Alkaline-assisted Microwave Pretreatment of *Tetraselmis suecica* Biomass for Fed-batch Enzymatic Hydrolysis

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Abstract. A two-part study on pretreatment and fed-batch enzymatic hydrolysis of pretreated *Tetraselmis suecica* using a high initial biomass concentration was conducted. First, the effect of different pretreatment processes, i.e. microwave (MC), dilute alkaline (AK), and microwave-alkaline assisted (MAK) pretreatment, on enzymatic hydrolysis of *T. suecica* biomass was evaluated. Furthermore, high initial biomass concentration enzymatic hydrolysis improvement via a fed-batch strategy was performed. Among the pretreatments tested, the MAK pretreatment produced the highest sugar concentration at 9.83 ± 0.24 mg/mL, corresponding to a conversion yield of up to 85.58% of carbohydrate content available in the pretreated biomass. The solid fraction generated after pretreatment was characterized using Fourier transform infrared (FTIR) spectroscopy. The FTIR analysis revealed a significant change in the functional hydroxyl and acetyl groups of the biomass, which is favorable for enzymatic hydrolysis. Introducing an initial microalgal biomass concentration beyond 15% (w/v) exhibited a low enzymatic hydrolysis yield. The fed-batch enzymatic hydrolysis strategy of the MAK pretreated *T. suecica* was further investigated by adding the substrate at different time intervals. The findings indicate that the fed-batch operation system could enhance sugar production and enzymatic hydrolysis yield one-fold.

Keywords: *biomass; biorefinery; biosugar; fed-batch hydrolysis; Tetraselmis suecica*.

1 Introduction

Recently, production of chemicals and biofuel from microalgae biomass has attracted much attention. Microalgal biomass is believed to have several advantages, such as being sustainable, abundantly available, cheap and environmentally friendly. To produce fine chemicals from this biomass, four main steps are involved, i.e. biomass preparation, pretreatment, hydrolysis, and fermentation [1]. The pretreatment and hydrolysis processes play a crucial role in ensuring the feasibility of biofuel production. According to Kumar *et al.* [2], these process both contribute approximately 60% to the overall production cost.

To date, many pretreatment methods microalgal biomass, including acid, alkaline, thermal, high-pressure and ultrasound pretreatment have been

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developed [3-5]. However, some limitations of the current pretreatment methods have been identified, such as thermal degradation, utilization of chemicals and time-consumingness, which make these pretreatments unfeasible for application in large-scale production. Microwave pretreatment has been suggested as a promising method to pretreat microalgae. This pretreatment method has also been reported to have advantages such as providing uniform heating and consuming less chemicals, with a short treatment time [6]. Dai, *et al*. [7] revealed that microwave pretreatment is able to enhance the lipid extraction process up to 30%. Microwave pretreatment with the presence of a catalyst agent such as alkali or acid can improve the efficiency of the process [8]. It was reported that the enzymatic hydrolysis of water hyacinth was higher than the conventional-heating sample.

A suitable pretreatment that is cheap and environmentally friendly and has a high conversion yield at high solid loading hydrolysis is considered to be the most promising approach to obtain high sugar concentrations [9]. Enzymatic hydrolysis using high initial biomass with a low enzyme concentration is the most efficient approach to produce high reducing sugar concentrations at low production cost. High initial biomass is required to obtain a high reducing sugar concentration and subsequently for production of highly concentrated fermentation products, which may substantially reduce the distillation cost during recovery [10]. However, using a high concentration of initial biomass concentration has been found to increase the viscosity of the hydrolysis system, which leads to low hydrolysis efficiency and increases energy consumption through a string process.

