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# Enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass for biosugar production and fed-batch bioethanol fermentation

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**Abstract**

This study investigated the factor that affect enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass and its potential as a bioethanol feedstock. The enzymatic hydrolysis optimization of dilute alkaline pretreated *Chlorella* sp. biomass was carried out prior to fermentation process. The enzymatic hydrolysis was performed using cellulase cocktail from *Trichoderma longibrachiatum*. The enzymatic hydrolysis parameters such as pH, temperature, enzyme and biomass concentrations that affect hydrolysis performance were also investigated. The optimization of enzymatic hydrolysis was carried out using the central composite design (CCD) approach. The study indicated that the highest reducing sugar $413.42 \pm 7.62$ mg $g_{biomass}^{-1}$, which corresponding to 84.33% hydrolysis yield was achieved when the hydrolysis was performed using 12.5 g $L^{-1}$ biomass at 45 °C and initial pH 5.0 for 96 h of incubation. The enzymatic hydrolysis kinetic parameters ($V_{max}$ and $K_m$) of the pretreated biomass were also been determined. The analysis indicated that $V_{max}$ and $K_m$ values for enzymatic hydrolysis of the biomass were 0.18 mgm$L^{-1}$$min^{-1}$ and 26.34 mg $mL^{-1}$ respectively. The hydrolysate generated from the enzymatic hydrolysis was further fermented for bioethanol production using *Saccharomyces cerevisiae* in batch and fed-batch fermentation modes. The fed-batch fermentation resulted slightly higher bioethanol concentration of 1008.48 mg $L^{-1}$, corresponding to bioethanol yield of 0.43 $gg_{sugar}^{-1}$ and 0.081 $gg_{biomass}^{-1}$ compared to batch fermentation. The results revealed that optimization of enzymatic hydrolysis process could enhance reducing sugar production from dilute alkaline pretreated *Chlorella* sp. biomass. The results obtained also demonstrated that fed-batch fermentation of alkaline pretreated *Chlorella* sp. hydrolysate from enzymatic hydrolysis process improve the efficiency of ethanolic fermentation in term of ethanol concentration production and yield.

**Keywords:** microalgae, pretreatment, sugar, optimization, fermentation, bioethanol.

## 1. Introduction

Production of biofuel and chemicals such as bioethanol from various types of feedstock including microalgal biomass has recently gained increasing attention [1, 2]. This is due to the fact that microalgae exhibits a high growth rate and is considered a renewable resource in which the biomass can be harvested within 10-20 days of cultivation [3]. Generally, the harvested microalgal biomass contains three major biochemical compounds, namely lipids, proteins and carbohydrates [4]. These biochemical compounds have a potential to be used as feedstock for a wide range of chemicals. For instance, microalgal-carbohydrates that contain complex carbohydrate polymers can be used for production of bioalcohol; such as bioethanol [5].

In order to produce bioethanol from microalgal biomass, it must undergo a series of processes including biomass production, pretreatment, sugar enzymatic hydrolysis and fermentation [1]. Pretreatment and enzymatic hydrolysis are among the most crucial steps in producing chemicals from biomass [6]. Generally, the rigid and complex microalgal cell walls need to be hydrolysed through pretreatment with either acid or alkali as the catalyst prior to enzymatic saccharification [7]. Several studies have been reported on the effects of pretreatment on enzymatic hydrolysis and bioethanol fermentation of microalgal biomass [8, 9]. Miranda et al. [8] compared the different pretreatment approach on enzymatic hydrolysis and fermentation of *Scenedesmus obliqus* biomass.

The report revealed that pretreatment has a significant effect on the subsequent hydrolysis and fermentation process. Other studies have also indicated that pretreatment of *Chlamydomonas mexicana*, *Scenedesmus obliqus* and *Micractinium reisseri* biomass using different pretreatment methods could significantly affect the enzymatic hydrolysis condition for sugar production [7].

To date, acid treatment is widely applied to disrupt the lignocellulosic material and microalgal cell walls prior to the enzymatic hydrolysis process [10, 11]. However, in comparison to the other chemical pretreatment methods, alkaline pretreatment has been reported to exhibit more advantages over acid treatment. One reason is, this method is less corrosive and can be performed at moderate temperatures, such as at ambient temperatures whereas to form less inhibitors for subsequently inhibiting the fermentation process [12]. On the other hand, this alkaline pretreatment has suggested being very selective and could cause changes on complex carbohydrates or cellulose structures by cleaving glycosidic bonds in the cell wall fraction. Subsequent release of the carbohydrate polymers in the medium without the break-down of polymers into simple sugars is also suspected [13]. The addition of an alkaline agent during the pretreatment process has a dual effect such as swelling of the substrate that leads to incrementing the surface area and degree alteration of polymerization in the complex microalgae cell walls [12, 13]. The selection of the most efficient pretreatment method is totally dependent on the substrate physical structure and chemical composition. According to Chen et al. [14], it was suggested that the alkaline pretreatment is more suitable for a substrate with low lignin content; such as herbaceous crop, hardwood and agro waste residue. Since microalgae are the most lignin-free lignin biomass, it is believed that the alkaline pretreatment method could offer an efficient method to disrupt the microalgal cellulose-based cell walls.

Enzymatic hydrolysis is one of the important steps in bioconversion for the process of biomass substrates into biofuel. Successively, the complex carbohydrate polymers in the substrate are converted into sugar platforms prior to the fermentation process [15, 16]. Hydrolysis of complex carbohydrate polymers involves a mixture of cellulase enzymes as a key biocatalyst for the formation of a simple sugar prior to the fermentation process [15]. In this process, the maximum conversion yield is important to ensure feasibility of the next fermentation process. Currently, the hydrolysis yield and bioconversion process of alkaline pretreated *Chlorella* sp. is not feasible due to the low conversion yield and reducing sugar production [17]. The efficiency of enzymatic hydrolysis could be attributed by several factors including the type of pretreatment and hydrolysis condition [10, 18]. According to Hernandez et al. [10] who reported that different types of pretreatments could affect the enzymatic hydrolysis performance due to the presence of degradation products produced during the pretreatment process [10]. Hence, many enzymatic hydrolysis optimization studies have been conducted to determine the optimal condition for maximum conversion yield and sugar production from various pretreated biomass [19, 20]. Another key factor that affects enzymatic hydrolysis performance is the process condition. Enzymatic hydrolysis parameters such as pH and temperature, enzyme and initial microalgal biomass concentration play a significant role on the hydrolysis process [18]. It was reported that these hydrolysis parameters are correlated and exhibit a synergistic effect on hydrolysis yield and sugar production.

Several studies probing the potential of microalgal biomass as a feedstock for bioethanol production have been published elsewhere [11, 19, 21]. Most of these studies have reported the utilization of acid pretreated hydrolysate microalgal biomass for enzymatic hydrolysis process prior to bioethanol production. However, to the best of our knowledge, a great deal of investigation has been conducted on microalgal pretreatment and hydrolysis. There is, however, a scarce amount of information on the enzymatic hydrolysis of microalgal biomass generated from the dilute alkaline pretreatment process. Thus, the aim of this present work is to optimize the hydrolysis conversion yield and sugar production from alkaline pretreated *Chlorella* sp. biomass. A central composite design was used to evaluate and determine the influence of hydrolysis process parameters that contribute to the maximum hydrolysis conversion yield and sugar production. The enzymatic hydrolysis kinetic parameters and its potential applications as a fermentation feedstock for bioethanol production via two different modes such as batch and fed-batch fermentation were also analyzed in this study.

