Optimization on fermentation of seaweed (Gracilaria sp.) as feedstock for bioethanol production by Saccharomyces cerevisiae

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Abstract. In this study, dried seaweed (Gracilaria sp.), a red algae was used as feedstock for the production of bioethanol due to its high carbohydrate content (76.67 %). Dried seaweed is hydrolysed into reducing sugar by using dilute acid hydrolysis pre-treatment at optimized conditions (0.1 M sulphuric acid (H2SO4), 121 °C, 30 minutes) and able to obtain 18.438 g/L of reducing sugar. This hydrolysate is converted into bioethanol by Saccharomyces cerevisiae via fermentation. Two significant factors, namely, fermentation temperature and medium pH were screened by performing One-Factor-At-A-Time (OFAT) analysis. The first factor manipulated was the fermentation temperature (40-50 °C at pH 4.5), followed by medium pH (pH 4.0-5.0 at 45 °C). Ethanol with concentration of 3.4202 g/L was successfully obtained from OFAT analysis under condition of 45 °C and pH 4.5. The significant factors were then statistically optimized using Central Composite Design (CCD) in Response Surface Methodology (RSM). From the data analyzed from Design of Experiment (DoE), at its optimum fermentation conditions (44.83 °C and pH 4.48), the maximal ethanol production obtained is 3.8395 g/L. A validation test with triplicate was performed to validate the actual with predicted value and result in ±1.49% deviation is accepted.

1. Introduction

Over the past century, rapid increased in the world population has drastically driven large amount of energy consumption. In concern to the matter, environmental matters like global warming as well as air pollution caused by processing of crude oil. The depletion of fossil fuels ultimately shifted the world energy consumption from depending on fossil energy resources towards creating alternative fuel sources from various bio-based renewable resources [1]. Production of bioethanol from renewable feedstock is the most convenient renewable energy source and an ideal substitution for gasoline. Bioethanol is considered clean, safe and bio-based energy to be utilized [2]. The global bioethanol production had risen rapidly in recent years to meet increasing energy demand.

The first generation bioethanol is yielded from feedstock like tubers, cereals or even high sugar content plants mainly from food crops. Consequently, it intense to cause the global food shortage and topple the food to population ratio [3].

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As the development of fuels from biomass continue without concerns, the consumption of sugars and edible crops has raised the food security, ethics issues, and human morality [4]. Meanwhile, the second generation bioethanol which refined from lignocellulosic biomass is developed to meet economic growth and morality requirements. Second generation bioethanol is manufactured from non-food raw material like tall grasses, woods, and other lignocellulosic materials. Lignocellulosic materials are used as feedstock to synthesis bioethanol products because comparatively bulky quantity and economic [5]. Lignocellulose is mainly constituted of hemicellulose, cellulose, lignin, extractives, and ashes. Sugars in lignocellulosic are difficult to extract due to compact structure arrangement, and extensive pre-treatment to secure the hydrocarbon polymers for proceeding fermentation. Among the technical barriers, delignification is the major obstacle as it consumes time and high cost [6].

Due to several obstacles in second generation bioethanol, a third generation bioethanol has been introduced and investigated is from macroalgae (seaweed). Seaweed contains low lignin and recalcitrant cell wall structure made the cellulose easy to hydrolyze without carry out lignin removal process [7]. Red algae is known to have high content of carbohydrates, typically galactose and glucose, in which both are considered as fermentable sugars for ethanol production by yeast [8]. Seaweed (Gracilaria sp.) is a type of red algae composed of carbohydrate (76.67 %wt), crude protein (16.0 %wt), crude lipid (1.2 %wt), ash (6.13 %wt) and crude fiber (7.87 %wt) based on the dried weight [9]. It is a fast-growing seaweed and produced abundantly as free-floating populations in marine water, often distributed in the shallow and warm-water embayment. In Gracilaria sp., the polysaccharide consists of carbohydrate is hydrolyzed to monosaccharide. Monosaccharide such as glucose, galactose and xylose are the potential compounds that can be converted to product such as bioethanol via fermentation process.