To date, several strategies have been proposed to overcome the problems with the hydrolysis of various lignocellulosic biomasses, such as a bench-scale helical bioreactor, a horizontal five chamber liquefaction reactor, a roller bottle reactor, prehydrolysis and fed-bach operation of simultaneous saccharification and fermentation (SSF) [10,11]. Fed-batch enzymatic hydrolysis was found to be among the most promising approaches to enhance enzymatic hydrolysis performance by adding the substrate or enzyme gradually to maintain biomass viscosity and enhance reducing sugar production [12]. This approach also exhibits several economic advantages, such as a low inhibitory effect, low water usage and by-product disposal, low operating cost because less energy is required for heating, cooling and purification, and low downstream processing cost because a highly concentrated product is generated from the process [13]. Yang, *et al.* [14], who conducted enzymatic hydrolysis via a corn stover fed-batch approach, were able to enhance the process up to 60% compared to a regular batch system. Another study showed that the fed batch system can increase reducing sugar production and hydrolysis conversion of alkaline pretreated sugarcane baggasse hydrolysis up to three-fold [15].

Even though there are some promising reports on the fed-batch enzymatic hydrolysis of various types of pretreated biomass, to the best of our knowledge there is only very limited information on the fed-batch enzymatic hydrolysis of microalgal biomass particularly generated from microwave pretreatment. Hence, this study was designed to enhance the enzymatic hydrolysis of pretreated *T. suecica* biomass at high solid concentration. The marine water *T. suecica* was selected for this study because this microalgae species has a great potential to be used in dual application CO_2 capture and fine chemical production [16,17]. Initially, different pretreatment approaches, i.e. microwave, alkaline and alkaline-microwave assisted pretreatment, to produce a high sugar concentration were evaluated. The effect of initial biomass concentration on the enzymatic hydrolysis conversion yield and sugar production was further investigated. Finally, the effect of high-solid fed-batch enzymatic hydrolysis on reducing sugar production was also investigated in this study in order to find the most efficient pretreated microalgal biomass.

2 Material and Methods

2.1 Microalgal Cultivation

Tetraselmis suecica, the marine water microalgal species used in this study was purchased from the Centre of Marine Science (COMAS), UPM. Cultivation of the microalgae was carried out using modified algae growth medium [18] consisting of 1.7 g/L sodium nitrate ($NaNO_3$), 0.49 g/L magnesium sulfate ($MgSO_4.7H_2O$), 0.14 g/L di-potassium phosphate (K_2HPO_4), 0.03 g/L calcium chloride ($CaCl_2.2H_2O$) and 35 g/L sodium chloride (NaCl). Incubation was performed in an incubator chamber, providing compressed air at 0.3 L/min, illuminated with a light intensity of 300 μmol/m/s and maintained at 30.0 ± 0.2 °C. The microalgae culture was harvested during the late logarithmic growth phase via centrifugation at 4500 rpm for 15 min. The resulting pellet was washed twice with distilled water and subsequently dried overnight at 60 °C. The dried biomass obtained was used for further study.

2.2 Chemical Composition

The chemical components in the biomass were determined according to Kassim, *et al.* [19]. Prior to the analysis, the culture was harvested and air dried at 60 °C for 24 h. For the total lipid content analysis, the lipid in the microalgal biomass was extracted using a mixture of hexane and isopropanol as the solvent via the Soxhlet extraction method [20]. The carbohydrate content was determined using the phenol-sulphuric acid method [21]. For this analysis, the total carbohydrate concentration present in the biomass was measured at 485 nm absorbance and was estimated using the glucose standard curve. The protein content in the

biomass was determined using the Lowry method, with bovine serum albumin (BSA) as the protein standard [22]. The protein present in the biomass was measured using a spectrophotometer at a wavelength of 750 nm.

2.3 Pretreatment

The microalgal biomass was pretreated prior to the enzymatic hydrolysis process. In this study, the pretreatment process was performed using 5% (w/v) of dried *T. suecica* biomass. The biomass was pretreated using different pretreatment approaches: alkaline (AK), microwave (MC) and microwave-assisted alkaline treatment (MAK) toward enzymatic hydrolysis of *T. suecica*.

The dilute alkaline (AK) pretreatment process was carried out as described by Kassim, *et al.* [4]. Briefly, a total of 5.0 g of dried microalgae biomass was suspended in 100 mL of 2% potasium hydroxide [23] at 120 °C for 2 h. After the pretreatment process, the suspension was centrifuged at 3500 rpm for a short period until the pH of the sample became neutral (pH ~7). The pellet was then dried at 70 °C for 24 h prior to the enzymatic hydrolysis process.

