Bioethanol production from microalgae *Chlorella sorokiniana* via simultaneous saccharification and fermentation

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**Abstract.** This study aimed to investigate the performance of a microalgal feedstock *Chlorella sorokiniana* in bioethanol production through simultaneous saccharification and fermentation (SSF). The major limiting factor of SSF is the compensation between optimum hydrolysis and fermentation parameters. Using a central composite design (CCD), three parameters were optimized: biomass concentration, temperature, and pH. For each run, the enzyme mixture (cellulase and amylase) was fixed at 25 g/L, fermentation time at 72 hrs, and inoculum size of *Saccharomyces cerevisiae* at 20% (v/v). The freeze-dried biomass is simultaneously mixed with the enzyme mixture and the inoculum and placed inside an incubator shaker. The microalgal biomass is found to contain 58.78% total reducing sugars (TRS). Highest ethanol yield obtained is 0.504 g ethanol/ g glucose. Using CCD helps navigate the design space and bioprocess.

1. Introduction

Alternatives for petroleum-based fuels are becoming a great deal of interest for research because they are generally more environment-friendly, renewable, and economical than petroleum-based counterparts. These characteristics may help alleviate problems on environment and energy security. A particular biofuel that is already commercially available is bioethanol. Many countries have already obligated their transportation sectors to include bioethanol blend in their petroleum products. However, improvement to the current production of bioethanol must be made to meet the global demands.

Bioethanol is produced from fermenting sugars. It can be classified into three groups based on where it was derived from: a) first-generation which is produced from crops, b) second-generation which is produced from dried biomass such wood, and c) third-generation which is produced from microalgae [1]. The first-generation feedstocks for bioethanol production are sugar canes and starchy crops which can be problematic as they compete with agricultural land and food. Meanwhile, second-generation bioethanol would be better alternatives because they are produced from agricultural waste products/byproducts such as stems, wood trimmings, peels, and leaves that are composed mostly of lignin, cellulose, and hemicellulose [2,3]. However, to convert them into bioethanol, the polysaccharides must be first enzymatically hydrolyzed into fermentable sugars often using costly enzymes [1,3]. Pretreatment of the lignocellulosic biomass also releases phenolic groups and other inhibitors that slow down the hydrolysis and decrease bioethanol yield [3]. Microalgae are good candidates for an alternative feedstock because they are easier and faster to grow, do not require land for cultivation, have higher photon conversion efficiency, and have higher carbon dioxide absorption [4,5]. Microalgae can accumulate large amounts of carbohydrate. Some species can produce up to 50% carbohydrate content per unit weight of dry biomass [6]. Microalgae do not have lignin which simplifies the process of extracting carbohydrates and fermentable sugars as compared to lignocellulosic biomass feedstock [7].

Several microalgal strains are in research and offer to be good options for bioethanol production. The microalga of interest is *Chlorella sorokiniana*. This species has a high growth rate and can be cultivated under both heterotrophic and mixotrophic conditions. *C. sorokiniana* is a resistant unicellular green alga.
that can grow under conditions that are unfavorable to other microalgae [8]. The highest specific growth rate reported for *C. sorokiniana* is 2.64 (day)\(^{-1}\) [9]. Furthermore, its primary intracellular storage material is starch. The carbohydrate content of *C. sorokiniana* can be as high as 57% (w/w) [10]. Starch synthesis is more energy efficient in storing carbon. The synthesis of fatty acids has a significant loss of carbon due to the conversion of pyruvate into acetyl-CoA. *C. sorokiniana* prefers to accumulate starch over lipid in almost all types of growth media. These characteristics make *C. sorokiniana* a robust option as a feedstock for bioethanol production and future industrial upscale.