## 2. Material and Methods

### *2.1 Microalgae cultivation condition*

The microalgae *Chlorella* sp. obtained from the CSIRO Algae Collection was used throughout this study. The medium used for the microalgae cultivation and seed preparation was modified algae growth (MLA) medium that contained of following components: 1.7 g $L^{-1}$ sodium nitrate ($NaNO_3$), 0.49 g $L^{-1}$ magnesium sulfate ($MgSO_4.7H_2O$), 0.14 g $L^{-1}$ di-potassium phosphate ($K_2HPO_4$) and 0.03 g $L^{-1}$ calcium chloride ($CaCl_2.2H_2O$) [22]. The cultivation was maintained at 30.0 ± 0.2 °C in an illuminated growth chamber provided with a photon intensity of 450 µmol $m^{-2}$ $s^{-1}$ aerated with an air pump at 0.3 L $min^{-1}$. Initially, the growth of microalgae culture was monitored up to 14 days until it reached the late logarithmic growth phase. At the end of cultivation period, the microalgal biomass was harvested by sedimentation and centrifugation at 4500 rpm for

15 minutes. The pellet obtained was then washed and subsequently, dried overnight in an oven at 60 °C. After the drying process the dried microalgal biomass was then ground using a mixer grinder and kept in closed container subject for further use.

*2.2 Biochemical composition determination*

The entire lipid content in the microalgal biomass were extracted via the Soxhlet extraction method [23]. Briefly, a total of 1.0 g of biomass was soaked in a mixture of hexane and isopropanol as the solvent. The extraction process was carried out for 15 fluxes and the process ended once the solvent became clear. Following the extraction process the solvent mixture was transferred into a pre-weight beaker and evaporation was carried out in a hot air oven at 80°C for 24-48 hours. The lipid content over dried biomass was calculated based on the weight difference before and after evaporation. For the carbohydrate content analysis, the compound was determined using the phenol-sulphuric acid method [24]. In this analysis, approximately 50 mg of dried pretreated *Chlorella* sp. was suspended in 2.5 mL of 2.5% (v/v) hydrochloric acid and incubated in a hot water bath for 30 min. Afterwards, the carbohydrate content was measured using a UV-visible spectrophotometer at 485 nm and was estimated using the glucose standard curve. The protein content was determined using the Lowry method [25]. For this analysis, a total of 20 mg of pretreated biomass was lysised using 10 mL lysis buffer prior to being suspended in sodium dodecyl sulphate. The mixture was then incubated in the dark condition for 30 minutes. The protein content was determined with the spectrophotometer at a wavelength of 750 nm. Bovine serum albumin (BSA) was used as a protein standard for this analysis.

*2.3 Dilute alkaline pretreatment of microalgal biomass*

The pretreatment of dried microalgae biomass was performed using sodium hydroxide as an alkaline agent as per described by Kassim and Bhattacharya [17]. Briefly, 1.0 g dried *Chlorella* sp. biomass was immersed in 100 mL of 2% (w/v) NaOH and was placed in an oven at 120 °C for 2 hours. After the pretreatment period, the sample was left at room temperature and the mixture was then separated through centrifugation at 3000 rpm for 10 minutes. The separated pellet obtained was then washed with hot water until the pH of the mixture became neutral. The neutralised pellet was dried prior for further enzymatic hydrolysis.

*2.4 Enzymatic hydrolysis*

For the enzymatic hydrolysis experiment, the mixture of the complex cellulase cocktail with an enzyme activity of 0.017 μmol mg $min^{-1}$ purchased from *Trichoderma longibrachiatum* (Sigma–Aldrich C9748) was used throughout this experiment. The hydrolysis reaction was performed in a 10 mM acetate buffer (pH 5.5) at 45 °C and an agitation rate of 150 rpm (Thermoline Scientific; Australia) for 96 hours. During the hydrolysis reaction period, an aliquot hydrolysate was withdrawn every 24 hours interval and heated at 100 °C to deactivate the enzyme activity prior to centrifugation at 3500 rpm for 5 minutes. SReducing ugar content in the hydrolysate was analysed following the DNS method as per described in next section. The hydrolysis yield for this experiment was calculated as:

$$\text{Hydrolysis yield (\%)} = \frac{(\text{Concentration of reducing sugar at time of t} \times 0.9)}{(\text{Carbohydrate content after pretreatment})} \times 100 \quad (1)$$

*2.5 Primary screening*

The effects of different enzymatic hydrolysis parameters including pH, temperature, initial enzyme and biomass concentration on reducing sugar production and hydrolysis yield was initially determined using the one-variable at a time design (OVAT) method. For this study, the enzymatic saccharification of pretreated *Chlorella* sp. biomass were carried out at a temperature ranging between 30-60 °C; a pH ranging between 3.5-6.5; an enzyme concentration between 5 to 25 FPU $g^{-1}$; and an initial biomass concentration between 5-30 g $L^{-1}$. A total of 1.0 mL of the hydrolysate sample was withdrawn and centrifuged in order to determine the reducing sugar concentration produced during the hydrolysis. The reducing sugar analysis was performed using the DNS method.

*2.6 Optimization of enzymatic hydrolysis*

The optimization of the enzymatic hydrolysis for reducing sugar production was performed using the central composite design (CCD) approach of the general response surface methodology approach (RSM). A total of 20 experimental runs with different conditions proposed by the CCD were conducted. For this study, the parameters taken for consideration were pH 3.5 - 6.5; a temperature ranging from 40-50°C and an initial

biomass concentration of 5 - 20 $gL^{-1}$. The enzyme concentration was kept constant for each experiment. After the hydrolysis reaction a total of 1 mL samples were withdrawn and centrifuged for the reducing sugars and hydrolysis yield estimation. The regression model for the full factorial design is expressed in the equation as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + X_1^2 + X_2^2 + X_3^2 \quad (2)$$

where Y is the hydrolysis yield (% of carbohydrate content), $\beta_0$ is the model coefficient, $\beta_1$, $\beta_2$, and $\beta_3$ represent the independent effect of each factor. $X_1$, $X_2$, and $X_3$ are code values for temperature, pH and initial biomass concentration. The fit of the model and the interaction effect of each variable were expressed by coefficient determination $R^2$.

*2.7 Enzymatic hydrolysis kinetic*

Enzymatic hydrolysis kinetic properties ( $V_{max}$ and $K_m$) of dilute alkaline pretreated *Chlorella* sp. were determined using Michaelis–Menten and Lineweaver–Burk plots. Extrapolation of the enzymatic reaction using Michaelis-Menten is described as follows:

$$r = \frac{V_{max}[S]}{K_m + [S]} \quad (3)$$

The rate of reaction (r) can be calculated from the rate of substrate and product formation. Thus,

$$r = \frac{dS}{dt} = \frac{dP}{dt} \quad (4)$$

$$\frac{dP}{dP} = \frac{V_{max}[S]}{K_m + [S]} \quad (5)$$

where $K_m$ denotes as the Michaelis constant ($g\ L^{-1}$), $V_{max}$ is rate of hydrolysis ($g\ L^{-1}\ h^{-1}$), t value is the time (h) of enzymatic hydrolysis process, S and P are the substrate and product produced ($mg\ L^{-1}$) respectively. Both $K_m$ and $V_{max}$ were determined from a linear equation of Lineweaver–Burk plots as follows:

$$\frac{1}{r} = \frac{1}{K_m} + \frac{K_m}{V_{max}} \left[\frac{1}{S}\right] \quad (6)$$

$V_{max}$ and $K_m$ was calculated based on the intercept $1/V_{max}$ and the slope $K_m/V_{max}$ .