The microwave (MC) pretreatment process was performed using 5% (w/v) of dried *T. suecica* biomass suspended in distilled water (dH_2O). The pretreatment was conducted at 1100 watt microwave power with 5 min of microwave exposure time. The pretreated biomass was centrifuged and washed for a short period until the sample became neutral (pH ~7) and dried in an oven for 24 h prior to further enzymatic hydrolysis.

For the microwave-assisted alkaline (MAK) pretreatment, the 5% (w/v) dried *T. suecica* biomass was suspended in 2% KOH and submitted for microwave pretreatment at microwave power 1100 watt for 5 min. After the pretreatment process, the sample was centrifuged and washed with hot water for a short period and dried in an oven for 24 h prior to the enzymatic hydrolysis process. All the described treatments were carried out in duplicate. The enzymatic hydrolysis of *T. suecica* biomass without pretreatment was used as control.

2.4 Batch Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated microalgal biomass was performed using a cellulase cocktail (0.017 μmol/mg/min) obtained from *Trichoderma longibrachiatum* (Sigma-Aldrich C9748). For this analysis, the 5% (w/v) pretreated microalgal biomass was soaked in 10 mM acetate buffer (pH 5.5) followed by incubation at 45 °C and 150 rpm (Thermoline Scientific) for 72 h.

For the study of the effect of various initial biomass concentrations of pretreated microalgal biomass, a batch enzymatic hydrolysis series was carried out using

different biomass concentrations, ranging from 5 to 30% (w/v). The samples were taken every 24 h and centrifuged at 3500 rpm for 5 min. The supernatant obtained was used for reducing sugar analysis. The reducing sugar estimation is described in the next section. The hydrolysis yield was calculated as follows:

$$\text{Hydrolysis yield} = \frac{[\text{sugar concentration at t}] \times 0.9}{[\text{carbohydrate content after pretreatment}]} \times 100$$

2.5 Fed-batch Enzymatic Hydrolysis

Fed-batch enzymatic hydrolysis was performed via substrate and enzyme feeding strategies. For this experiment, a fixed-volume fed-batch hydrolysis system was applied throughout the process. In the substrate feeding strategy, the substrate was fed into the system via three different approaches: (a) batch enzymatic hydrolysis with a total final biomass concentration of 20% (w/v) biomass; (b) Strategy A: enzymatic hydrolysis started with 10% (w/v) biomass and 5% (w/v) of fresh substrate and fed twice after 12 h and 24 h to get a final biomass concentration of 20% (v/w); (c) Strategy B: enzymatic hydrolysis started using 2.5% (w/v) pretreated biomass and another subsequent 2.5% (w/v) fresh substrate fed thrice after 12 h, 24 h and 36 h to get a final biomass concentration of 20% (w/v).

2.6 Sugar Analysis

The reducing sugar concentration released during hydrolysis was determined using the 3, 5 dinitrosalysilic acid (DNS) method [24]. The supernatant obtained was added with 1 mL DNS reagent and boiled in a water bath for 10 min. After the boiling process, the sample was allowed to cool down at room temperature and the absorbance was measured at 540 nm using a UV spectrophotometer (Hach, DR-5000). The result was expressed in milligram of reducing sugar per gram of dry microalgal (DM) biomass.

2.7 Fourier-Transform Infrared Spectroscopy (FTIR)

In the IR study, approximately 3 mg of pre- and post-treated microalgal biomass was mixed with potassium bromide (KBr) and ground to less than 5 mm fine powder prior compression to 7000 prf to form a thin transparent pellet. The analysis was performed using an attenuated total reflectance (ATR) accessory on a Thermo Nicolet Avatar 360 FTIR spectrometer. The single beam spectra of each sample were collected by 32 scans at a resolution of 4 cm^{-1} from 4000 to 900 cm^{-1}.

2.8 Statistical Analysis

All experiments were performed in triplicate and an ANOVA test was carried out to identify the significant differences between the responses.