The conversion of sugars in bioethanol feedstock typically assisted by suitable microbe. Saccharomyces cerevisiae (S. cerevisiae) is one of the widely studied and used yeast for the fermentation process that applied in many industrial sectors. S. cerevisiae is well-known to be superior to bacteria, other yeasts, or filamentous fungi commonly in physiological characteristics concerning to ethanol production. S. cerevisiae sustains to wide range of pH (4.0-5.0), which often makes the fermentation less susceptible to infection. S. cerevisiae also known as generally regarded as safe (GRAS) on its various applications.

The production of bioethanol from dry seaweed made a considerable choice for commercial application of alternative fuels. However, the studies regarding optimization of ethanol production from dried seaweed (Gracilaria sp.) by using S. cerevisiae is limited. Hence, it is worthwhile to study on the optimization condition of S. cerevisiae to produce ethanol for seaweed since seaweed contains high carbohydrate and S. cerevisiae is capable to convert reducing sugars to bioethanol.

2. Materials and Methods

2.1. Raw Materials

Simple and branched blades of fresh seaweed, namely, Gracilaria sp., was collected from seaweed farmer at Sungai Petani, Kedah and transported to Microbiology Laboratory in Universiti Malaysia Perlis, Perlis to be stored. For sample preparation, the seaweed was washed thoroughly with tap water in order to remove impurities and dried in an oven at 45 °C for 1.5 hour. 10 g of clean dried seaweed was blended by using blender. The blended seaweeds were stored in air-tight container for pre-treatment process later.

2.2. Dilute Acid Hydrolysis Pre-treatment of Dried Seaweed

10 g of blended dried seaweed was mixed with 200 mL of 0.1 M H₂SO₄ in a round bottom flask. A proper set-up of apparatus installed with water condenser was placed inside the fume hood for acid hydrolysis purpose. At optimum condition, the pre-treatment required 30 minutes for each run at 121 °C [10]. After cooling down for 15 minutes, 50 mL of seaweed hydrolysate was centrifuged at 4000
rpm for 20 minutes to separate the supernatant and pellet layers. The supernatant layer was obtained by pipetted out leaving the pellet. Few drops of 5 M sodium hydroxide (NaOH) was added to neutralize the hydrolysate before stored in the chiller to avoid further reaction.

2.3. Proximate Analysis
The carbohydrate content was analyzed using Anthrone method [11]. For Anthrone reagent preparation, 100 mg of Anthrone was dissolved in 50 mL of ice-cold crude 96 % H₂SO₄. For total carbohydrate measurement, 4 mL of Anthrone reagent was added to 1 mL of hydrolysate and slowly mixed. The test tubes were caped to minimize volume loss due to evaporation. The mixture was boiled at 100 °C in water bath for 10 minutes. After cooled to room temperature, the absorbance was determined at 630 nm wavelength [11]. Similar preparation was performed for blank except for 1ml of hydrolysate was replaced with 1ml of distilled water. The reducing sugar content was analyzed by using 3,5-Dinitrosalicylic acid (DNS) method [12]. For DNS reagent preparation, 1 g of dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite were dissolved in 100 mL of 1 % NaOH by continuous stirring in an aluminium-wrapped conical flask. The solution was stored in chiller at 4 °C prior before use. For total reducing sugar measurement, 3 mL of DNS reagent was added to 1 mL of hydrolysate and slowly mixed. The test tubes were caped to avoid the loss of liquid due to evaporation. The mixture was heated at 90 ºC for 5 minutes in water bath to develop the red-brown colour. 1 mL of 40 % potassium sodium tartrate was added to stabilize the colour formed. The mixture was let to cool down at room temperature before the absorbance was recorded using UV-Vis spectrophotometer at wavelength of 540 nm [13]. Similar preparation was performed for the blank except for 1ml of hydrolysate was replaced with distilled water.

2.4. Preparation of Suspension Inoculum
*S. cerevisiae* from the stock culture was streaked and cultured on Potato Dextrose Agar (PDA) plate and incubated at 30 ºC for 48 hours [14]. One full loop of *S. cerevisiae* was transferred into 100 mL of yeast extract peptone-dextrose (YPD) broth and incubated at 30 ºC and 150 rpm for 24 hours.