*2.8 Batch and fed-batch fermentation*

Bioethanol production from enzymatic hydrolysis of *Chlorella* sp. hydrolysate was performed in batch and fed-batch system using *Saccharomyces cerevisiae*. The yeast culture was cultured in glucose yeast extract-peptone-glucose medium consisting of 20 g $L^{-1}$ glucose, 20 g $L^{-1}$ peptone and 10 g $L^{-1}$ yeast extract. For the batch fermentation, approximately 10% (v/v) of active *S. cerevisiae* was added to a 250 mL shake flask that contained 100 mL hydrolysate in which the fermentation was carried out for 92 hours. Whilst, for the fed batch fermentation, the experiment was initially performed in batch condition until the substrate level showed a reduction level up to 20% prior to feeding new substrate. Similarly, 10% (v/v) of the active yeast was added in the hydrolysate at early fermentation stage. The substrate feeding strategy was applied by starting the experiment with an initial of substrate of 50% of the total volume followed by substrate feeding for three times (20:20:10) at 36, 48 and 60 hours. The entire fermentation process was conducted at pH 7, a temperature of 30°C and centifuged at 100 rpm for 96 h. The fermentation sample was withdrawn at every 24 hour interval for bioethanol and reducing sugar analytical monitoring. All current experiments were conducted in triplicate (n=3).

*2.9 Analytical methods*

*2.9.1 Reducing sugar estimation*

The 3, 5 dinitrosalysilic acid (DNS) method was used to analyse the total reducing sugar produced during the hydrolysis [26]. Briefly, the supernatant obtained from the harvested sample after hydrolysis was added with 1 mL of DNS reagent and then kept in boiling water (temperature ~90°C) for 10 min. After the boiling process, the reaction mixture was removed and cooled at room temperature. The reducing sugar concentration produced

was measured using a UV spectrophotometer at an absorbance of 540 nm (DR5000, Hach Canada). The result was expressed per milligram of reducing sugar per gram of dry microalgal (DM) biomass.

*2.9.2 High performance liquid chromatography (HPLC)*

The reducing sugar composition ( glucose, xylose, maltose, and galactose) in microalgal hydrolysate production during hydrolysis was analysed using high performance liquid chromatography ( HPLC) ( Waters, USA). The HPLC was equipped with a RH sugar-pack column ( 6.5 mm X 300 mm: Waters, USA ) and a refraction index detector (RID). The analysis was performed using ultra pure water as a mobile phase with an injection volume of 10 µL at a flow rate of 0.6 mL $min^{-1}$. The column and detector temperature was set at 75 °C and 45 °C respectively.

*2.9.3 Gas Chromatography*

The bioethanol concentrations produced from the fermentation process was determined using gas chromatography equipped with a flame ionization detector (Shimadzu GC-FID 2010). Helium gas was used as a carrier gas and the column used for this analysis was a 50 m capillary RT-Q-BOND column (inner diameter of 0.32 mm and 30 m long) (Restek, USA). The oven temperature was set to increase from 50 °C to 200 °C at a rate of 20 °C $min^{-1}$. The injector and detector temperature was programmed at 200 °C respectively. The flow rate and operating pressure was set at 21.9 mL $min^{-1}$ and 71.1 kPa respectively. For this analysis, an aliquot of 1 mL fermentation sample was withdrawn periodically every 24 h for analysis and centrifuge at 3000 rpm for 10 mins. The supernatant obtained was then filtered using 0.45 um cellulose acetate filter prior to subject for GC analysis.

*2.9.4 Statistical analysis*

All the samples were prepare in triplicate and data obtained was analysed statistically using one way analysis of variance ( ANOVA) to determine the significant differences among parameters and variables. The results were considered as significant if the P value was less than 0.05 ($P < 0.05$).

**3. Results and Discussion**

*3.1 Microalgae species*

Table 1 shows the initial percentage of lipids, proteins and carbohydrates in *Chlorella* sp. biomass. Protein is considered the most dominant chemical compound in *Chlorella* sp., followed by carbohydrate and lipid. Based on the chemical composition obtained, *Chlorella* sp. has a high potential to be used for a variety of applications including cosmetics, health foods, animal feed, chemicals and biofuels. The biochemical percentage in this current cultivation condition is different with other *Chlorella* sp. reported elsewhere. Generally, the biochemical distribution in microalgal biomass varies depending on growth medium and cultivation conditions. The protein content obtained in *Chlorella* sp. biomass is slighly higher than those reported in previous studies. Chen et al. [27] reported olegenous *C. vulgaris* had a protein content of 41% (dry weight). However, the protein content (46.24%) obtained is comparable to those reported for *Chlorella* sp. cultivated in different cultivation conditions [28]. The second highest biochemical component of 38.83% in *Chlorella* sp. biomass is carbohydrate. The carbohydrate fraction obtained from this present study is similar with the carbohydrate content reported in *Chlorella* sp. [28]. A substantial amount of carbohydrate content for this *Chlorella* sp. indicated that this microalgal biomass has a high potential to be used as fermentation feedstock for bioethanol and other chemical products via the fermentation process. In relation to lipid content, the present study indicates that only 14.92% of lipid content was available in the *Chlorella* sp. biomass. The lipid content obtained was comparable with other studies and found to be higher than that reported on the *Chlorella vulgaris* [29]. According to Mata et al. [30], the common range of lipid content for *Chlorella* sp. is 5-58%.

**Table 1** Main chemical compositions in untreated *Chlorella* sp. biomass

| Chemical composition | Percentage (wt %) on dry weigh basis |
|---|---|
| Carbohydrate | 38.83 |
| Protein | 46.24 |
| Lipid | 14.92 |

*3.2 Pretreatment and enzymatic hydrolysis*

Initially, the *Chlorella* sp. was pretreated using a 2% (w/v) dilute alkaline agent as a catalyst at 120 °C for 30 mins in order to alter the biomass structure and increase its surface area prior to enzymatic hydrolysis. Table 2 shows that a slight increase of carbohydrate content (2%) was observed in the pretreated *Chlorella* sp. biomass. Similar observations have been reported on changes of the chemical composition after the pretretament process [31&32]. A relatively higher carbohydrate content after the treatment process might be predominantly attributed by the removal of lipids and a protein fraction released during the pretreatment.

**Table 2** Carbohydrate content before and after alkaline pretreatment for microalgal biomass

| | Percentage (%) on dry weight basis |
|---|---|
| Before pretreatment | 38.83 ± 2.39 |
| After pretreatment | 41.44 ± 1.71 |

Preliminary enzymatic hydrolysis was carried out using a cellulase cocktail mixture that consists of xylanase, mannase, pectinase, amylase, protease and β-glucosidase activity. Note that the reducing sugar released from the hydrolysis was derived from the total carbohydrate content in the microalgal biomass. As shown in Figure 1, as expected, the reducing sugar produced from enzymatic hydrolysis of diluted alkaline pretreated *Chlorella* sp. was more than that of the untreated samples. The higher reducing sugar produced from the pretreated *Chlorella* sp. biomass demonstrated that the pretreatment process could enhance the hydrolysis and provide easy accessibility for cellulase enzyme to attack carbohydrate in the biomass.