3 Results and Discussion

3.1 Chemical Composition

It can be observed that the protein composition in *T. suecica* is relatively high (52.33%). The lipid and carbohydrate contents of *T. suecica* are 23.11% and 17.41% respectively. Overall, the present results exhibited a similar trend in the biochemical distribution in the *T. suecica* biomass [25,26]. The lipid content in this study was in agreement with the lipid content range between 13-18% for *T. suecica* cultivated under different salinity conditions [25]. In contrast, the carbohydrate demonstrated different percentages in comparison with other studies. It should be noted that the biochemical composition distribution in microalgal biomass can be attributed to several factors, particularly the cultivation conditions. The differences in the chemical composition percentage observed in this study may be due to the cultivation conditions applied in this study.

The biochemical compounds in microalgal biomass have many potential applications. It is known that microalgal lipids from microalgae can be used to produce biodiesel via transesterification. Meanwhile, the carbohydrate of the microalgae can be used as reducing sugar feedstock to produce a wide range of fine chemicals through fermentation [16]. The high carbohydrate content in the *T. suecica* biomass exhibits potential to be used as a feedstock for biofuel or fine chemical feedstock through fermentation.

3.2 Effect of Pretreatment on Enzymatic Hydrolysis

A suitable pretreatment approach and hydrolysis conditions in sugar production as a chemical platform are essential in order to ensure the feasibility of fine chemical production. In the present study, the effect of four different pretreatment methods, i.e. dilute alkaline (AK), microwave (MC), alkaline-assisted microwave (MAK) pretreatment, of *T. suecica* and its carbohydrate conversion were evaluated. The results are shown in Figure 1.

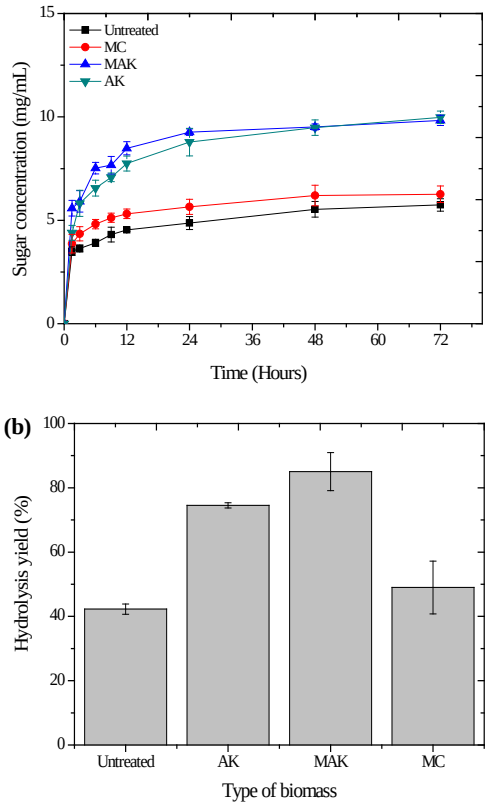

Figure 1 Sugar concentration and hydrolysis yield of microalgal biomass from different pretreatment approaches. (a) Sugar concentration produced from different pretreated samples; (b) hydrolysis conversion yield of *T. seucica* biomass from different pretreatments. AK = alkaline, MC = microwave. MAK = alkaline-assisted microwave.

As expected, a higher sugar concentration and hydrolysis yield were observed for the pretreated microalgae biomass compared to that of the untreated sample (Figure 1). Higher sugar concentrations (9.81 and 9.98 mg/mL) were observed for MAK and AK respectively. Only 6.26 mg/mL of sugar was produced from the sample that had been treated with microwaves, corresponding to a 9% increment compared to the untreated sample. Even though the sugar concentrations obtained from MAK and AK were comparable, it was found that the dilute alkaline pretreatment required a longer period (~120 min) to a obtain similar conversion yield as the MAK pretreatment. The longer biomass pretreatment period requires more energy and and as a result increases the production cost. This study also clearly indicates that pretreatment had a

significant effect on the carbohydrate conversion. The maximum conversion yield was observed for the MAK treatment, followed by AK, with conversion yields of 85.58% and 74.54% respectively. This study also indicates that the conversion yield for the sample pretreated with microwaves (MC) was 49.01%, i.e. only 5% higher than the untreated sample. Overall, this study demonstrated that the effectiveness of the pretreatment clearly influences the enzymatic hydrolysis efficiency and reducing sugar production.

