient of variation (C.V.% = 6.57), standard deviation (SD = 0.44) and predicted residual sum of squares (PRESS = 16.22) were relatively low, which demonstrated that the model had a good precision and the experiments were reliable.

Considering the ANOVA analysis, seven model terms, A, C, D, A^2, B^2, C^2 and D^2 with $p < 0.05$ were found highly significant influencing the fermentation rate. The following second order polynomial equation was found to explain the production of bioethanol by fermentation of glucose from *E. cottonii* in terms of coded factors.

Final equation in terms of coded factors:

Table 4
Experimental design matrix (actual and coded) and results of Central Composite Design for optimization of fermentation for bioethanol production.

Run	Independent variables				Dependent variable	
	Inoculum concentration % (v/v)	pH	Temperature (°C)	Fermentation time (h)	Bioethanol concentration (g/L)	
					Experimental	Predicted
1	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	8.95	9.02
2	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	8.59	9.02
3	15.00 (+1)	5.00 (−1)	40.00 (+1)	72.00 (+1)	8.66	8.87
4	15.00 (+1)	5.00 (−1)	30.00 (−1)	72.00 (+1)	9.78	9.84
5	5.00 (−1)	5.00 (−1)	30.00 (−1)	48.00 (−1)	4.04	3.70
6	15.00 (+1)	6.00 (+1)	30.00 (−1)	48.00 (−1)	5.63	5.75
7	5.00 (−1)	6.00 (+1)	40.00 (+1)	48.00 (−1)	4.37	4.04
8	15.00 (+1)	6.00 (+1)	40.00 (+1)	48.00 (−1)	4.88	5.23
9	15.00 (+1)	5.00 (−1)	40.00 (+1)	48.00 (−1)	5.07	5.10
10	15.00 (+1)	6.00 (+1)	30.00 (−1)	72.00 (+1)	9.07	9.02
11	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	8.73	9.02
12	15.00 (+1)	6.00 (+1)	40.00 (+1)	72.00 (+1)	8.51	8.59
13	5.00 (−1)	5.00 (−1)	40.00 (+1)	48.00 (−1)	3.14	3.12
14	5.00 (−1)	6.00 (+1)	30.00 (−1)	48.00 (−1)	4.36	4.23
15	5.00 (−1)	5.00 (−1)	30.00 (−1)	72.00 (+1)	7.23	6.95
16	15.00 (+1)	5.00 (−1)	30.00 (−1)	48.00 (−1)	5.83	6.02
17	5.00 (−1)	6.00 (+1)	40.00 (+1)	72.00 (+1)	6.96	6.83
18	5.00 (−1)	6.00 (+1)	30.00 (−1)	72.00 (+1)	7.36	7.07
19	5.00 (−1)	5.00 (−1)	40.00 (+1)	72.00 (+1)	6.69	6.32
20	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	9.07	9.16
21	10.00 (0)	5.50 (0)	25.00 (−2)	60.00 (0)	7.28	7.45
22	10.00 (0)	6.50 (+2)	35.00 (0)	60.00 (0)	7.67	7.70
23	10.00 (0)	5.50 (0)	45.00 (+2)	60.00 (0)	6.33	6.32
24	0.00 (−2)	5.50 (0)	35.00 (0)	60.00 (0)	0.00	0.85
25	10.00 (0)	5.50 (0)	35.00 (0)	84.00 (+2)	8.51	8.72
26	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	8.63	8.41
27	10.00 (0)	5.50 (0)	35.00 (0)	60.00 (0)	8.95	8.41
28	20.00 (+2)	5.50 (0)	35.00 (0)	60.00 (0)	5.58	4.92
29	10.00 (0)	4.50 (−2)	35.00 (0)	60.00 (0)	7.29	7.48
30	10.00 (0)	5.50 (0)	35.00 (0)	36.00 (−2)	2.13	2.11

$$\text{Bioethanol concentration} = 8.72 + 1.02A + 0.061B - 0.29C + 1.65D - 0.20AB - 0.084AC + 0.14AD + 0.10BC - 0.10BD - 0.013CD - 1.38A^2 - 0.21B^2 - 0.38C^2 - 0.75D^2$$

where A (inoculum concentration), B (pH), C (temperature) and D (fermentation time).