2.5. Bioethanol Fermentation with Pre-treated Seaweed Hydrolysate
50 mL of the neutralized hydrolysate in 250ml shake flasks were adjusted to desired pH before autoclaved for 15 minutes at 121 °C. After cooling down, 10 % (v/v) inoculum was inoculated into each flask and cultivated at 30 ºC and 150 rpm for 18 hours [14].

2.6. Analytical Method
The fermentation samples were centrifuged at 4000 rpm for 15 minutes. The supernatant layer was separated from pellet by pipetting into test tubes. For dichromate reagent preparation, 10 g of potassium dichromate was dissolved in 100 mL of 5 M H₂SO₄. The ethanol concentration was estimated by potassium dichromate method [15]. To measure the ethanol concentration, 1 mL of dichromate reagent was added to mix with 3 mL of the fermentation sample. The test tubes were caped prior to prevent the loss of volume due to evaporation. The test tubes were placed in water bath at 90 ºC for 10 minutes and cooled to room temperature. The absorbance was measured at 595 nm wavelength using UV-Vis spectrophotometer. Similar preparation was made for the blank except for 3ml of fermentation sample was replaced with hydrolysate.

2.7. Screening of Factors Affecting Bioethanol Production
Factors that affect bioethanol production were screened by performing OFAT experiments. Two parameters were manipulated as fermentation temperature and medium pH. The range of temperatures, 30, 35, 40, 45, and 50 ºC, respectively for the screening study. Meanwhile, the range of medium pH, 4.0, 4.5, 5.0, and 5.5 were chosen. All samples were performed in triplicates.
2.8. Response Surface Methodology for Bioethanol Production

The optimization of fermentation parameters was performed based on OFAT results using RSM. CCD method was used to study the interaction based on two parameters that affect the bioethanol production. The variables studied in this model were fermentation temperature \((X_1)\) and medium pH \((X_2)\). Table 1 shows the selected range and level for fermentation process variables designed in CCD table.

| Parameter | Independent variables | Unit | Level  |
|-----------|-----------------------|------|--------|
| \(X_1\)   | Fermentation temperature | °C   | -α 37.93 40 45 50 52.07 α |
| \(X_2\)   | Medium pH             | pH   | -α 3.79 4.0 4.5 5.0 5.21 α |

Optimization of the significant factors was carried out using CCD in order to obtain a maximal bioethanol production. A total set of 11 experimental runs including three replicated centre points used to develop correlation between the fermentation parameters were formed. Each parameter was divided into three levels: low \((-1)\), medium \((0)\), high \((+1)\) and \(±α\).

3. Result and Discussion

3.1. Carbohydrate and Reducing Sugar Contents

Glucose standard curve was used as calibration tool for both findings of total carbohydrate and reducing sugar contents. Table 2 shows the carbohydrate and reducing sugar contents of dried seaweed hydrolysate obtained through dilute acid hydrolysis pre-treatment.

| Carbohydrate content (g/L) | Reducing sugar content (g/L) |
|----------------------------|----------------------------|
| 20.840 ± 0.26              | 18.438 ± 1.036             |

3.2. Range Screening of Fermentation Parameters of Seaweed Hydrolysate by Saccharomyces cerevisiae

The range screening of parameters were done by using OFAT to determine significant range of parameters for optimization study later. There are two parameters involved, namely fermentation temperature \((^°\text{C})\) and medium pH \((\text{pH})\). The significant level of each parameter was determined by analyzing the optimal range obtained from the plotted curve based on parameter effect. The effect of the two parameters on production of ethanol are represented in Figure 1.

![Figure 1](image.png)

**Figure 1.** The effect of two parameters on production of ethanol, (a) The effect of fermentation temperature on ethanol concentration and (b) The effect of medium pH on ethanol concentration.