The high reducing sugar concentration obtained from the MAK pretreatment can be explained by the fact that this microwave irradiation causes rapid heating and generates heat from direct contact between the molecules of the biomass and the electromagnetic waves, which results in fragmentation of the microalgal biomass structure [27]. Also, the presence of alkaline as catalyst assists the swelling and degradation of the microalgal biomass, providing more surface area for the enzymatic hydrolysis process.

Microwave irradiation pretreatment studies on different types of biomass have been reported by previous researchers [28,29]. According to Lu, *et al.* [30], the glucose yield produced increased up to 56.2% when rape straw was pretreated using a microwave pretreatment approach. Another study reported that an alkaline assisted microwave pretreatment of empty fruit bunches using NaOH could significantly increase enzymatic hydrolysis up to 5.8-fold and was able to remove more lignin and hemicellulose from the EFP sample [31]. Hence, according to the present study, the MAK pretreatment method is the most suitable approach to be applied for obtaining high reducing sugar concentrations and conversion yield of *T. suecica* biomass.

3.3 FTIR Analysis

Generally, hydrolysis efficiency is correlated with the functional group changes of the biomass [32]. Thus, the biomass obtained post-pretreatment was subjected to an FTIR analysis. The effect of pretreatment on the microalgal biomass composition was evaluated based on the relative changes in absorbance at specific bands. The result is presented in Figure 2. Significant changes of the peaks for the band 900-2000 cm^{-1} were observed for the alkaline, the alkaline assisted microwave and the microwave pretreated samples. The relative change of the bands at 900 cm^{-1} and 1200 cm^{-1}, representing polysaccharide and C-O-C adsorption, suggests cleavage of the cell wall structure during pretreatment using the alkaline, microwave and alkaline-assisted microwave treatment methods. Moreover, significant reduction was also observed in the protein peaks of the band between 1390 cm^{-1} and 1650 cm^{-1}, demonstrating degradation of proteins in the biomass sample during the pretreatment process. The FTIR spectrum also demonstrated that the AK and MAK treatment caused further

changes in the band between 1500 cm^{-1} to 1700 cm^{-1}, which represent the removal of lipid content from the biomass. These bands represent the N-H stretching for protein amine I and II [33]. The considerable decrease of the band within these ranges indicates a reduction of protein content in the biomass after pretreatment. Removal of protein components from the microalgal biomass during pretreatment is most probably due to thermal degradation and deterioration during the process [34].

Based on the FTIR spectra analysis it is clear that the pretreatments of the microalgal biomass applied had as significant effect on the functional groups of the biomass, and degradation and swelling of the biomass, leading to higher enzymatic hydrolysis efficiency.

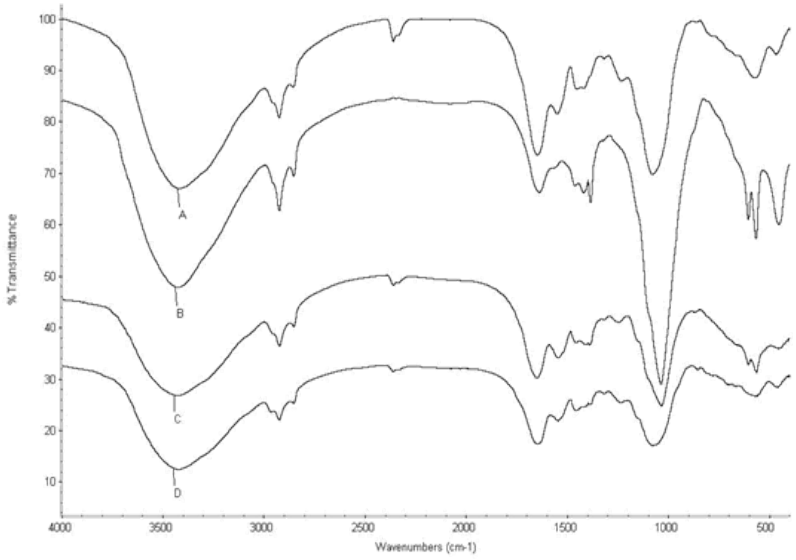

Figure 2 FTIR result of microalgal biomass: (A) untreated; (B) alkaline pretreatment (AK); (C) alkaline-assisted microwave pretreatment (MAK); and (D) microwave pretreatment (MC).