Bioconversion of polysaccharides into ethanol is done in two steps: hydrolysis of the polysaccharide and fermentation of the fermentable sugars/monosaccharide. Hydrolysis is the process of breaking down polysaccharides into simpler sugars like glucose which can be fermented to ethanol. It can be chemical (acidic or basic) or enzymatic. The chemical method is usually used as a pretreatment for lignocellulosic biomass; however, microalgal cell wall is lignin-free thus it becomes a hydrolysis step [11]. Using dilute sulfuric acid is mostly used because higher concentrations will easily degrade the carbohydrates [12]. However, many limitations make chemical hydrolysis impractical to be scaled up. It operates at high temperature, needs high energy input, very corrosive and produces inhibitory compounds subsequently reducing bioethanol yield [11,12]. Neutralization and the removal of the toxic compounds are also required before going to fermentation, thereby increasing the number of steps in bioethanol production. Enzymatic hydrolysis, on the other hand, uses enzymes such as amylase and cellulase to break down the polysaccharides. It operates at mild conditions, produces fewer by-products, safer to the environment and has higher glucose yield than chemical hydrolysis [11,12].

There are two major bioconversion processes: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The difference between the two is that the enzymatic hydrolysis and fermentation occur at the same time in the same bioreactor in SSF while they occur separately in SHF. Because acid hydrolysis produces toxic compounds, it cannot be performed in SSF because the compounds will kill the fermenting organism. Enzymatic hydrolysis is best done in SSF. The advantage of SHF over SSF is that each step can be performed at their respective optimum conditions. However, inhibitory effects occur because of the accumulation of glucose which ultimately reduces the efficiency of the enzyme used in the process. Meanwhile, in SSF, glucose produced during saccharification is immediately fermented into ethanol. No accumulation of glucose occurs which improves the ethanol yield [13].

The major limiting factor in SSF is the enzymatic hydrolysis. The optimum temperatures for fermentation and hydrolysis are often far apart. Enzymatic hydrolysis occurs typically on 50 °C while fermentation using *Saccharomyces cerevisiae* occurs optimally at 30 °C [14]. Therefore, a key solution is to find a compromise between the temperatures that yield the maximum bioethanol yield. Another problem is the operating pH. A yeast strain that has the same optimum pH as that of the enzyme is preferable. Recycling of the enzyme and the yeast is also difficult to perform.

2. Methodology

2.1. Raw materials and reagents

The *C. sorokiniana* was bought as freeze-dried powder from Taiwan Chlorella Manufacturing Co Ltd. It is an isolated strain grown in a fresh pond. Cellulase and amylase were also purchased from Xi’ian Rongsheng Biotechnology Co Ltd. and Xi’an Salus Nutra Biotech Inc., respectively. The cellulase was produced from *Trichoderma reesei* and has an activity of 300,000 u g\(^{-1}\). Meanwhile, the amylase was produced from *Bacillus subtilis* and has an activity of 50,000 u g\(^{-1}\). The yeast, *Saccharomyces cerevisiae Type II*, was purchased from Sigma–Aldrich, St. Louis, MO, USA. Analytical grade glucose monohydrate, peptone, and yeast extract were also used. Other special reagent needed was dinitrosalicylic acid (DNS) for carbohydrate content determination.

2.2. Inoculum preparation

A yeast extract, peptone and dextrose (YPEP) medium was used for the nutrient medium. For this method, 10 g/L yeast extract, 10 g/L peptone, and 50 g/L glucose. This was all mixed and dissolved with DI water in a 500 mL Erlenmeyer flask and was heated in a hot plate if needed. Once thoroughly mixed, the media was autoclaved at a setting of 121 °C and 15 psi for 15 minutes. After this, 5 g of the freeze-dried *C. sorokiniana* was inoculated in the nutrient medium. This was incubated for 18 hours at 30 °C.
while being stirred at 150 rpm. Spectrophotometry was done to ensure same yeast cell population at OD$_{575}$ = 2.6.

2.3. Simultaneous saccharification and fermentation (SSF)

Potassium phosphate buffer solutions were prepared from pH 5.5 – 6.5. Then, nutrient media containing 10 g/L yeast extract and 10 g/L peptone was mixed in 200 mL of the buffer solution in 500-mL Erlenmeyer flask. Different amounts of the freeze-dried *C. sorokiniana* was added to each medium according to the central composite design (CCD) experiments. The flasks were then autoclaved at 121 °C and 15 psi for 30 minutes. The media were cooled to room temperature. Then, 25 g/L cellulase and amylase were added simultaneously with 40 mL of the yeast inoculum. The flasks were placed inside incubators set at the corresponding temperature setting in the CCD. The shaker was at 150 rpm and the fermentation took place for 72 hrs. At the end of SSF, the fermentation broth was centrifuged at 3750 rpm for 10 min. The supernatant was filtered through a 0.22 µm PTFE disc filter. The samples were stored in 4 °C for further analysis.