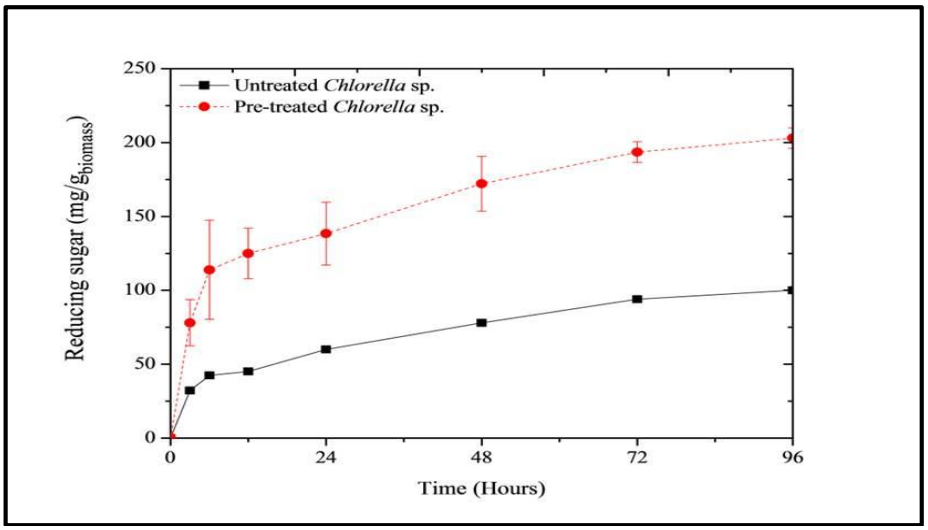


**Figure 1** Reducing sugar concentration from untreated and pretreated *Chlorella* sp. obtained from enzymatic saccharification at pH 5.5 and 40°C.

*3.3 Primary screening of enzymatic hydrolysis parameters of pretreated Chlorella sp. biomass*

To optimize reducing sugar production and conversion yield, the first step involves determining the most suitable parameters that can affect the response. For this study, initial screening was conducted using varying hydrolysis conditions. Various hydrolysis conditions that could affect the level of sugar production such as pH, temperature, enzyme concentration and initial biomass concentration were screened via the one variable at one time (OVAT) approach. Figure 2 shows the production of reducing sugar at different hydrolysis conditions. The present study indicates that the range of maximum reducing sugar production was obtained when the enzymatic hydrolysis was carried out between pH values of 4. 5-5. 5, temperatures ranging from 40-50 °C, an initial biomass concentration of 20-30 g $L^{-1}$ and an enzyme concentration of 20 to 25 FPU $g^{-1}$ respectively. The results obtained clearly indicated that pH, temperature, initial biomass and enzyme concentration have a significant effect ($P < 0.05$) on reducing sugar and hydrolysis yield of the pretreated *Chlorella* sp. biomass. Figure 2a shows that the maximum reducing sugar was obtained at pH 5.5 with 349.17 mg$g_{biomass}^{-1}$. Even though reducing sugars obtained from hydrolysis using pH 5.5 were comparable, the study indicated that there was no statistical difference on reducing sugar production between pH 5.5 and 4.5 ($P=0.256$). The results also indicated that an increase of enzyme concentration could increase sugar production (Figure 2c). As can be seen from figure 2c,

the maximum reducing sugar of 354.15 mg $g_{biomass}^{-1}$ was obtained when the hydrolysis was carried out using a high enzyme concentration of 20 $FPUg^{-1}$. Further increase of enzyme concentration to 25 $FPUg^{-1}$ was found to decrease reducing production. According to figure 2d, further increase of reducing sugar production was observed with the increasing of initial microalgal biomass concentration from 5 to 30 $gL^{-1}$. Nevertheless, a further increase of initial biomass concentration was found to significantly reduce hydrolysis yield. These results clearly suggested hydrolysis at high initial biomass concentration is not efficient, which subsequently could affect the final fermentation product's concentration. Low reducing sugar production at high initial biomass concentration indicates that not all the carbohydrate content in the biomass was fully converted into reducing sugars which may be contributed to the increase of viscosity and reduce the surface contact between enzyme and substrate during the process [33].

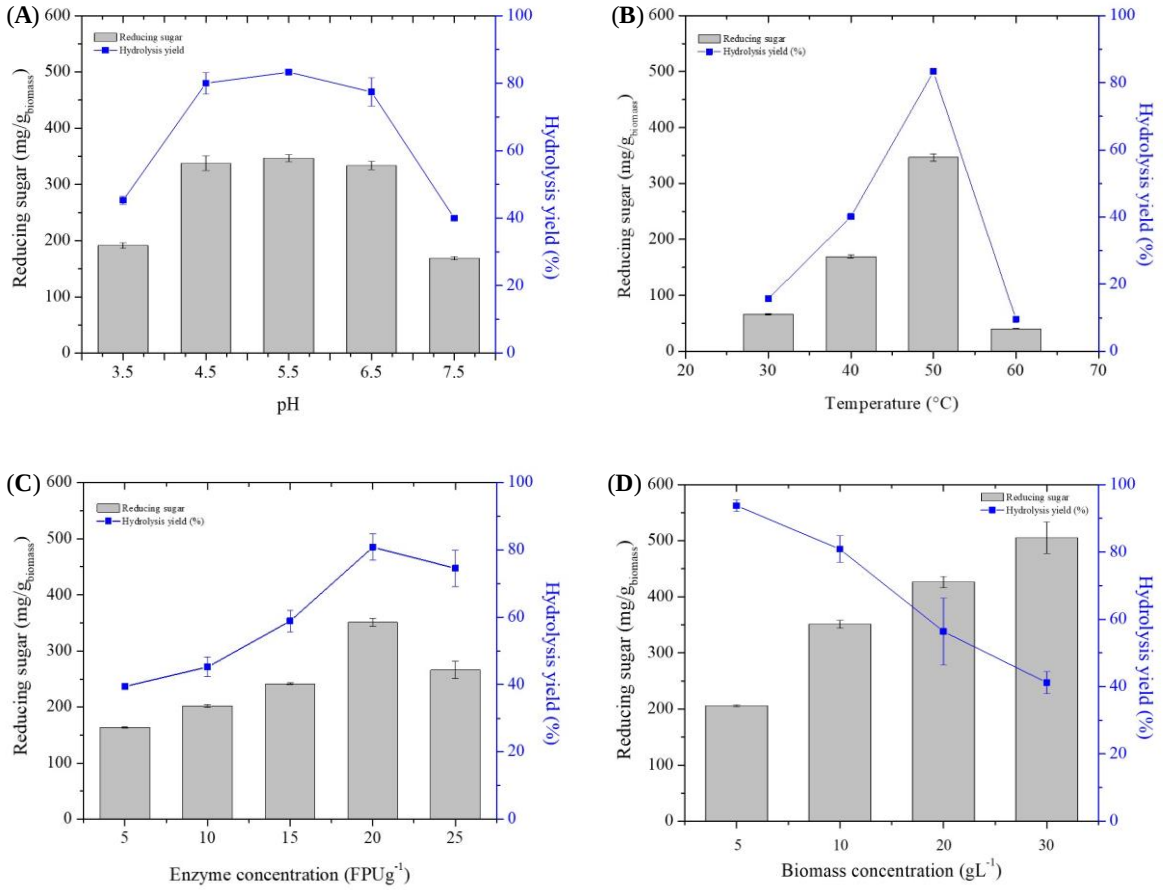


**Figure 2** Screening of hydrolysis parameters. (A) pH, (B) Temperature, (C) Enzyme concentration (D) Biomass concentration loading.