3.4 Effect of Initial Biomass Concentration

To produce a high sugar concentration from microalgal biomass, a high initial biomass concentration is required for enzymatic hydrolysis. However, it is known that enzymatic hydrolysis using a high initial solid biomass will lead to a low conversion yield due to the inhibitory effect of high viscosity during the process [35]. In the present study, the effect of initial biomass concentration was investigated to determine the inhibitory effect and hydrolysis yield when hydrolysis was performed using a high *T. suecica* biomass concentration.

Figure 3 shows the enzymatic hydrolysis of MAK pretreated *T. suecica* at different initial biomass concentrations ranging from 5 to 30% (w/v). The results indicate that the initial biomass concentration had a positive influence on the reducing sugar production from MAK pretreated *T. suecica* biomass. The reducing sugar production increased with the increment of the initial biomass concentration. As shown in Figure 3, the findings indicate that the highest reducing sugar production of 16.90 ± 2.44 mg/L was observed for hydrolysis using 30% (w/v) biomass. As expected, a higher initial biomass concentration will produce a higher reducing sugar concentration. Nevertheless, an increase in initial biomass concentration from 10 to 30% (w/v) of biomass resulted in a relatively low hydrolysis yield. A low conversion yield was observed for hydrolysis at biomass concentrations of 20 and 30% (w/v). Hence, hydrolysis using a high initial biomass concentration has a negative impact on the hydrolysis yield and reducing sugar production.

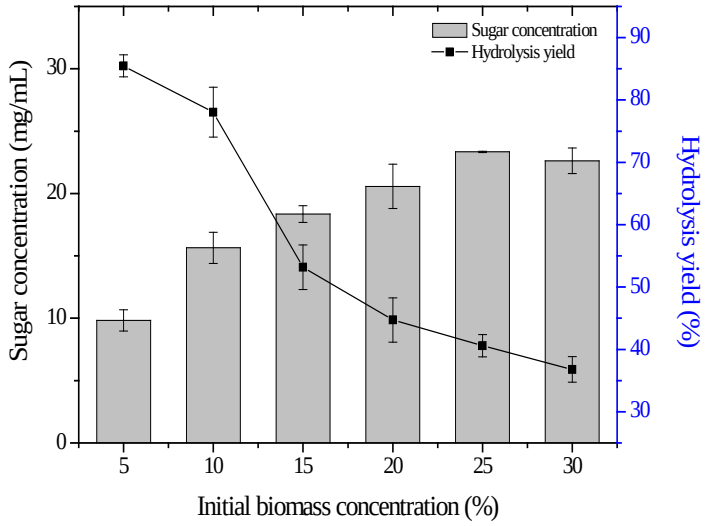

Figure 3 Conversion yield and reducing sugar concentration of microwave-alkaline assisted pretreated *T. suecica* at different initial solid loadings.

The low conversion yield at high initial biomass concentration is contributed by several factors, including limited mass transfer rate and enzyme inhibition by inhibitors, leading to reducing enzyme activity [36]. In the present study, a 37% decrease in hydrolysis yield was observed when the solid loading was increased from 10% to 30% (w/v). Several previous investigations have shown approximately 10% reduction of conversion or hydrolysis yield when the initial

biomass concentration was increased from 2% (w/v) to 20% (w/v) biomass loading during enzymatic hydrolysis [37].

Generally, there was free moisture or free water in the reactor system that could assist the hydrolysis reaction when the initial biomass concentration was lower than 15% compared to hydrolysis using biomass higher than 15% (w/v). Operating at this condition causes the biomass structure to degrade more slowly and form a thick paste that hinders the hydrolysis reaction [38]. According to Ioelovic & Morag [39], who mention that low enzymatic efficiency at high initial switchgrass biomass concentration could lead to diminution of the product solution owing to its absorption and retention by the residual non-hydrolyzed biomass, which results in a reduced productivity and enzymatic hydrolysis process.

