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FRACTIONATION OF HYDROLYZED MICROCRYSTALLINE CELLULOSE BY ULTRAFILTRATION MEMBRANE

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Abstract

Bioethanol process using cellulosic materials have been emerging an interesting field with a high potential of replacing petroleum-based fuel, as a future alternative. This work emphasised on improvement of enzymatic hydrolysis of alkaline NaOH-pretreated cellulose by applying an ultra-filtration membrane 10 kDa cutoff in order to minimise sugar inhibition on enzymes, reuse enzyme in hydrolysis and recover sugar for the subsequent fermentation. An improvement in the methodology of the enzymatic hydrolysis with ultrafiltration was made that the membrane was installed at the end of a tube connecting with a peristaltic pump to continuously remove glucose from hydrolysis reaction hence sugar was unable to inhibit enzyme activity and enzyme was retained inside the reactor for the reusing purpose. The combination of NaOH 1M alkaline pretreatment, enzymatic hydrolysis of cellulose with the optimum 3% enzyme dosage, ultrafiltration 10 kDa cutoff was evaluated to obtain the highest sugar concentration at 9 mg/ml after 6 hour hydrolysis. In comparison between hydrolysis with ultrafiltration and hydrolysis without ultrafiltration, the sugar concentration in hydrolysis with ultrafiltration was very much higher than that in hydrolysis without ultrafiltration in all enzyme dosages (1.5%, 3%, 6%). The hydrolysis with filtration produced a time profile in six hours with continuously significant increase in the sugar concentration. Only a small reduction initially for 1.5% dosage and no reduction in sugar concentration in 3% and 6% dosages. Hence the effect of product inhibition in hydrolysis was minimised as a result. In addition, a direct relationship between sugar concentration inside hydrolysis reactor, enzyme dosage and rate of sugar removal was observed during the hydrolysis process. Higher enzyme dosage in hydrolysis required a higher rate of sugar removal sufficiently to avoid inhibition in hydrolysis reaction.

Keywords: Enzymatic hydrolysis of cellulose, Product inhibition, Ultrafiltration, Reuse of enzyme cellulases, Alkaline pretreatment, Cellic Ctec enzymes.

1. Introduction

Technology of cellulose-based ethanol production has been continuously advanced over the past decades with progressive efforts of making the process more cost-effective and reachable for large-scale industrial production. Hence, nowadays bioethanol is considered as a very potential alternate to conventional fossil fuel usage causing environmental concerns such as green-house effects and global warming. Feedstock containing cellulose to produce bioethanol comes from two main sources, i.e., the first generation (sugar, and starch like corn, wheat, soya, and grains) and the second generation (agricultural residues like palm empty fruit bunches, sugar bagasse and corn stover). However, it was argued that the first generation feedstock causes a dilemma and a debatable issue on food versus fuel due to its negative impacts on regional water source, biodiversity, soil quality [1-5]. In contrast, the second generation feedstock from non-food source is able to avoid such issues and create a newly environmental-friendly way to reuse cellulosic biomass by producing a value-added ethanol product.

The production of bioethanol from cellulose consists of sub-processes namely a) pretreatment of cellulose for more susceptibility as well as increase in enzymatic accessibility, b) hydrolysis of cellulose to sugar, c) fermentation of sugar to ethanol and subsequent product purification. Research and development have been on the progress to manage and optimise these sub-processes in order to improve its both technological and economic feasibilities [6]. Pretreatment of cellulose plays an important role, which has an impact on hydrolysis later. Without pretreatment, only 20% out of total cellulose are converted to glucose [7]. The pretreatment is to remove lignin from biomass by breaking down the recalcitrant structure of biomass into lignin, hemicellulose and cellulose. On the other hand, pretreatment also helps to decrease the degree of polymerization and crystallinity of the cellulose component, causing it to swell, hence making cellulose after pretreatment more susceptible for enzymes to attack in hydrolysis step [8-10]. Methods of pretreatment are various and depend on the economic factor, type of feedstock, severity degree and so on. Unlike acid pretreatment causing a severe corrosion on equipment and generating undesirable inhibiting byproducts, alkaline pretreatment is safer to use, minimise corrosion and more preferable in the industry of bioethanol production [11, 12]. In addition, hydrolysis by using enzymes named cellulases is more preferable in cellulosic bioethanol process due to its advantages in term of lower energy consumption, limited formation of inhibiting byproducts in subsequent fermentation, high sugar yield, operating under mild conditions as compared to acid hydrolysis [8, 13]. However, two major disadvantages existing as obstacles in enzymatic hydrolysis of cellulose are accessibility for enzyme to cellulose and sugar inhibition on the enzymes. These result in an increase in overall production cost.