The optimization analysis was done to locate the optimum conditions for the fermentation process. From the optimization analysis of the experimental data, the suggested optimum levels of all the variables determined by the quadratic model of CCD in this study were 12% (v/v) inoculum concentration, pH 5.2, temperature 32 °C and 72 h of fermentation time. Under these optimum conditions, the predicted bioethanol concentration reached up to 9.98 g/L. Fig. 2 shows the optimum response surface plots (3-D) of the CCD model.

3.5.1. Effect of inoculum concentration

Fig. 2 (A, B and C) shows the interaction of inoculum concentration to the other three studied parameters. It can be seen from the interaction that increase in the inoculum concentration subsequently increases the concentration of bioethanol produced. Most of the previous studies on bioethanol production from seaweed feedstock have employed 5–10% (v/v) of *S. cerevisiae* inoculum during the fermentation [34–37].

All of these studies have achieved more than 80% of conversion efficiency at these ranges of inoculum concentration. This has proven that higher inoculum concentration is needed to enhance production of bioethanol. However, further increase in the inoculums concentration up to 20% (v/v) did not show any significant increase in the bioethanol production.

3.5.2. Effect of pH

It can be seen from Fig. 2 (A, B and D) that slightly acidic conditions are the most preferable ones for yeast especially *S. cerevisiae*. Unfortunately, at certain cases, these conditions tend to retard the growth of yeast cells as it easily contaminates the fermentation medium. In most of the cases, yeast cells preferred more acidic conditions than the neutral and basic environments [38]. Based on the results of this research, 5.2 was the optimum pH for the fermentation process. This indicates that the *S. cerevisiae* ATCC® 200062 employed in this study preferred a slightly acidic condition to grow.

3.5.3. Effect of temperature

Fig. 2 (B, D and F) shows the interaction of temperature with other parameters. A slight increase or decrease in temperature during the fermentation process significantly affect the yield of bioethanol [39]. Increase in temperature will subsequently increase the yeast metabolism as well as growth rate [33]. Based on the previous study by Ref. [40], *S. cerevisiae* ATCC® 200062 have shown high growth rate between the temperatures of 25–35 °C. For a comparison, this present study has found that 32 °C was the optimum temperature for the fermentation by *S. cerevisiae* ATCC® 200062.

3.5.4. Effect of fermentation time

Fermentation time is one of the most important parameters in the production of bioethanol as it determines the economical part of the whole production process as well as the viability of the