The Figure 1 (a) depicted an increasing trend of graph. The lowest concentration of ethanol was obtained at 30 °C with 2.6130 g/L. The ethanol concentration was observed to increase steadily until it
reached at maximum concentration of 3.4202 g/L at 45 °C. Typically, *S. cerevisiae* is found to produce ethanol optimally at the range of 30 - 40 °C [17] and started to decrease the growth at temperature above 40 °C, thus may not obtained optimum ethanol at respective thermophilic temperature condition [18]. Nonetheless, in this study the highest ethanol production was at 45 °C indicates the strains of *S. cerevisiae* used were thermotolerant. However, at 50 °C, the ethanol concentration started to decrease to 3.3195 g/L. In ethanol fermentation, temperature at 50 °C may not preferred as changes in the transport system caused from the build-up of toxin together with ethanol in the cell and the denaturation of enzymes and ribosomes and membrane fluidity problems may cause decrease in ethanol production [17].

From Figure 1 (b), the ethanol concentration increase from 3.1871 g/L to 3.2845 g/L at pH 4.0 and 4.5 respectively. The increment is due to preferable on acidic environment by *S.cerevisiae* whereby the state of acidic amino acid that help to determine the three-dimensional shape of the protein has not been altered and lead to correct shape to allow the fermentation process [19]. The significant decrease of ethanol production was observed at pH of 5.0 and 5.5 with 3.0893 g/L and 2.9323 g/L respectively. Exceed pH 5.0, the concentration of ethanol was considerably low after the denaturation of *S. cerevisiae* when the production of butyric acid was favoured [17].

### 3.3. Optimization of Fermentation Parameters of Dried Seaweed Hydrolysate by *S. cerevisiae*

The optimization of experiment design was carried out by using CCD under RSM by DoE software. Summary of the Analysis of Variance (ANOVA) for response surface quadratic model is presented in Table 3. The ANOVA was primarily used to identify the significance of the model. The significance of each coefficient was determined by whether the value of “Prob > F is less than 0.0500. Contrarily, the variable with “Prob > F” value greater than 0.1000 was considered not significant. The *F*-value is the measurement of variation of the data about the mean. The high *F*-value and very low probability indicate that the model is in good prediction of the experimental results [16]. From Table 3, the model *F*-value of 16.01 implied that the model was significant. There was only 0.43 % chance that a “Model *F*-value” could occur due to noise. In this case, A² and B² shown the *p*-value of 0.0003 and 0.0336, respectively which indicated they were significant model terms. The “Lack of Fit *F*-value” of 2.09 implied that the Lack of Fit is not significant relative to the pure error. However, there is a 34.02% of chance that a “Lack of Fit *F*-value” could occur due to noise. The not significant of Lack of Fit indicated that the model was significant and fit.

| Source | Sum of Squares | df | Mean Square | F Value | *p*-value Prof > F | Remark |
|--------|----------------|----|-------------|---------|-------------------|--------|
| Model  | 0.35           | 5  | 0.070       | 16.01   | 0.0043            | significant |
| A- Temperature | 2.020E-003 | 1  | 2.020E-003 | 0.46    | 0.5271            |        |
| B- pH  | 2.833E-004     | 1  | 2.833E-004 | 0.065   | 0.8093            |        |
| AB     | 2.061E-003     | 1  | 2.061E-003 | 0.47    | 0.5231            |        |
| A²     | 0.35           | 1  | 0.35        | 78.98   | 0.0003            | significant |
| B²     | 0.037          | 1  | 0.037       | 8.45    | 0.0336            | significant |
| Residual | 0.022          | 5  | 4.37E-003  |         |                   |        |
| Lack of Fit | 0.017          | 3  | 5.529E-003 | 2.09    | 0.3402            | not significant |
| Pure Error | 5.300E-003   | 2  | 2.650E-003 |         |                   |        |
| Cor Total | 0.37           | 10 |             |         |                   |        |

*Note: Cor Total = Corrected Total
df = Degree of freedom

### 3.4. Validation of Statistical Model

The accuracy of the fit of the model was checked by multiple correlation coefficients (R²). Table 4 shows the statistical variables obtained from ANOVA. The closest the correlation R² to value of 1.0,
the higher the quality of the model. From the results of this study, the value of $R^2$ was found to be 0.9412 indicated that there was a high correlation of experimental value towards predicted value. A reasonable covenant between the predicted $R^2$ (0.6512) and the adjusted $R^2$ (0.8825) indicated a good consistency between both the experimental and predicted values for ethanol concentration. Additionally, a relatively low value of the coefficient of variation (1.81 %) also showed a significant reliability of the experiments.