To overcome the obstacles in the usage of enzyme cellulases in hydrolysis, research and development in two strategies have been conducted, firstly to decrease the crystallinity of cellulose with strong solvents hence improve enzyme accessibility and secondly to reduce product inhibition by the removal of sugar from the hydrolysis process [6]. This paper will focus on the second strategy of reducing product inhibition.

The application of membrane separation in hydrolysis to continuously remove sugar was proven to be very suitable and effective in a number of research papers especially, the use of ultrafiltration membrane 10 kDa cutoff (1 Da = 1 g/mol) [6, 14-16]. Enzyme cellulases as protein with large molecular weight from 35 to 65 kDa will be retained, whereas glucose molecules (150-180 Da) will be penetrated through membrane barrier [6, 17]. Thus, the high potential of ultrafiltration membrane is applied for reducing sugar inhibition, the recovery of sugar and reuse of enzymes. When sugar product is concentrated after hydrolysis and enzyme is reused, cost saving in subsequent downstream separation and enzyme consumption in hydrolysis will make the overall bioethanol process more cost-effective.

Experiments studied the combination of ionic liquid pretreatment, hydrolysis by a synergy of enzymes, plus different membrane filtration configurations (side membrane reactor, 5kDa or 10 kDa cutoff membranes, nanofiltration, electrolysis) showed very supportive results about the application of membranes in hydrolysis of cellulose [16,11,12]. 90% conversion of cellulose to glucose with 98% of enzymes retained were achieved [16]. In addition, testing with a ceramic membrane with higher mechanical strength under the same condition was showed its feasibility to use in industrial scale up [11]. Other research targeted at 50% conversion using a 10kDa cutoff side membrane reactor managed to reduce inhibition on enzyme and increase conversion [12]. Hence the suitability and effectiveness of membrane filtration were sufficiently proven to solve the limitations in saccharification of cellulose to sugar by the reduce in product inhibition, recycle of enzymes and recovery of sugar for the subsequent fermentation process.

However the membrane filtration has its own disadvantages due to the occurrence of concentration polarization, membrane fouling. This resulted in a decrease in productivity again when a certain permeate flux was exceeded [15]. An explanation to this phenomenon is due to the accumulated cellulose on the membrane surface, consequently preventing hydrolysis. Plus the simultaneous loss of cellobiose - an intermediate of sugar into the permeate of the membrane as a limiting factor [18]. Moreover, the use of ionic liquid as a pretreatment method is not preferred in industrial production of bioethanol because of the very high cost as being a cost driver taking 33% of total production cost [16]. All of these factors make the application of ultrafiltration membrane difficult to reach fully commercialization in the bioethanol sector, which still prefers acid or alkaline pretreatment because of its low cost and high sugar conversion.

Hence the focus of this research is about combination of alkaline pretreatment, enzymatic hydrolysis with high activity of β-glucosidase and ultrafiltration membrane 10 kDa cutoff to evaluate the performance of hydrolysis and feasibility in applying for industry.

2. Objectives and scopes

This research aimed at studying the feasibility of the combination of alkaline NaOH pretreatment, hydrolysis of cellulose with high activity in β-glucosidases, and ultrafiltration membrane 10 kDa cutoff to reduce the sugar inhibition on enzyme, recover glucose for the subsequent fermentation and reuse enzyme.

The scope of this research is on studying the enzymatic hydrolysis of cellulose by using enzymes Cellic CTec with enzyme dosages in the range from 3% to 6% (w/w) and solid loading of 10% (w/v).

