# Fermentation of *Chlorella sorokiniana* Biomass for Bioethanol Production

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**Abstract:** Bioethanol is a renewable fuel derived from fermentation of biomass containing carbohydrates. In this study, ethanol is produced from the fermentation of *Chlorella sorokiniana* biomass and the effects of temperature and inoculum size on the fermentation were investigated. Microalgae is a viable third generation source of carbohydrates that is fermentable by yeast (*Saccharomyces cerevisiae*), and the *Chlorella* sp. is a notable candidate microalgae biomass with high carbohydrate content that can be found in its cell wall. Extraction of carbohydrates was done using dilute acid hydrolysis. The total carbohydrates yield and ethanol content were determined using HPLC analysis. The final ethanol yield was characterized in terms of ethanol per gram of glucose fed. Under anaerobic condition, a temperature of 30˚C and an inoculum size of 10% (v/v), the highest ethanol yield of 0.1208 g ethanol/g glucose fed was obtained. An ANOVA analysis was carried out to determine the significance of the effects of temperature and inoculum size, and the result shows that both parameters are significant with a p-value < 0.05; however, the interaction of the two parameters is found to be insignificant. This study has shown that *Chlorella sorokiniana* could be used as substrate for ethanol fermentation.

**Key Words:** bioethanol; fermentation; microalgae

## 1. INTRODUCTION

Bioethanol is a renewable fuel derived from the fermentation of carbohydrates through the use of yeasts. Bioethanol in particular is very promising because its manufacture is dependent on glucose supply and is also easily renewable given the recent advancements in agriculture. It has also been reported as a good alternative for conventional fuels and fuel additive because of its many advantages such as its high octane rating and higher volumetric efficiency (Walker, 2010).

In the production of bioethanol, algae as source of carbohydrates are of interest because they do not compete with food supply, are abundant in nature, has high carbohydrate content, and has a high rate of biomass production (Sirajunnisa & Surendhiran, 2016; Ho et al., 2013). There are many species of algae and carbohydrate contents also vary from species to species. In previous works done, it was determined that microalgae species that fall in the genera such as *Chlamydomonas, Dunaliella, Scenedesmus, Tetraselmis* and *Chlorella* are likely to contain high concentration of carbohydrates (Ho, et al., 2013), which are very favorable for fermentation process.

The genus *Chlorella*, with its vast range of species, is said to have the simplest form of reproduction and structure among the algae. It is described as a unicellular, green, aquatic and motionless microalgae with a very high and promising potential of producing different compounds like pharmaceutical compounds, enzymes, vitamins, pigments, proteins, lipids, and polysaccharides. One

specific species of *Chlorella* is the strain *Chlorella sorokiniana*. This species has a glucosamine cell wall, which is made up of polysaccharide matrix (Takeda, 1991).

The carbohydrates in the cell wall of microalgae can be extracted via acid hydrolysis. The carbohydrates can then be fermented using Baker's yeast (*Saccharomyces cerevisiae*) to produce bioethanol. Temperature and inoculum size are some factors that affect the ethanol production by yeast, so it is in the interest of this study to determine the effects of these parameters on the fermentation of sugars derived from *Chlorella sorokiniana* biomass.

## 2. METHODOLOGY

### *2.1 Materials*

Dry powdered form of *Chlorella sorokiniana* microalgae were obtained from a manufacturing company in Taiwan named Taiwan Chlorella Manufacturing Co Ltd. The biomass was kept in cool dry conditions prior to carbohydrate extraction using dilute acid hydrolysis.

The yeast culture, Saccharomyces cerevisiae BIOTECH 2118, used in the experiment was purchased from Philippine National Collection of Microorganisms (PNCM) Biotech. It was maintained in a test tube with YEPD media.

### *2.2 Bioethanol production*

In the study, *Chlorella sorokiniana* biomass were first subjected to dilute acid hydrolysis to release the carbohydrates to be used as feedstock for bioethanol production. During dilute acid hydrolysis, a solid-to-liquid ratio of 4% (w/v) using *Chlorella sorokiniana* and dilute 4% sulfuric acid were used based on the study done by Sim et al. (2009). The mixture of acid and biomass was placed in an autoclave set at 121˚C and 0.1MPa. Used biomass is separated using vacuum filtration. The supernatant containing the sugars was adjusted to pH 5-6 using sodium hydroxide pellets and added with yeast extract (10g/L and peptone (10g/L) before fermentation.