2.4. Analytical methods

The total carbohydrate content of the biomass was determined using the laboratory analytical procedure provided by the National Renewable Energy Laboratory, USA [15]. A sample of 25 ± 2.5 mg of the freeze-dried *C. sorokiniana* was mixed thoroughly with 72% (w/w) sulfuric acid in a test tube. The glass tube was immersed in a water bath at 30 °C for an hour while being vortexed. Seven mL of deionized water was added to the sample to dilute the sulfuric acid to 4%. The sample was then autoclaved for an hour at 121 °C in the slow setting. Three mL was taken from this solution and reacted with 3 mL of DNS. The mixture was then heated in 90 °C water bath for 5 mins. One mL is transferred to a disposable cuvette and is analyzed using UV-VIS spectrophotometry at λ=540 nm. For the calibration curve, standard glucose solutions of different concentrations were prepared. One mL of a standard solution was reacted with 1 mL of DNS. The mixture is immersed in a 90 °C water bath for 5 mins. The mixture is then diluted to 10 mL with deionized water. One mL was transferred to a disposable cuvette. This was also analyzed UV-VIS spectrophotometry at λ=540 nm. A calibration curve was made thereafter.

The bioethanol content from SSF was determined using gas chromatography. A standard was made by diluting 5 mL of 95% ethanol in 20 mL water. Ethanol solutions with 5%, 10%, 30%, 50%, and 70% of the original concentration were made by diluting the original stock solution. A calibration curve was constructed by running these standards in the GC. The column temperature is at 45 °C while the flame ionization detector is at 250°C. The column used was Stabilwax®-DA with internal diameter of 0.32mm and film size of 0.25µm. After performing each analysis, the ethanol yield was calculated using Eq. 1:

$$Y_E = \frac{E}{B \times 0.5544 \times 0.5111}$$  (1)

where $Y_E$ is the ethanol yield, $E$ is the weight of ethanol (g) in the fermentation broth, $B$ is the weight of biomass (g) used, 0.5544 is the glucose coefficient of the biomass, and 0.5111 is the stoichiometric coefficient of glucose to ethanol.

2.5. Experimental design

A response surface methodology (RSM) was preferred over factorial designs because it is more geared towards optimization and it requires fewer experimental runs. A central composite design with $\alpha = 1.316$ was used for the experiments. Three independent variables were tested: biomass concentration, pH, and temperature. Table 1 shows the experimental setup. Each factor has five levels. There is a total of 20 experimental runs and each run will be replicated twice. There are 6 center point runs and 6 corner point runs. The values are based from previous studies. The biomass concentration levels are based from previous study [11] which studied the effect of substrate loading on the enzymatic hydrolysis of C. *vulgaris*. The pH levels and temperature values are ranges wherein *S. cerevisiae* grows. The fermentation time is 3 days. Design Expert 11 was used to generate the matrix and was also used for data analysis.
Table 1. Central composite experimental design with practical $\alpha$ value.

| Independent variables | Symbol | Range and levels |
|-----------------------|--------|-----------------|
| Temperature ($^\circ$C) | $X_1$  | -1.316 -1 1 1.316 |
| Biomass loading (g/L) | $X_2$  | 22.63 25 32.5 40 42.37 |
| pH                   | $X_3$  | 5.34 5.5 6.0 6.5 6.66 |