3. Methodology

3.1 Materials

Microcrystalline cellulose purchased from R&M Chemicals has a degree of polymerization 210-270, able to undergo pH 5.5-7.0. Alkaline was sodium hydroxide NaOH purchased from Friendemann Schmidt Chemical, dissolving in water to make a concentration of 1 M. For the use of Cellic Ctec as enzyme cellulases for the hydrolysis of cellulose, the activity of enzymes is not provided by the manufacturer Novozymes. However a recommendation of Novozymes is to test the hydrolysis from 1.5% to 30% (w/w) enzyme dosages (enzyme to substrate ratio) in order to find an optimal enzyme dosage which is suitable for the designed process and conditions. Detail of the instruction for using Cellic Ctect can be found in the application sheet issued by Novozymes [19]. Ultrafiltration membrane manufactured by Sartorius Stedim Biotech GmbH, Germany has molecular weight cutoff of 10 kDa, and made of regenerated cellulose.

3.2 Method

3.2.1 Pretreatment

Microcrystalline cellulose (50 g) was soaked in aqueous sodium hydroxide (1 M) 8% (w/v) solid loading at 100^0C in a water bath. After 3 hours pretreatment, the liquid portion was drained out, left the pretreated cellulose preserved in distilled water. For every subsequent hydrolysis experiment, pretreated cellulose was filtrated by Whatman filter paper to remove water.

3.2.2 Enzymatic hydrolysis and ultrafiltration

Hydrolysis of pretreated cellulose was tested with different enzyme dosages (1.5%, 3%, 6% and/or 30%) with and without ultrafiltration at 45^0C, pH 5.0 citrate buffer under magnetic stirring for 6 hours at 10% (w/v) solid loading (mass of cellulose (g)/total volume (ml)). Samples were taken from the hydrolysis reactor and at the permeate for total reducing sugar quantification by Dinitrosalicylic acid (DNS) method. Readings of absorbance for each sample were concordant and obtained by repetition of twice for each measurement. From the concordant readings of absorbance, sugar concentrations were converted and considered as preliminary data for this experiment.

A membrane was attached at the end of a tube used to remove sugar solution by a peristaltic pump and thus enzymes and cellulose were retained inside the reactor. After the first 2-3 hours, ultrafiltration was started to perform. Figure 1 shows the experimental set up of hydrolysis with ultrafiltration.

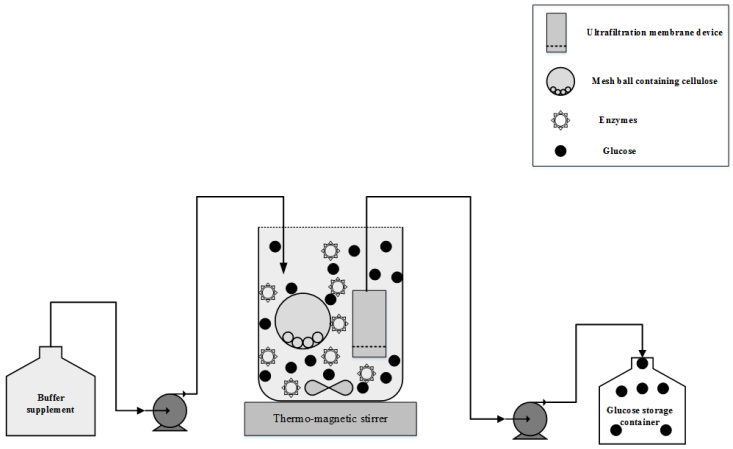

Fig. 1. Experimental setup of enzymatic hydrolysis with ultrafiltration.

4. Results and discussion

The experiments were divided into two parts. In part one, hydrolysis of pretreated microcrystalline was carried out without ultrafiltration at four different enzyme dosages (1.5%, 3%, 6% and 30%). In part two, the same hydrolysis experiments were conducted with ultrafiltration membrane 10kDa cutoff. Plus the pretreated cellulose was contained inside a sieve ball, hanging suspended inside the hydrolysis reactor.

Glucose liberated from hydrolysis reaction was measured in term of sugar concentration for every hour inside the reactor. As can be seen in Fig. 2, the sugar concentration is directly proportional to the enzyme dosages. For higher enzyme dosage or enzyme loading, there are more cellulases accessing to polymeric chains of cellulose to release glucose monomers, thus results in higher sugar concentration [8]. However, it is observed that for all four dosages there is the same trend occurring. After the first two or three hours in hydrolysis, sugar concentration started to decrease. This trend became obvious with a significant reduction in sugar released for hydrolysis with 6% and 30% dosages of enzymes. An explanation for this phenomenon is due to the product inhibition on the enzymes. The presence of released glucose causes inhibition on cellulases, or reduces the enzymatic activity of cellulases [14, 12]. Therefore the presence of sugar have an effect on the rate of glucose released and the concentration of sugar dropped after the second hours during hydrolysis (refer to Fig. 2).