3.5 Fed-batch Enzymatic Hydrolysis

As discussed above, enzymatic hydrolysis at high initial biomass concentration can reduce hydrolysis yield. Thus, applying fed-batch enzymatic hydrolysis is an approach that could be used to enhance the hydrolysis yield in a high initial biomass enzymatic hydrolysis process.

3.5.1 Effect of Substrate Feeding

Figure 4 shows the sugar production profiles for different substrate feeding strategies. It is clear that fed-batch enzymatic hydrolysis can enhance reducing sugar from MAK treated *T. suecica* biomass. As shown in Figure 4, there is a significant difference in hydrolysis rate for batch and fed-batch enzymatic hydrolysis in the first 24 h. A high hydrolysis rate was observed for the batch operation in the early stages of the reaction, however, it starts to slow down after 12 h of incubation. In contrast, the sugar concentration started to increase for both fed-bach operations after 12 h, when the first feeding was applied to the system. Further, the sugar production kept increasing when the substrate was fed into the system. It was found that the sugar production for Strategy C started to reach the equilibrium phase after 36 h, which indicates the inhibitory effect occurred during this stage. Nevertheless, a slight increment in sugar production was observed for Strategy B after 36 h of incubation.

The highest sugar production (58.55 ± 8.08 mg/mL) was obtained from hydrolysis via Strategy B followed by hydrolysis using Strategy C with 47.39 ± 2.13 mg/mL of sugar. It had over 55.17% higher enzymatic hydrolysis and more than 32.53 mg/mL more sugar was obtained compared to batch enzymatic hydrolysis.

The higher reducing sugar concentration observed for Strategy B may be because most of the biomass was already liquefied at an early stage, i.e. within 12 h of hydrolysis. Typically, a fed-batch operation allows time for the slurry to liquefy before feeding either substrate or enzyme in order to keep the low initial insoluble solid substrate in the system. Addition of a small portion of substrate was found to have no significant influence on the viscosity and inhibitory effect of the hydrolysis.

The results obtained from this study are in agreement with fed-batch enzymatic hydrolysis using various types of biomass [40,41]. According to Jung, *et al.* [42], who reported that the substrate feeding could affect the enzymatic hydrolysis of rice straw performance with a highest hydrolysis yield of 76%, observed for hydrolysis using three times feeding. On the other hand, fed-batch hydrolysis of alkaline-pretreated wheat straw and sugarcane baggase could improve the reducing sugar production and slow down the enzymatic reaction rate, which reduced the product inhibition and enzyme activation [43]. Hence, this study suggests that fed-batch biomass feeding after 12 and 24 h is a suitable strategy to improve enzymatic hydrolysis of *T. suecica* biomass at high initial biomass concentration.

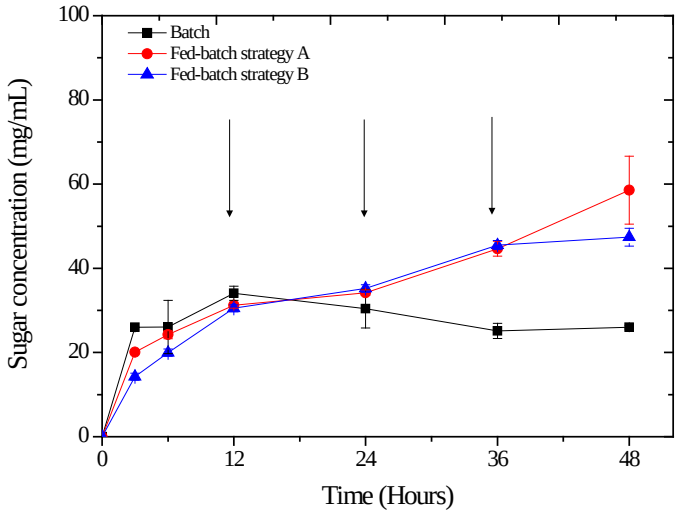

Figure 4 Reducing sugar production using different substrate feeding strategies.