Table 2. Ethanol yield of SSF runs

| Runs | Variables in Coded levels | Ethanol Yield |
|------|---------------------------|---------------|
|      | $X_1$ | $X_2$ | $X_3$ | Experimental | Predicted |
| 1    | -1   | -1   | 1    | 0.220        | 0.215     |
| 2    | 0    | 0    | -1.33| 0.430        | 0.428     |
| 3    | 0    | 0    | 0    | 0.343        | 0.338     |
| 4    | 0    | 0    | 1.33 | 0.390        | 0.379     |
| 5    | 1    | 1    | 1    | 0.484        | 0.485     |
| 6    | 1.33 | 0    | 0    | 0.247        | 0.238     |
| 7    | 1    | 1    | 1    | 0.497        | 0.485     |
| 8    | 1    | -1   | 1    | 0.251        | 0.259     |
| 9    | -1   | -1   | -1   | 0.296        | 0.313     |
| 10   | 1    | -1   | -1   | 0.485        | 0.485     |
| 11   | 0    | 0    | 0    | 0.116        | 0.119     |
| 12   | 0    | 0    | 0    | 0.483        | 0.485     |
| 13   | -1   | 1    | -1   | 0.218        | 0.215     |
| 14   | 0    | 0    | 0    | 0.499        | 0.485     |
| 15   | 0    | 0    | 0    | 0.407        | 0.408     |
| 16   | 0    | 0    | 0    | 0.487        | 0.485     |
| 17   | -1.33| 0    | 0    | 0.502        | 0.512     |
| 18   | 0    | -1.33| 0    | 0.254        | 0.254     |
| 19   | 0    | 1.33 | 0    | 0.431        | 0.437     |
| 20   | 1    | 1    | -1   | 0.458        | 0.468     |

The obtained values are then inputted in Design Expert 11. The quadratic model was chosen as it closely fits the data based on the $R^2$, adjusted $R^2$, and predicted $R^2$ coefficients. The linear, quadratic, and interaction effects were calculated as well as the significance of each coefficient. Contour plots were constructed to depict effects of the variables to the ethanol yield. Then, the maximum ethanol yield was calculated, and duplicate confirmatory runs were performed. Prediction of the optimum point is expressed as:

$$Y = b_0 + \sum_{i=1}^{k=3} b_i x_i + \sum_{i=1}^{k=3} b_{ij} x_i^2 + \sum_{i < j}^{k=3} b_{ij} x_i x_j + e$$

(2)

where $Y$ is the predicted response, $b_0$ is a constant coefficient, $b_i$ is the linear coefficient, $b_{ij}$ is the quadratic coefficient, $b_{ij}$ is the interaction coefficient, and $e$ is random error.

### 3. Results and discussion

#### 3.1. Optimization of process variables for SSF

Temperature, biomass loading, and pH level were the process variables studied because they are important factors that affects the final ethanol yield using SSF. The results of each run are shown in Table 2. The data was analyzed using Design Expert and a quadratic model was chosen (Eq. 3). Another column reflecting the model prediction of the ethanol yield was also added for easy comparison.
Temperature highly influences the structure and rigidity of cells. At high temperatures, the cell becomes more fluid and at lower temperatures, it stiffens. Outside the upper and lower functional temperature bounds of the yeast, cell growth and metabolism seize. It is either because it has lost its structure or become completely impermeable to extracellular nutrients. Maintenance energy is the energy expenditure of the yeast for cellular repairs, nutrient transfers, motility, and osmolarity adjustment. When the temperature is above the optimum growth temperature range, the maintenance energy requirement becomes higher. This lowers the product yield coefficient because more energy is being used for cellular maintenance instead of product formation [16]. Specifically, for fermentation, temperature affects the rate-limiting step. Fermentation is an enzyme-catalyzed process so increased

\[ Y = 0.4853 + 0.0224X_1 - 0.0959X_2 - 0.0117X_3 + 0.0074X_1X_2 + 0.0297X_1X_3 - 0.0063X_2X_3 - 0.1164X_1^2 - 0.0577X_2^2 - X_3^2 \pm 0.011 \]  

Eq. 3 is the coded model equation. The coefficients in Eq. 3 shows the magnitude of change in ethanol yield when that factor is changed. Positive coefficients increase ethanol yield while negative ones decrease it. Temperature has a positive linear effect but a negative quadratic effect. This means that ethanol yield increases with increasing temperature but will start to decrease when the quadratic effect becomes more evident than the linear effect. Biomass concentration and pH has both a negative linear effect and negative quadratic effect. Increasing these factors, decreases the ethanol yield.