*3.4 Effect of variables on enzymatic hydrolysis yield*

Further enzymatic hydrolysis optimization using the central composite design (CCD) approach was applied to examine the parameters that influence the hydrolysis process. In this present study, the enzymatic hydrolysis yield was used as an output of the experiment. A total of 20 designated experiments were performed. The hydrolysis yields as a function of pH, temperature and biomass concentration all which have been obtained are shown in the Table 3.

Based on the results, the fit summary analysis of a sequential model sum of squares and model statistical analysis is performed. It is suggested that the quadratic model is the most suitable for optimization design. Furthermore, analysis of variance (ANOVA) and the F-test for the selected model was performed and presented in Table 4. The analysis shows that the correlation coefficient ($R^2$) of the model is calculated to be 0.95 (Adj $R^2$= 0.90), which is close to 1. This indicates that the model's prediction is in agreement with the actual hydrolysis yield value obtained from different conditions of pH, temperature and biomass concentration. On the other hand, the low *P* value ($P < 0.05$) obtained indicates that the model is significant and exhibits a good fit to the quadratic model. The lack of fit value of 0.1173 (not significant) indicates that the model fits well and there

is a significant effect on the enzymatic hydrolysis parameters on hydrolysis yield. The regression equation of the quadratic model that represents the correlation between variables and actual enzymatic hydrolysis yield can be written as follows:

$$Y\,(\%) = 203.98 + 8.13X_1 + 20.79X_2 + 6.30X_3 + 0.49X_1X_2 + 0.16X_1X_3 - 0.59X_2X_3 - 0.15X_1^2 - 2.99X_2^2 - 0.39X_3^2 \quad (7)$$

Where Y is the hydrolysis yield (% of carbohydrate content), $X_1$, $X_2$ and $X_3$ are temperature, pH, and initial biomass concentrations respectively.

**Table 3** Hydrolysis yield (%) from hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass

| Run | Temperature (°C) | pH | Biomass concentration (g $L^{-1}$) | Hydrolysis yield (%) | |
|---|---|---|---|---|---|
| | | | | Predicted | Actual |
| 1 | 50.00 | 3.50 | 5.00 | 13.92 | 11.55 |
| 2 | 45.00 | 5.00 | 12.50 | 79.29 | 83.32 |
| 3 | 36.59 | 5.00 | 12.50 | 72.88 | 65.44 |
| 4 | 45.00 | 5.00 | 12.50 | 79.29 | 79.88 |
| 5 | 40.00 | 6.50 | 5.00 | 61.35 | 65.88 |
| 6 | 45.00 | 5.00 | 0.00 | 8.99 | 0.00 |
| 7 | 45.00 | 5.00 | 12.50 | 79.29 | 80.29 |
| 8 | 40.00 | 6.50 | 20.00 | 47.33 | 52.56 |
| 9 | 45.00 | 7.52 | 12.50 | 75.06 | 70.25 |
| 10 | 40.00 | 3.50 | 20.00 | 50.44 | 46.88 |
| 11 | 45.00 | 2.48 | 12.50 | 45.39 | 46.14 |
| 12 | 45.00 | 5.00 | 25.11 | 26.58 | 31.35 |
| 13 | 45.00 | 5.00 | 12.50 | 79.29 | 80.58 |
| 14 | 53.41 | 5.00 | 12.50 | 65.16 | 68.55 |
| 15 | 45.00 | 5.00 | 12.50 | 79.29 | 69.32 |
| 16 | 50.00 | 6.50 | 20.00 | 62.13 | 56.35 |
| 17 | 50.00 | 6.50 | 5.00 | 52.32 | 58.75 |
| 18 | 50.00 | 3.50 | 20.00 | 50.29 | 48.63 |
| 19 | 45.00 | 5.00 | 12.50 | 79.29 | 82.69 |
| 20 | 40.00 | 3.50 | 5.00 | 37.9 | 46.55 |

**Table 4** Analysis of variance (ANOVA) for enzymatic hydrolysis of *Chlorella* sp. biomass.

| Regression statistic | R square | Adjust R-square | Standard error | | |
|---|---|---|---|---|---|
| Value | 0.95 | 0.90 | 7.38 | | |
| | | | | | |
| ANOVA | Df | SS | MS | F | Prob >F |
| Regression | 9 | 9459.02 | 1051 | 19.32 | <0.001 |
| Residual | 10 | 543.96 | 54.4 | | |
| Lack of fit | 5 | 412.61 | 82.52 | 3.14 | 0.1173 |
| Pure of error | 5 | 131.35 | 26.27 | | |
| Total | 19 | 10002.98 | | | |
| Variables | Coeffecient | Std error | P-value | | |
| Intercept | 79.29 | 3.01 | | | |
| $X_1$ | -2.3 | 2.00 | 0.277 | | |
| $X_2$ | 8.82 | 2.00 | 0.001 | | |
| $X_3$ | 5.59 | 2.00 | 0.019 | | |
| $X_1X_2$ | 3.74 | 2.61 | 0.182 | | |
| $X_2X_3$ | 5.96 | 2.61 | 0.045 | | |
| $X_1X_3$ | -6.64 | 2.61 | 0.029 | | |
| $X_1^2$ | -3.63 | 1.94 | 0.091 | | |
| $X_2^2$ | -6.74 | 1.94 | 0.006 | | |
| $X_3^2$ | -21.96 | 1.96 | 0.000 | | |

Df: Degree of freedom, SS: Sum of square, MS: Mean square, $X_1$:Temperature, $X_2$: pH and $X_3$: Biomass concentration

The interaction of the variables on enzymatic hydrolysis yield is displayed in a 3D response plot. Figure 3 shows the interaction of the pH value, temperature and initial biomass concentration on enzymatic hydrolysis yield of dilute alkaline pretreated *Chlorella* sp. biomass. The plots indicate the interaction of two variables with one variable being kept constant at its optimal condition. The results have indicated that a clear maximum peak for hydrolysis yield can be observed in the designed boundary for each interaction plot; indicating that the maximum hydrolysis yield can be obtained from the hydrolysis performed inside the designed boundary.

The interaction of pH and temperature on the hydrolysis yield of microalgal biomass is shown in Figure 3a. For this experiment, the biomass concentration of 12.5 $gL^{-1}$ was kept constant. The result indicated that the maximum hydrolysis yield is observed at a low pH value and temperature. The maximum hydrolysis yield was obtained at a pH value of 4.5 at 45°C. Significant reduction of the hydrolysis yield was noted when the enzymatic hydrolysis was performed beyond pH 5 and 47°C. Low hydrolysis yield observed beyond optimum conditions (>50°C ) could be due to low biocatalyst activity. According to Andreous et al. [34], hydrolysis at high temperatures will change the substrate and unfold cellulase structure, subsequently hinder the modified substrate from binding to the enzyme's active site making it unable to undergo a biocatalysis reaction.