To reduce the inhibition effect of sugar on enzyme cellulases, ultrafiltration membrane was applied in the second part of the experiment by means of continuously removing sugar from hydrolysis.

For the 1.5% dosage, the hydrolysis experiment (Fig. 3), which ultrafiltration started after two hours, the sugar concentration inside the reactor continuously increased significantly by two folds from 0.14 mg/ml to nearly 0.35 mg/ml due to the effective removal of sugar by the membrane, thus minimized the sugar inhibition on cellulases.

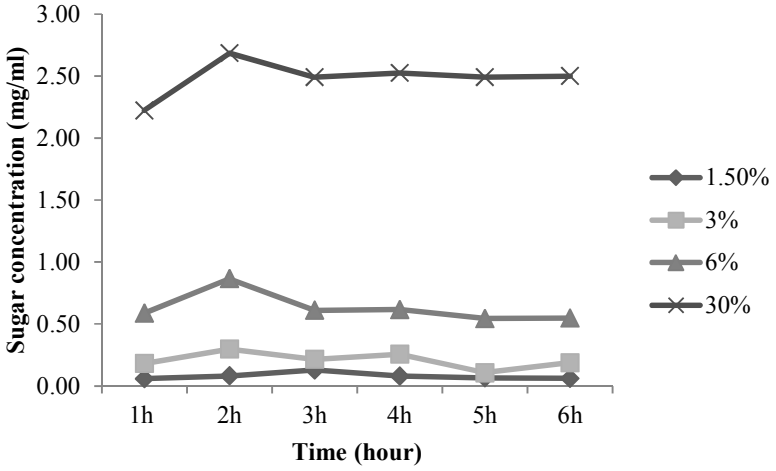

Fig. 2. Time profile of hydrolysis for different enzyme dosages without ultrafiltration.

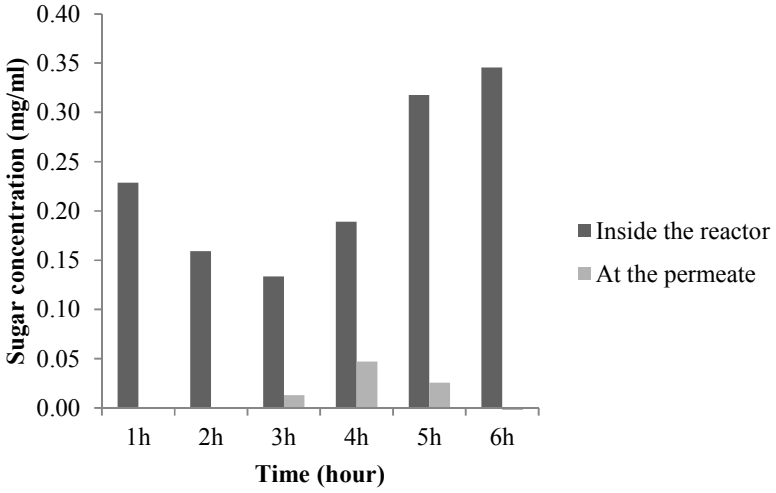

Fig. 3. Time profile of 1.5% dosage in hydrolysis with ultrafiltration and 3 ml/min rate of sugar removal.

The following hydrolysis with ultrafiltration at 3% and 6% enzyme dosages respectively are shown in Figs. 4, 5 and 6 with increasing rates of sugar removal. No reduction in sugar concentration occurred throughout the 6 hours experiments (Fig 5 and 6), except the case of 3% enzyme dosage with sugar removal rate at 3 ml/min, which there is a small reduction in glucose concentration (Fig. 4). A rapid increase was observed in the sugar concentration inside the reactor from nearly 2.5 mg/ml up to 9 mg/ml for 3% dosage with a flow rate controlled at 10 to 15 ml/min (Fig. 5). Although hydrolysis with 6% enzyme dosage (Fig.6) shows