After the quadratic model was chosen, an ANOVA study was made to determine the significance of the factors. Table 3 shows the ANOVA results. P-values less than 0.05 means that the factor is significant. The model is significant while the lack of fit is insignificant which means that model can be used to predict the behavior of SSF system. The corresponding R², adjusted R², and predicted R² are 0.9503, 0.9034, and 0.8567, respectively. These are good metrics for the goodness of fit thus the model is reliable for prediction.

### Table 3. ANOVA study for the quadratic model.

| Source      | Sum of Squares | df | Mean Square | F-value | p-value |
|-------------|----------------|----|-------------|---------|---------|
| Model       | 0.2770         | 9  | 0.0308      | 255.46  | < 0.0001|
| X₁-Temp     | 0.0057         | 1  | 0.0057      | 47.69   | < 0.0001|
| X₂-Biomass  | 0.1054         | 1  | 0.1054      | 874.96  | < 0.0001|
| X₃-pH       | 0.0016         | 1  | 0.0016      | 12.99   | 0.0048  |
| X₁X₂        | 0.0004         | 1  | 0.0004      | 3.60    | 0.0870  |
| X₁X₃        | 0.0071         | 1  | 0.0071      | 58.52   | < 0.0001|
| X₂X₃        | 0.0003         | 1  | 0.0003      | 2.65    | 0.1346  |
| X₁²         | 0.0944         | 1  | 0.0944      | 783.63  | < 0.0001|
| X₂²         | 0.0232         | 1  | 0.0232      | 192.72  | < 0.0001|
| X₃²         | 0.0024         | 1  | 0.0024      | 20.09   | 0.0012  |
| Residual    | 0.0012         | 10 | 0.0001      |         |         |
| Lack of Fit | 0.0010         | 5  | 0.0002      | 3.73    | 0.0873  |
| Pure Error  | 0.0003         | 5  | 0.0001      |         |         |
| Cor Total   | 0.2782         | 19 |             |         |         |

### 3.2. Effect of temperature and pH on ethanol yield

Model contour plots were generated from the data to show the interactive effects of two variables at a time while the other variable is held at middle level. It can be seen from Fig. 1a that increasing the fermentation temperature also increases the ethanol yield. However, at a certain temperature, further elevation will lead to a decrease of the ethanol. This is the trend for all the pH levels. Meanwhile, the effect of pH is highly dependent to the temperature. When the temperature is held at a low level (T=25 °C), increasing the pH decreases the ethanol yield. The inverse is seen when the temperature is held at a high level. At the middle level, the system is resistant to pH changes and seems to be only affected by the temperature. This conforms to the ANOVA study wherein the temperature has a higher degree of significance that of pH.
temperatures will increase the rate of product formation until the thermally-sensitive enzymes denature [17].

The hydrogen ion concentration (pH) also affects the growth of the yeast. Protein configuration and enzyme activity are dependent on pH. The ions affect the state of the functional groups attached to the enzymes which can change the shape or charge properties [18].

3.3. Effect of temperature and biomass loading on ethanol yield

Fig. 1b shows that lower biomass levels the ethanol yield is higher and decreases with increasing biomass concentration. Meanwhile at higher biomass loading, increasing the temperature decreases the ethanol yield. However, at lower biomass concentration, increasing the temperature also increases the ethanol yield at a certain temperature range. The decrease of the ethanol yield from the increase of biomass concentration maybe attributed to the overdosing effect. The over-dosing effect is a phenomenon wherein excessive amounts of the substrate becomes inhibitory for the microorganisms. For yeast, glucose concentration above 200 g/L is inhibitory for ethanol fermentation because of water activity reduction. Substrate far over the stoichiometric amounts limits cellular function but too low concentration does not activate cell growth [17]. Increasing the temperature also increases the enzymatic
activity thus leading to faster hydrolysis of the sugars in the system [19]. At high temperature and high biomass loading, the amount of glucose being hydrolyzed is very large and at a faster rate. However, this led to a sudden increase of the substrate concentration in the system thus leading to the overdosing effect. But at high temperature but low biomass loading, the amount of yeast in the system can effectively convert the hydrolyzed glucose immediately to ethanol thus leading to a higher ethanol yield.