Figure 3b shows the interaction between temperature and initial microalgal biomass concentrations on enzymatic hydrolysis yield. The maximum hydrolysis yield of 83% was obtained when the hydrolysis was carried out within a temperature range of 42°C-47°C and the initial microalgal biomass of 8.5-15.5 $gL^{-1}$. The present study indicated that enzymatic hydrolysis at high temperatures beyond 47°C and an initial biomass concentration of 12.5 $gL^{-1}$ will produce low sugar and hydrolysis yield. High reducing sugar and hydrolysis yield observed at high temperatures and high initial biomass concentrations may occur due to the hydrolysis that was performed at high temperatures. This in turn reduces the biomass mixture's viscosity which leads to more contact between the enzyme and the substrate during the reaction process. According to Adebowale et al. [35], reductions of solid material viscosity at high temperatures could be contributed by increased temperatures that will increase shear rates leading to a reduction of molecule to molecule interaction. As a result, it will reduce the viscosity of the samples. On the other hand, low reducing sugar release at high temperatures could change the enzymatic structure and deactivate the enzyme; reducing the enzyme activity. This may cause low hydrolysis yield obtained from the hydrolysis performed beyond optimum conditions [19]

The effect of initial biomass loading and pH on the enzymatic hydrolysis yield has also been evaluated. The results are shown in Figure 3c. Higher hydrolysis yield was achieved as the hydrolysis was performed at the middle of the designed boundary. The present study indicated that the maximum hydrolysis yield was obtained when the reaction was performed using a pH 4.5 and 12.5 $gL^{-1}$ microalgal biomass. Low hydrolysis yield was obtained under high levels of initial biomass concentration and pH value. A low degree of conversion of carbohydrates to reducing sugars at high initial biomass concentrations and pH value could be attributed to the increment of biomass viscosity under this reaction's conditions. Benoit et al.[36] reported that an increase of pH levels can increase the viscosity and sample dispersion. Thus, reducing the enzymatic and substrate interaction that could lead to low enzymatic hydrolysis reactions.

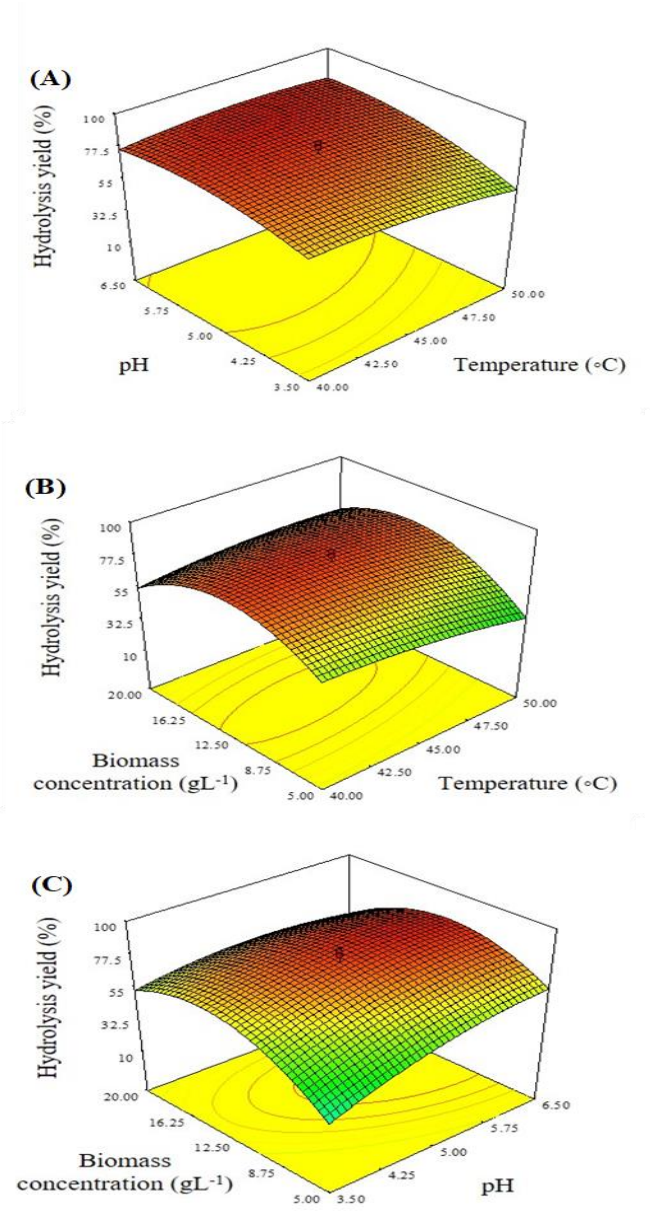


**Figure 3** Response surface interaction of reducing sugar production from pretreated *Chlorella* sp. biomass at different variables. ( A) pH and temperatures. ( B) Biomass concentration and temperature. ( C) Biomass concentration and pH.

A validation of enzymatic hydrolysis of the pretreated *Chlorella* sp. biomass at pH 5.0, at 45°C using initial a biomass concentration of 12.5 $gL^{-1}$ was performed in triplicate. Approximately 4.1 $gL^{-1}$ of reducing sugars corresponding 84.53% hydrolysis yield was obtained at this enzymatic hydrolysis condition. This has shown to be 2-fold higher than the amount of reducing sugar obtained from unoptimised conditions. The experimental value obtained is consistent with the predicted value ( Figure 4a) . The reducing sugar composition in the hydrolysate was also analysed using HPLC. The results have indicated that the major reducing sugars present in the hydrolysate were glucose at 50% , followed by xylose at 45% . A small amount of maltose has also been detected in the microalgal hydrolysate ( Figure 4b) . High glucose and xylose concentration in the hydrolysate have revealed the potential of the dilute pretreated *Chlorella* sp. biomass as a feedstock for liquid fuel such as bioethanol production via a fermentation process.

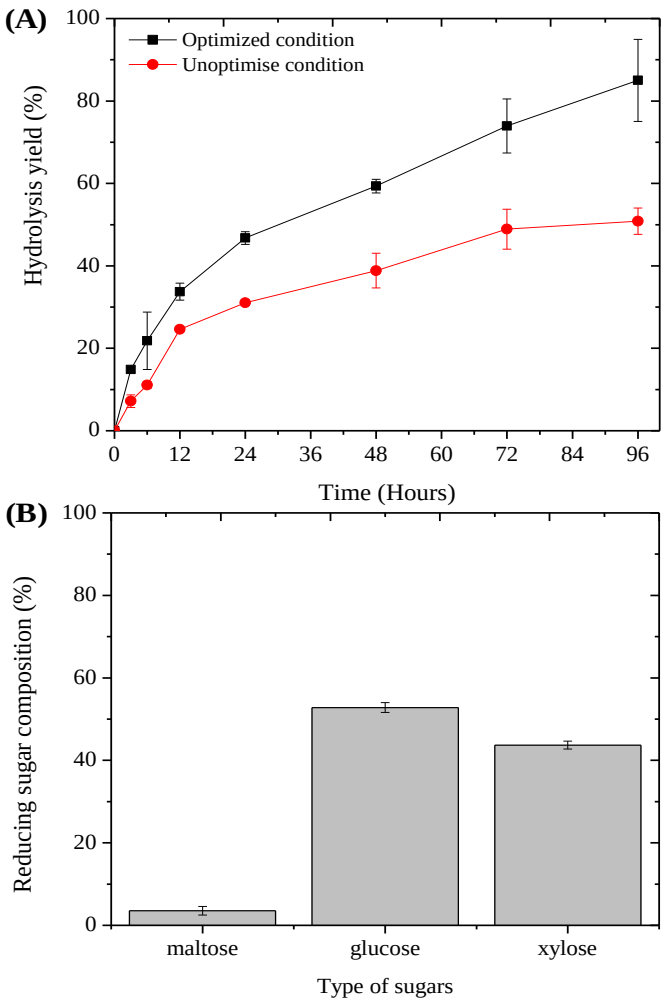


**Figure 4** Enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass ( A) . Reducing sugar production at optimum and unoptimum conditions (B). Reducing sugar composition.