For the fermentation, temperature was varied using a water bath shaker at temperatures of 20˚C, 30˚C, and 40˚C to check if optimal growth of yeast strain used is at around 30ºC like in the study of Kovacevic, (2015). Yeast inoculum were prepared and used when the yeast are in the logarithmic phase. The inoculum was introduced at varying sizes of 5%, 10% and 20% (v/v) based on the study of Ueno et al (2002) that found 5% inoculum size to have good ethanol yield. Fermentation was conducted for 72 hours. Samples were collected before and after each fermentation and analysis of the fermentation broth for ethanol and carbohydrates was done. The broth was filtered with a 0.45um syringe filter and analyzed using high performance liquid chromatography (HPLC) with a SHODEX SH1011 column, with a mobile phase of 0.005M sulfuric acid solution, at a flowrate of 1mL/min. To determine the significance of the effect of temperature and inoculum size under anaerobic conditions, two-way ANOVA analysis was done.

## 3. RESULTS AND DISCUSSION

The total carbohydrate content of the *C. sorokiniana* biomass used in the study was first determined and found to be 0.5878 g glucose/g biomass based on the National Renewable Energy Laboratory (NREL) method that uses Dinitrosalicyclic Acid (DNS). This shows that there is a high amount of carbohydrates in *C. sorokiniana* biomass that could be used for bioethanol production. During dilute acid hydrolysis, the extraction yield showed an average of 0.5148 g glucose/g biomass, which is about 88% of the total present in the biomass.

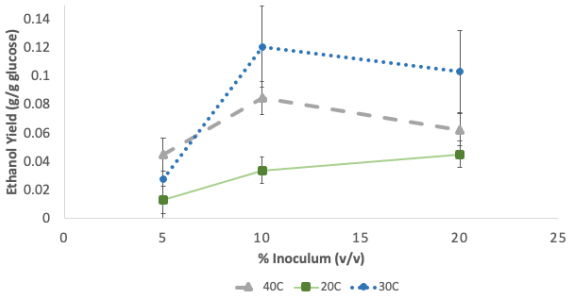


Fig. 1. Effects of Inoculum and Temperature on Ethanol Yield

Fermentation of the carbohydrates from *C. sorokiniana* biomass was carried out under anaerobic conditions at varying temperatures of 20˚C, 30˚C, and 40˚C, and inoculum size of 5%, 10% and 20% (v/v). The

effects of these parameters on ethanol yield, expressed as g ethanol/g glucose fed, is shown in Figure 1.

### *3.1 Effects of Temperature*

In the experiment, the temperature values were varied from 20˚C, 30˚C, and 40˚C. As shown in Figure 1, the highest ethanol yield occurred at 30˚C while lowest yield at 20˚C. Further increase from 30˚C to 40˚C resulted to decrease in ethanol yield. This is because the optimal growth temperature of yeast is reported to be at about 30˚C (Kovacevic, 2015), and that at elevated temperatures above the optimal temperature, yeast cells would experience increased stress which could result to a higher death rate of cells (Schuler & Kargi, 2002).

At a temperature of 20ºC, the results showed that ethanol yield was only 0.03053 g ethanol/g glucose fed, which is lower compared to that for other temperatures. This is because at 20˚C, the lag phase could be longer as observed in the study of Torija et al. (2003) in which at 15˚C to 20˚C, yeast cells growth was slow and had longer lag phase. The increase in duration of lag phase experienced by the cell leads to the decrease in ethanol yield because at the lag phase, only cell mass is increased and there is little product formation that is happening.

At 5% inoculum, a deviation from the trend can be seen in Figure 1 in which the highest ethanol yield was observed at 40˚C. At low inoculum size, lag phase is usually longer (Schuler & Kargi, 2002, Torija et al, 2003). With the increase in temperature, fermentation with low inoculum size of 5% probably had the shortest lag phase that led to higher ethanol yield.

Therefore, at the temperature range that was chosen for this study of 20ºC to 40ºC it is shown that there is an increase in ethanol yield up to a point until cell death causes a decrease in yield except for low inoculum size in which increase in temperature of up to 40˚C still increases yield. The study also shows that the best temperature for ethanol fermentation using *S. cerevisiae* is at about 30ºC.