3.4. Effect of pH and biomass loading on ethanol yield
The interactive effects of the pH levels and biomass loading is seen on Fig. 1c. It can be inferred that at all pH levels, increasing the biomass loading decreases the ethanol yield. The system is more resistant to biomass loading changes at higher pH levels. When the biomass loading is held at low concentrations, increasing the pH decreases the ethanol yield. From the ANOVA table, biomass loading is a more significant factor than pH. Thus, the effect of the pH level was only observed at low biomass loading. The effect of the biomass concentration overcomes the effect of the pH. It is recommended to further increase the scope of the study by increasing the pH range so that the effects of pH may be seen more clearly.

3.5 Optimum ethanol yield
The maximum ethanol yield was calculated to be 0.507 g ethanol/g glucose at T= 33.94 °C, pH= 5.55, and biomass concentration = 16.95 g/L. Two confirmation runs were performed. The mean ethanol yield was 0.504 g/g (5.65 g/L) – an error of only 0.59%. This indicates that the model can predict the SSF with great accuracy. Table 4 also shows that the result from this study is comparable to others at a basis of the mass of microalgae used. A reason for this is that none of the other studies studied the optimum values for ethanol fermentation. This is critical especially for SSF because the optimum parameter values for fermentation and enzymatic hydrolysis are different. A compensation between these values must be made.

Table 4. Ethanol yield of different microalgae feedstock using SSF or SHF.

| Feedstock       | Mode | Fermenting Organism | Parameters                                      | Ethanol yield (g ethanol/g algae) | Ref. |
|-----------------|------|---------------------|-------------------------------------------------|-----------------------------------|------|
| C. vulgaris     | SHF  | B. custersii        | Temperature: 30 °C pH: 5.5 Biomass: 100 g/L     | 0.037                             | [20] |
| Scenedesmus sp. | SHF  | Saccharomyces cerevisiae | Temperature: 30 °C pH: 6.0 Biomass: 50 g/L | 0.079                             | [21] |
| Mixed culture  | SHF  | S. cerevisiae       | Temperature: 30 °C pH: 6.5 Biomass: 50 g/L      | 0.109                             | [22] |
| C. vulgaris     | SHF  | Zymomonas mobilis ATCC 29191 | Temperature: 30 °C pH: 6.0 Biomass: 20 g/L | 0.178                             | [11] |
| C. vulgaris     | SSF  | Z. mobilis ATCC 29191 | Temperature: 30 °C pH: 6.0 Biomass: 20 g/L     | 0.214                             | [11] |
| C. mexicana     | SHF  | S. cerevisiae       | Temperature: 30 °C pH: 5.0 Biomass: 38.1 g/L   | 0.223                             | [23] |
| Chlamydomonas  | SSF  | S. cerevisiae       | Temperature: 30 °C pH: 5.0 Biomass: 38.1 g/L   | 0.276                             | [23] |
| C. sorokiniana | SSF  | S. cerevisiae       | Temperature: 33.94 °C pH: 5.55 Biomass: 16.95 g/L | 0.292                             | This study |
4. Conclusion and recommendation

Using SSF on microalgae feedstock like Chlorella sorokiniana is an effective approach on ethanol production. By optimizing the temperature, pH level, and biomass concentration, a maximum ethanol yield of 0.504 g ethanol/g glucose was achieved at T= 33.94 °C, pH= 5.55, and biomass concentration = 16.95 g/L. This is close to the model prediction of 0.507 g/g. The central composite design is a robust experimental design that can be used to determine effects of the variables for fewer experimental runs.

A recommendation for further studies is to include the effect of the yeast concentration. The yeast concentration was held constant for all runs but increasing the yeast population may overcome the overdosing effect experienced for higher biomass loading. This may further increase the ethanol content of the final fermentation broth. Another recommendation is to use a thermotolerant strain of S. cerevisiae and to increase the range of pH being studied.

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