3.5.2 Effect of Enzyme Concentration

Enzymatic hydrolysis of a high solid biomass concentration using a low enzyme concentration with a suitable feeding mode is important to ensure low

production cost. Thus, the effects of different enzyme concentrations, ranging from 100 FPU/g, 80 FPU/g, 60 FPU/g, 40 FPU/g to 20 FPU/g, on fed-batch enzymatic hydrolysis of MAK pretreated *T. suecica* biomass were investigated. In this study, the whole enzyme was added in the initial enzymatic hydrolysis process. Figure 5 shows the reducing sugar production profiles from enzymatic hydrolysis using different enzyme concentrations.

Among these enzyme concentrations, the maximum sugar concentration was obtained with the hydrolysis conducted using 80 FPU/g of enzyme with a final concentration of 56.55 ± 8.08 mg/mL within 48 h of incubation (Figure 5). As can be seen in Figure 5, the hydrolysis rate for the fed-batch operation using a high enzyme concentration was higher compared to that using a low enzyme concentration. A slight reduction in sugar production was observed for the hydrolysis that used 40 FPU/g. As illustrated in Figure 5, only 46.33% and 27.06% of the carbohydrate content was converted to sugar when the hydrolysis was carried out using 60 and 40 FPU/g cellulase respectively. The low sugar concentration achieved in this study is in agreement with other hydrolysis experiments under these conditions [44]. At low enzyme concentration, the low enzymatic hydrolysis yield observed may be due to the enzyme concentration not favoring carbohydrate hydrolysis in the microalgal biomass. In fact, the presence of many substrate molecules competes with the enzyme's active sites, blocking these sites and thus preventing other substrates from occupying them [45]. As a result the enzymatic reaction rate is reduced.

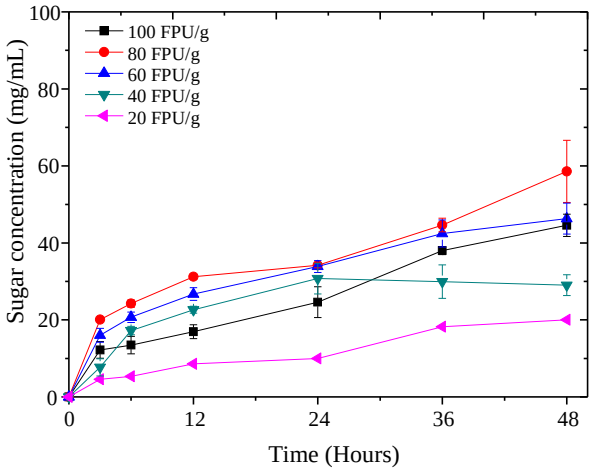

Figure 5 Sugar concentration produced from enzymatic hydrolysis of alkaline assisted microwave pretreated *T. suecica* biomass using different enzyme concentrations via fed-batch substrate feeding strategy. The hydrolysis was

performed using a final biomass concentration of 20% pretreated biomass at pH 5.5 and 45 °C.

4 Conclusion

The effects of different pretreatment methods for maximum reducing sugar production and conversion yield were evaluated. This study demonstrated that the type of pretreatment has a significant effect on the sugar production and hydrolysis yield. The microwave-alkaline assisted pretreament (MAK) was the most favorable pretreatment method, producing the highest reducing sugar concentration and conversion yield from *Tetraselmis suecica* biomass.

The fed-batch enzymatic hydrolysis strategy was successfully enhanced, reducing sugar production of hydrolysis yield of MAK pretreated *T. suecica* when the hyrolysis was performed using a high initial biomass concentration. This study also demonstrated that the fed-batch strategy via biomass feeding strategy could improve reducing sugar production and hydrolysis yield of hydrolysis using 20% (w/w) pretreated biomass. Under this condition, the total sugar concentration in the hydrolysate and hydrolysis yield were 58.55 mg/mL and ~90%, corresponding to one-fold higher than the batch process. The results obtained from this study can be used to improve the reducing sugar and subsequent biofuel production.

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