Table 4. The statistical variables obtained from ANOVA.

| Variables                | Response     | Variables                | Response     |
|--------------------------|--------------|--------------------------|--------------|
| Standard deviation       | 0.066        | $R^2$                    | 0.9412       |
| Mean                     | 3.66         | Adjusted $R^2$           | 0.8825       |
| Coefficient of variation (%) | 1.81        | Predicted $R^2$          | 0.6512       |
| PRESS                    | 0.13         | Adequacy precision       | 10.587       |

To predict the optimal values for ethanol concentration after fermentation, a polynomial model was fitted to the experimental result for the ethanol concentration by DoE software as in equation (1):

$$Y = -20.66423 + 0.84674A + 2.49242B + (9.08 \times 10^{-3})AB - (9.89758 \times 10^{-3})A^2 - 0.32366B^2 \quad (1)$$

where ethanol concentration ($Y$), is a function of fermentation temperature ($A$) and medium pH ($B$). Response surface and the contour lines are presented in Figure 2, and they were used to estimate the ethanol concentration over the independent variables which were fermentation temperature ($A$) and medium pH ($B$).

Figure 2 shows the effect of fermentation temperature and medium pH on the production of ethanol. The increased in both parameters had significantly improved the ethanol production to a maximum limit of 3.8750 g/L at temperature of 45 °C and medium pH of 4.5 during fermentation process. Perform the fermentation out of the optimum fermentation conditions (temperature and medium pH) may not significantly enhance the ethanol production. This phenomenon might be due to at high temperature, it will denatured specific enzyme in *S. cerevisiae* while less acidic environment is not preferable by *S. cerevisiae*.

The interaction between AB exhibited a small effect on the ethanol production as the $p$-value was 0.5231 which indicates a weak correlation between fermentation temperature and medium pH. It was due to individual effect of temperature and medium pH during fermentation. In fact, both of them play
vital role in increasing enzyme activity in *S. cerevisiae* for converting reducing sugar to ethanol. However, they act individually to achieve that role. A rise in temperature is associated with increased molecular vibrations and kinetic energy and thus results in successful collision and high enzyme-catalyzed reactions [20]. Variations of medium pH may modify the ionic form of the enzyme active site and changes the activity of the enzyme and also the reaction rate [19]. Doubling the effect of each temperature and medium pH (A² and B²) had significantly affect towards bioethanol production.

3.5. Optimization Design
The DoE software generated the optimum conditions with fermentation temperature at 44.83 °C and medium pH of 4.48 was predicted to produce 3.8976 g/L of ethanol concentration with desirability of 0.902. To compare with the value predicted, a validation test with triplicate of the optimum condition was done. The result by using suggested condition showed the actual value of 3.8395 g/L which was closely to the predicted result. This indicated that the optimization achieved in the present study was reliable. Table 5 shows the predicted and actual concentration of ethanol.

| Table 5. Predicted and actual concentration of ethanol. |
|-----------------------|-----------------------|-----------------------|
| Predicted ethanol concentration (g/L) | Actual ethanol concentration (g/L) | Deviation (%) |
| 3.8976 | 3.8395 | ±1.491 |

4. Conclusion
The carbohydrate and reducing sugar contents after pre-treatment were obtained at concentrations of 20.840 g/L and 18.438 g/L, respectively. The range of two fermentation parameters were screened by OFAT analysis followed by optimization using CCD in RSM. The optimum condition for bioethanol production from *S. cerevisiae* was obtained at temperature of 44.83 °C and medium pH of 4.48 with 3.8395 g/L of ethanol concentration. This actual ethanol concentration is closed to the predicted value which was 3.8976 g/L with ±1.491% of deviation.

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