### *3.2 Effect of Inoculum Size*

To study the effects of inoculum size, the ratio of the volume of inoculum to the fermentation volume was varied from 5%, 10% and 20%, respectively. The effect of inoculum size on ethanol yield can be seen on Figure 1. It is shown that the ethanol yield increases as the inoculum size increases to 10% inoculum size but decreases as the inoculum size was further increased to 20%. This is probably because at higher inoculum size, cells over crowd each other and their accumulated wastes eventually slow or inhibit their growth due to product limiting microbial fermentation effect of ethanol on *S. cerevisiae* (Levenspiel, 1999). However, at the temperature of 20˚C, increasing inoculum size to 20% still increased yield, this is probably because no overcrowding occurred because at low temperature, a longer lag phase occurred such that the 20% inoculum size is still not much after 72hrs of fermentation.

From the inoculum size range chosen in this study which is from 5% to 20%, it can be said that the best inoculum size could be at about 10% which gave the highest yield of about 0.1208 g ethanol/g glucose. The best inoculum size shows that too low cell density leads to lower yield probably because of longer lag phase but too high number of cells could prove to be counterproductive to the alcohol production because it leads to crowding of the yeast cells due to over population and higher accumulation waste at the early stage of fermentation (Harun & Danquah, 2011).

### *3.3 ANOVA Analysis of the Effects of Temperature and Inoculum*

The experimental data found in Table 1 is used to determine the statistical relevance of the data compared to the population. A two-way ANOVA Analysis is done to show if; (1) temperature has an effect in the ethanol yield; (2) inoculum size has an effect on ethanol yield; (3) the interaction of temperature and inoculum size has an effect on ethanol yield. Table 1 show the results of the analysis of variance and from the p-values, it shows that that temperature and inoculum size has a significant effect

on the ethanol yield at 95% confidence level, however the interaction of the two parameters is insignificant.

Table 1. ANOVA analysis of Effect of Parameters

| Source of Variation | Sum of Squares | *df* | Mean Square | F-ratio | p-value |
|---|---|---|---|---|---|
| Temperature | 0.008442 | 2 | 0.004221 | 15.6543 | <0.01 |
| Inoculum Size | 0.008741 | 2 | 0.004370 | 16.2079 | <0.01 |
| Within | 0.003365 | 9 | 0.000841 | | |
| Interaction | 0.002427 | 4 | 0.000270 | 3.1202 | 0.072 |
| Total | 0.022976 | 17 | | | |

From Table 1 the F ratio of temperature and inoculum is very high, which means that we can reject the null hypothesis for these two cases. It is apparent from Figure 1 that when these two parameters are varied, there is a significant increase in ethanol yield. It is also observed that inoculum size has a higher F-ratio which suggests that it has a higher degree of effect on yield compared to temperature, but both values are relatively close to each other so degree of effect of both seems to be equal in magnitude. In terms of the interaction of the two parameters however, it shows an F-ratio that does not make it to the 95% confidence level and only shows a p-value of 0.072, which accepts the null hypothesis for this case that, the combination of the two parameters does not lead to any significant change in the ethanol yield.

## 4. CONCLUSIONS

On the effects of parameters with the fermentation process, this study was able to show that as the fermentation temperature is increased ethanol yield is increased at inoculum size of 5%. However, at higher inoculum size of 10% and 20% it is shown that ethanol yield is highest at a temperature of 30˚C. It has also shown that as the inoculum size is increased, ethanol yield increases as well. In terms of which has more effect on bioethanol yield, statistical analysis shows that inoculum size affects the yield more compared to temperature. However, both showed a very close range of p-value to one another suggesting that both parameters have similar degree of effect on yield.

Using *Chlorella sorokiniana* biomass, in the fermentation process, as substrate by yeast, lead to a bioethanol yield of 0.1208 g ethanol/g glucose fed. At the temperature and inoculum ranges chosen in this study, the temperature of 30˚C and inoculum size of 10% (v/v) gave the highest ethanol yield per gram of glucose fed.

This study has shown that *Chlorella sorokiniana* could be used as substrate for ethanol fermentation however, in the parameters studied, a low yield of ethanol in comparison to previous studies using microalgae as substrate was obtained.

With the low ethanol yield obtained in this study, it is recommended to analyze the hydrolysate prior to fermentation for presence of inhibitors so that they can minimized or removed so that the maximum amount of yeast activity could be achieved on the fermentation. Also, it might be recommended to have a higher concentration of sugars in the hydrolysate by using higher solids to liquid ratio during the acid hydrolysis to provide yeast with more carbohydrates to ferment.

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