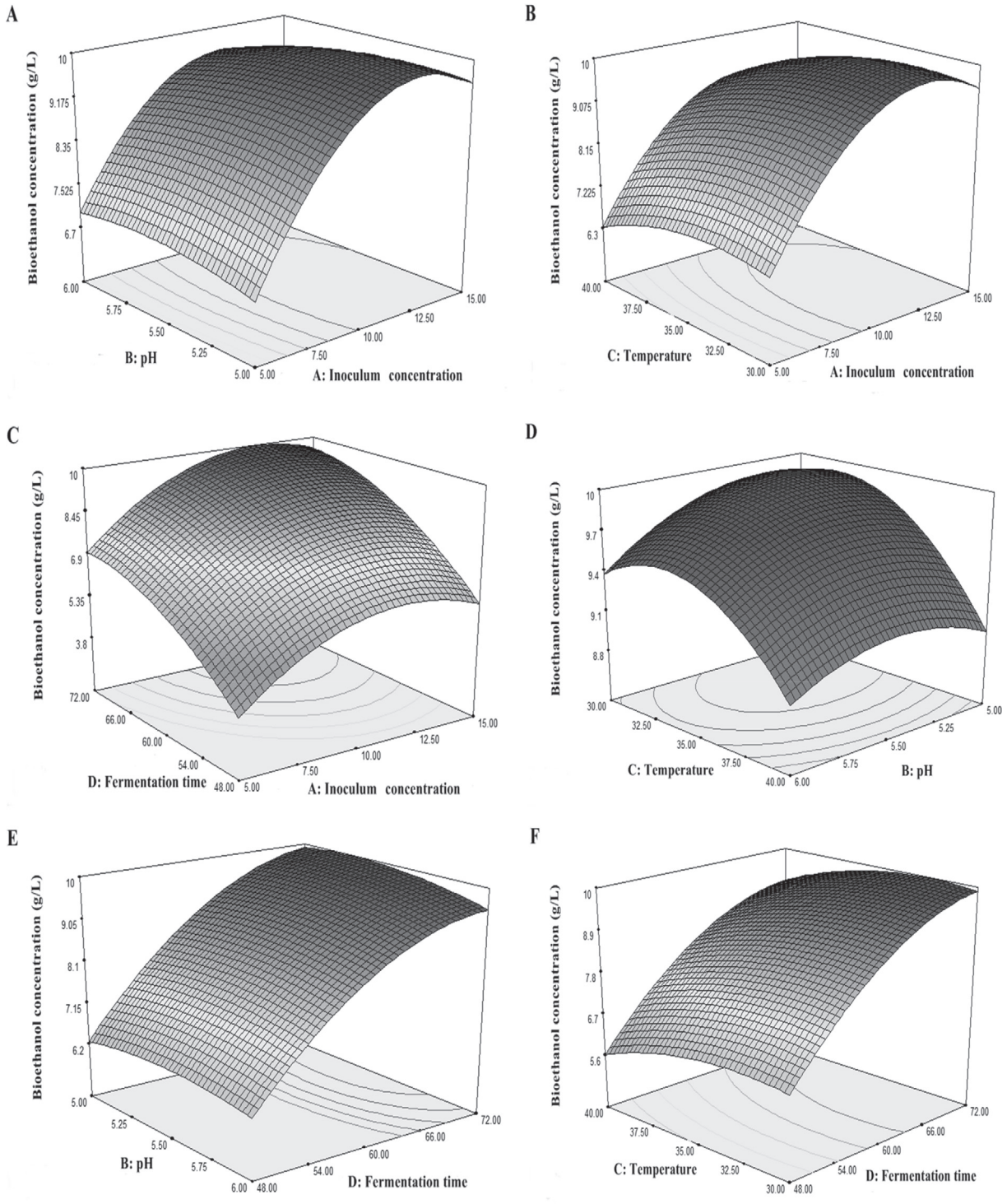

Fig. 2. The 3-D surface plot of interaction between the parameters on bioethanol production, **A)** inoculum concentration and pH, **B)** inoculum concentration and temperature, **C)** inoculum concentration and fermentation time, **D)** pH and temperature, **E)** pH and fermentation time and **F)** temperature and fermentation time. (**Note:** The darker region represents the area of optimum conditions for fermentation).

fermentative microorganisms employed [41]. According to [42], increasing the fermentation time will eventually lead to higher production of bioethanol. It was reported that the maximum bioethanol yield can be obtained after 60–72 h of fermentation as shown in this study (Fig. 2 (C, E and F). Different types of reducing sugars have different optimum fermentation time due to distinct metabolic regulation for each of them. As for comparison, glucose consumption by *S. cerevisiae* was faster than other types of sugars [43].

3.6. Validation of CCD model of fermentation for bioethanol production

The validation of fermentation experiment was performed

under optimum conditions of 12% (v/v) inoculum concentration, pH 5.2, temperature 32 °C and 72 h of fermentation. The bioethanol concentration with the sources of 24.24 g/L of glucose after undergoing fermentation under optimized conditions was 9.77 g/L (0.40 g/g yield, 78% conversion efficiency) which was in close agreement with model prediction value of 9.98 g/L. The difference between the predicted and experimental value was only 2.05%. Hence, it can be concluded that the response surface generated in this study could be reliably used to predict the production of bioethanol from fermentation process. The optimization of fermentation alone employing RSM software is hardly found which make this present study as one of the pioneer reference for future research. The analysis of the experimental data of this study was able to locate the optimum conditions for fermentation of glucose from *E. cottonii* residue through fermentation. Thus, it can be said that the RSM is an efficient approach in optimizing the fermentation of the third generation bioethanol feedstock as compared to its conventional method.

4. Conclusion

The optimization of enzymatic hydrolysis and fermentation of *E. cottonii* using CCD of RSM was evaluated in this study. It was found that 3% (w/v) of cellulose loading, 4% (v/v) of enzyme loading and 54 h of incubation time were the optimum conditions that gave the highest glucose production from enzymatic hydrolysis of *E. cottonii*. On the other, the highest bioethanol concentration was obtained when fermentation was conducted with 12% (v/v) of inoculum concentration, pH 5.2, 32 °C and 72 h of fermentation time. From the results, it can be concluded that the experimental data obtained based on the optimized conditions was in close agreement with the RSM model prediction. This RSM approach of optimization has a promising potential to be employed for a better bioethanol production in the future. In addition, the potential of *E. cottonii* seaweed as the main feedstock may contribute to the development of bioethanol industry in Malaysia.

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