A comparison was made between the enzymatic hydrolysis yield obtained from this study with other previous enzymatic hydrolysis processes with different types of microalgal biomass found summarized in Table 5. Most of the studies have reported that acid pretreatment was often applied to the microalgal biomass prior to the enzymatic hydrolysis process. The present study revealed that the alkaline pretreatment was able to enhance the enzymatic hydrolysis yield up to 80-85% ; similar levels to the yield obtained from the acid pretreatment approach. The enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. at low temperatures obtained from this study has had additional advantages in terms of environmental friendliness. Due to this particular process less chemicals and energy are required as a result of low temperature reactions.

**Table 5** Comparison of enzymatic saccharification reported for different types of microalgae biomass within the present study

| Feedstocks | Pretreatment | Solid loading ($gL^1$) | Enzymatic hydrolysis conditions | Yield | References |
|---|---|---|---|---|---|
| *Dunaliella tertiolecta* | 1.2% HCl at 121°C for 15 min | 50 | pH 5.5 at 55°C for 12 h | [b]80.9 | [19] |
| *Chlamydomonas reinhardtii* | Acid treatment 3% $H_2SO_4$ at 110°C for 30 min | 50 | pH 5.5 at 55°C | [a]58 % | [37] |
| Microalgae | 5.3% $H_2SO_4$ at 90°C for 30 min 9.4% NaOH at 90°C for 30 min | 100 | Acid slurry | [a]8.92 % | [38] |
| *Chlorella vulgaris* FSP-E | 1% $H_2SO_4$ at 120°C for 20 min | 20 | Enzyme mixture pH 6 at 45°C | [b]90% | [21] |
| *Chlorococcum humicola* | Ultrasonication | 10 | pH 4.8 at 40°C for 72 h | [a]68.2 % | [15] |
| *Chlorella* sp. | 2% NaOH at 120°C for 120 min | 10 | pH 5.5 at 45°C for 72 h | [b]84% | Present study |

[a] based on the residual biomass, [b] based on the total amount of carbohydrates of the residual biomass

*3.5 Enzymatic hydrolysis kinetic analysis*

A study on kinetic parameters ($V_{max}$ and $K_m$) of enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass was determined using typical Michaelis–Menten and Lineweaver–Burk plots. The $V_{max}$ and $K_m$ values were obtained from the intercept and slope of the Lineweaver-Burk plot by varying the microalgal biomass concentrations from 5 to 30 g $L^{-1}$ in acetate buffer (Figure 5). Generally, the $V_{max}$ value represents the rate of reaction when the enzyme is saturated with substrate, while, $K_m$ represents the concentration of substrate which allows the enzyme to reach half $V_{max}$. An enzyme with a high $K_m$ has a low affinity for its substrate, which requires a high substrate concentration to achieve $V_{max}$.

The study has indicated that the hydrolysis rate of the dilute alkaline pretreated *Chlorella* sp. biomass increased its non-linear behavior with an increased substrate concentration up to 20 $gL^{-1}$. The $V_{max}$ and $K_m$ values for enzymatic hydrolysis of dilute alkaline pretreated *Chlorella* sp. biomass were 0.18 mg $mL^{-1}$ $min^{-1}$ and 26. 34 mg $mL^{-1}$ respectively. The $V_{max}$ value obtained was higher than those reported on the enzymatic hydrolysis of *Chlorococcum humicula* (0.035 mg $mL^{-1}$ $min^{-1}$) and mixed microalgae (0.082 mg $mL^{-1}$ $min^{-1}$) [15, 39]. On the other hand, this present study indicated that the $K_m$ value obtained was higher than the $K_m$ value of 15.18 mg $mL^{-1}$ for *C. humicula* indicating that a higher substrate concentration is required to achieve $V_{max}$ [15]. Even though, a high $K_m$ was obtained the value is comparable with the $K_m$ value for other types of lignocellulosic material such as sugarcane bagasse and empty fruit bunches with $K_m$ value of 54.81 mg $mL^{-1}$ and 171.8 mg $mL^{-1}$ respectively [40 & 41]. Overall, this study showed that the $K_m$ and $V_{max}$ values obtained varied from other studies reported elsewhere. According to Carvalho et al. [42], the different values obtained from the studies were attributed by the substrate nature and the process conditions used during the hydrolysis process. Typically, the $K_m$ value for cellulase from *Trichoderma* sp. –substrate complex was with the parameters of 54.81 mg $mL^{-1}$ to 209.99 mg $mL^{-1}$ [41].

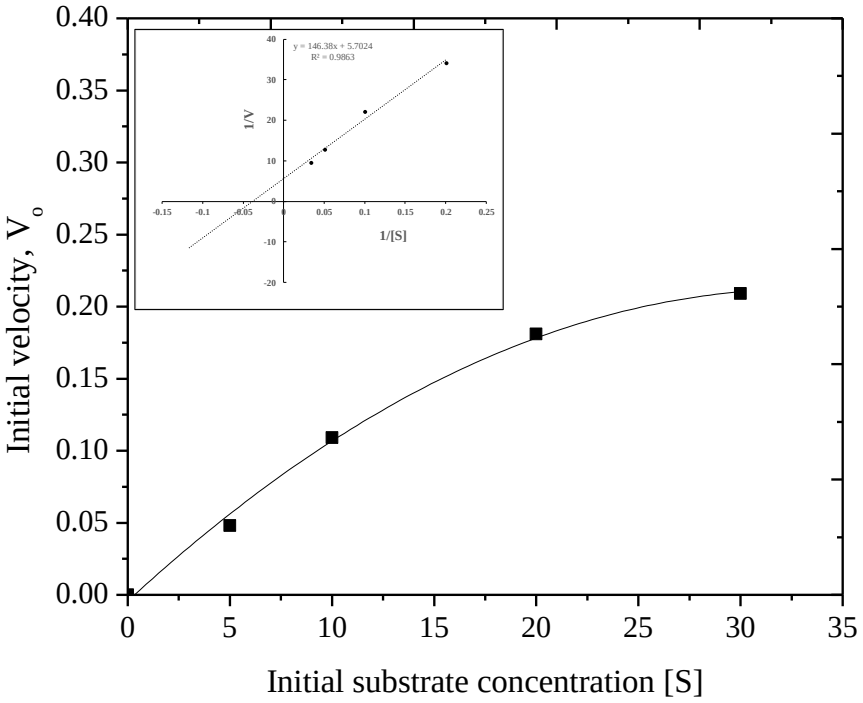


**Figure 5** Michaelis–Menten and Lineweaver–Burk plots for enzymatic saccharification of alkaline pretreated *Chlorella* sp.