the same trend with a moderate increase in sugar released at a higher removal rate of 30 ml/min, sugar concentration with 6% dosage at 3.5 mg/ml was actually lower more than half in comparison to that of 3% dosage hydrolysis at 9 mg/ml after six hours hydrolysis reactions. It was explained that when a certain permeate flux was reached due to a small amount of pretreated cellulose accumulated on the retentate side of the membrane and the loss of cellobiose as intermediate sugar before completely converting to glucose into permeate of membrane [6, 15]. In addition, considering the cases in Figs. 4 and 5, although the same enzyme dosage of 3% was applied on both, an increase in the rate of sugar removal from 3 ml/min to 10-15 ml/min led to the avoidance of glucose inhibition since there was no fluctuation or reduction in sugar concentration as can be seen in Fig. 5. A summary table to track the occurrence of inhibition of glucose on enzymes is shown in Table 1. It is also notified that the sugar concentration inside the reactor is different with that at the permeate of the membrane. At low to medium product removal flow rate from 3 to 15 ml/min, glucose concentration in the permeate is substantially lower than that inside the reactor (Figs. 3, 4 and 5). Whereas increasing the flow rate of product removal to a higher level of 30 ml/min (Fig. 6), the concentration of glucose at both sides of the membrane are nearly equal.

Table 1. Occurrence of glucose inhibition on enzymes at various dosages and sugar removal rate.

Enzyme dosage	Sugar concentration at 6h (mg/ml)	Rate of removing sugar	Sugar inhibition on enzymes
1.5%	13	3 ml/min	No
3%	**23.6**	**3 ml/min**	**Yes**
3%	**266.5**	**10-15 ml/min**	**No**
6%	105.2	30 ml/min	No

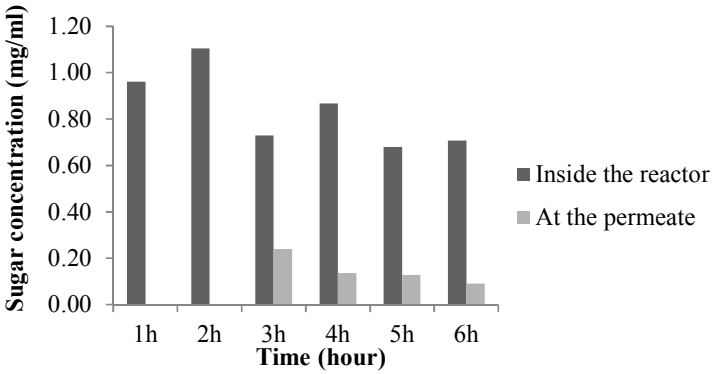

Fig. 4. Time profile of 3% dosage in hydrolysis with ultrafiltration and 3 ml/min rate of sugar removal.

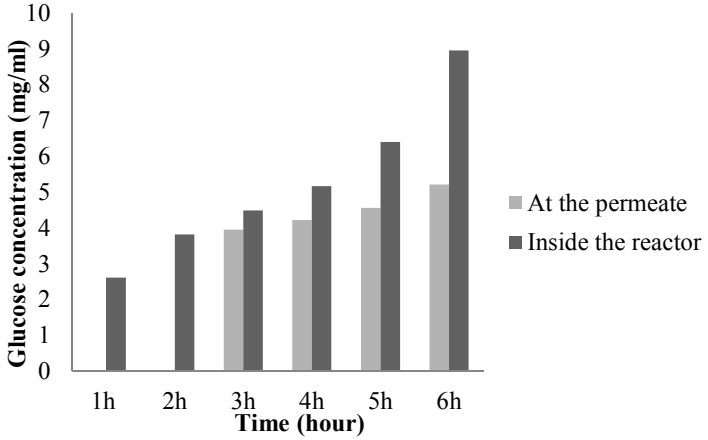

Fig. 5. Time profile of 3% dosage in hydrolysis with ultrafiltration and 10-15 ml/min rate of sugar removal.