3.6 Batch and Fed-batch fermentation

The *Chlorella* sp. hydrolysate obtained from the enzymatic hydrolysis process was fermented for bioethanol production using *S. cerevisiae* through two different fermentation modes, namely batch and fed-batch fermentation. Be noted that the carbohydrate content in the pretreated *Chlorella* sp. biomass is 23%. The amount of sugar concentration after enzymatic hydrolysis for batch and fed-batch fermentation from 83% of hydrolysis convertion was 2346.19 mg $L^{-1}$ and 2358.33 $mgL^{-1}$ respectively. For the fed-batch fermentation study, the addition of hydrolysate was conducted periodically, with the second, third and last feeding being performed at 36, 48 and 60 hours respectively. Figure 6 shows the bioethanol production and reducing sugar consumption profile from the ethanolic fermentation of *Chlorella* sp. hydrolysate in a batch and fed-batch fermentation process.

It can be noted that higher bioethanol production and yield was obtained from the fermentation of dilute alkaline pretreated *Chlorella* sp. hydrolysate via fed-batch fermentation system. The results have shown that bioethanol concentration for the batch and fed-batch fermentation was 921.38 $mgL^{-1}$ and 1008.8 $mgL^{-1}$ respectively. Bioethanol yield ($Y_{ps\ sugar}$) for both batch and fed-batch fermentation was 0.38 and 0.43 $gg_{sugar}^{-1}$, corresponding to 81% and 85% of the theoritical value for total sugar (Table 6). The bioethanol yield ($Y_{psbiomss}$) of 0.07 $gg_{biomass}^{-1}$ and 0.08 $gg_{biomass}^{-1}$ was observed from batch and fed-batch fermentation process, which is lower than those reported in other previous studies. A low bioethanol production yield obtained may be contributed by the low carbohydrate content available in the *Chlorella* sp. biomass. Thus, further optimization and enhancement of carbohydrate content in microalgal biomass during the cultivation process is required to ensure the feasibility of bioethanol production. The results obtained was in agreement with other previous studies which reported on the efficiency of fed-batch fermentation process [43 & 44]. According to Laopaiboon et al. [45], the fed-batch fermentation of sweet sorgum juice produced a higher bioethanol concentration and yield of 0.49 $gg^{-1}$ compared with those batch fermentations. On the other hand, bioethanol yields ($Y_{ps}$) obtained from this study were comparable with other bioethanol productions from various types of microalgal biomass such as *Chlorella* sp., *Dunaliella* sp and *Chlamydomonas* sp. [19, 21]. According to Ho et al. [21], the bioethanol yield obtained from the fermentation of *Chlorella* sp. KR-1 is 0.44 $gg_{sugar}^{-1}$. Similar bioethanol yield of 0.42 $gg_{glucose}^{-1}$ was also obtained from fermentation of *Duniella tertiolecta* [19].

As shown in Figure 6, approximately 21% and 15% of reducing sugars were still remaining in batch and fed-batch after 96 hours of the fermentation process. This may be attributed due to some reducing sugars in the

hydrolysate ( disaccharides, oligosaccharide and polysaccharide) which can not be utilised by microorganisms [46]. Other studies also reported that fermentation by products such as acetaldehyde, glycerol, butanediol, lactic and acetic acids produced during ethanolic fermentation could inhibit *S. cerevisiae*, and subsequently, reduce the ethanol production process [47].

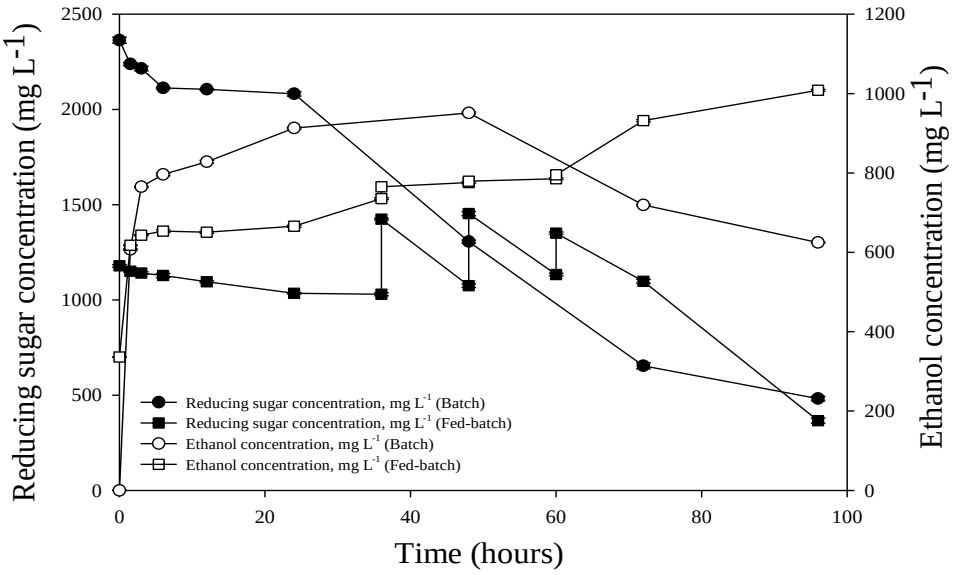


**Figure 6** Reducing sugar consumption and ethanol production profile for batch and fed-batch fermentation of alkaline pretreated *Chlorella* sp. biomass.

**Table 6** Comparison of the fermentation parameters of alkaline pretreated *Chlorella* sp. hydrolysate in batch and fed-batch system

| Fermentation mode | Reducing sugar (mg $L^{-1}$) after hydrolysis | Sugar consumption (%) | Ethanol concentration (mg $L^{-1}$) | Ethanol yield, $Y_{ps\ sugar}$ (%) | Conversion (%) |
|---|---|---|---|---|---|
| Batch | 2346.19 | 80 | 921.38 | 0.39 | 81.10 |
| Fed-batch | 2358.33 | 84 | 1008.48 | 0.43 | 85.52 |

## 5. Conclusions

The effects of enzymatic hydrolysis parameters on reducing sugar production and yield from dilute alkaline-pretreated *Chlorella* sp. was investigated using a mixture of cellulase from *T. longibrachiatum*. The maximum reducing sugars and hydrolysis yield of $413.42 gg_{biomass}^{-1}$ and 84.33% were obtained when the enzymatic hydrolysis was performed using $12.5\ gL^{-1}$ pretreated biomass at pH 5 and 45°C. Hydrolysis condition parameters such as temperature, pH and biomass concentrations found could significantly affect ($p < 0.05$) the reducing sugar production and hydrolysis yield from dilute alkaline pretreated *Chlorella* sp. biomass. Further comparison between the batch and fed-batch fermentation system of *Chlorella* sp. hydrolysate obtained from enzymatic hydrolysis process has indicated that fed-batch fermentation proved to enhance bioethanol production and yield ($Y_{pssugar}$). The information generated from this study may be useful for further investigation on up-scale bioethanol production using microalgal biomass. Further work on the optimization of fed-batch fermentation for higher bioethanol production from alkaline pretreated microalgal biomass to ensure the feasibility production is also currently being carried out.

## 6. Acknowledgements

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