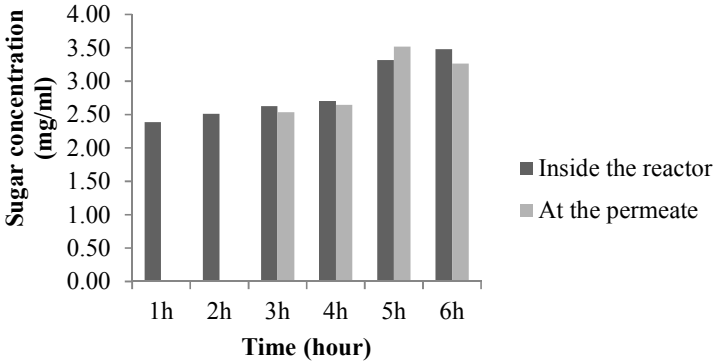

Fig. 6. Time profile of 6% dosage in hydrolysis with ultrafiltration and 30 ml/min rate of sugar removal.

From Table 1, the two experiments at 3% are in bold. The effectiveness of setting a suitable flow rate of removing the released sugar from hydrolysis reaction would probably help to prevent the inhibition effect, hence produce more sugar. In other words, the rate of sugar removal might become a deciding factor playing a key role in the occurrence of glucose inhibition. Thus, it is hypothesised that there could be existing a relationship between occurrence of inhibition, enzyme dosage, sugar concentration inside the hydrolysis reactor and the removal rate of released sugar by the ultrafiltration membrane. Enzyme cellulases have a function to increase the rate of hydrolysis at a specific enzyme loading/dosage, and the presence of glucose released from cellulose causes the inhibition on this enzyme. But only at a certain level of the concentration of sugar inside hydrolysis reactor can cause this inhibition, and the sugar removal rate should be sufficiently high to avoid inhibition as observed in case of 3% enzyme dosage, 10-15 ml/min sugar removal rate. If the rate of sugar removal is low, for example the case 3% dosage 3 ml/min as shown Table 1, concentration of sugar inside hydrolysis

reactor was still high enough to cause inhibition. Therefore, further study is suggested to look inside into this phenomenon in order to find out the relationship of these parameters (enzyme dosage, sugar concentration in hydrolysis and rate of removing sugar) and to determine a minimum rate of sugar removal to avoid inhibition of sugar on enzymes.

From Figs. 7, 8 and 9, a comparison was made for sugar concentration profile during 6 hours between with ultrafiltration (UF) and without ultrafiltration according to each enzyme dosage at 1.5%, 3% and 6% respectively. Clearly showing in graphs that in all cases, hydrolysis with ultrafiltration produced more sugar, or showed a very much higher sugar concentration without any reduction compared to that without ultrafiltration except for the case of 1.5% dosage which a small decrease occurred during first 2 hours, due to the continuous removal of sugar by the membrane and the ability to retain cellulases inside the reactor.

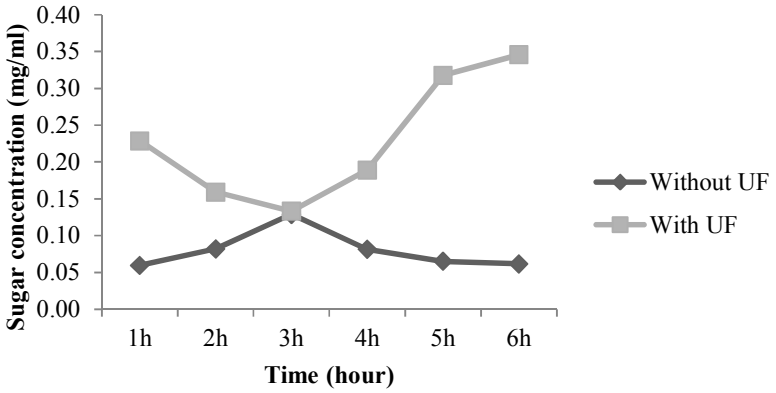

Fig. 7. Comparison of hydrolysis with and without ultrafiltration (UF) at 1.5% dosage.

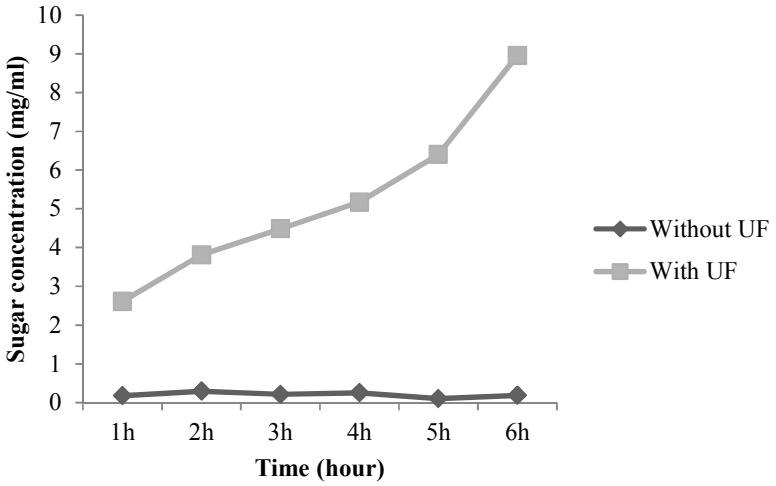

Fig. 8. Comparison of hydrolysis with and without UF at 3% dosage.

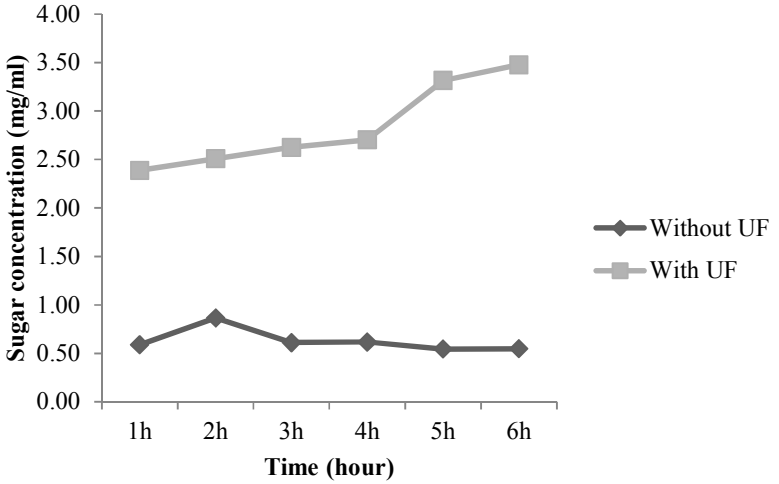

Fig. 9. Comparison of hydrolysis with and without UF at 6% dosage.

In these research experiments, among three enzyme dosage 1.5%, 3% and 6% used in hydrolysis with ultrafiltration, the 3% dosage is the optimal enzyme dosage, which always results in a relatively higher glucose concentration during 6 hours hydrolysis of pretreated cellulose compared to 1.5% and 6% dosage (Fig. 10). Therefore, the conditions applied in these experiments (alkaline-pretreated cellulose, hydrolysis at 45^0C and pH 5.0, under magnetic stirring, 10% solid loading and membrane ultrafiltration for sugar removal at flow rate 10-15 ml/min), was able to handle a low enzyme dosage of 3% as the optimal dosage giving the highest sugar concentration at almost 9 mg/ml compared to 1.5% and 6% at approximate 0.35 mg/ml and 3.5 mg/ml respectively after 6 hours.

Comparing the research on enzymatic hydrolysis of cellulose without application of membrane filtration conducted by Rabelo's group, the system could only handle a low solid loading at 3% to give 91% conversion of cellulose to sugar. A decrease by half in enzyme loading resulted in a moderate drop in glucose produced and cellulose conversion. When 20% solid loading with the same enzyme loading was applied, a significant drop in conversion from 97.9% to 44.4%. This substantial drop was due to the increase in sugar released from cellulose, but the presence of sugar in the hydrolysis reaction itself was an inhibition factor to affect enzyme cellulase activity subsequently [11,12]. Whereas hydrolysis in this experimental work, was able to handle high solid loading at 10%, with an enzyme dosage of 3% for producing a high concentration of sugar by continuously removing sugar product from hydrolysis to avoid inhibition on enzymes.

In comparison with the results of Abels's in 2013, in which membrane filtration was applied in hydrolysis, the effect of sugar inhibition was minimized to give constant concentration of sugar at permeate and retentate at both sides of the membrane to obtain 90% conversion but at very low solid loading (1% and 2%) and high enzyme loading (700 U/g for cellulase) with an addition of a filtration unit after the hydrolysis reactor [6], whereas for this research, the

ultrafiltration membrane was directly installed at the end of the tube connected with a peristaltic pump to fractionate sugar released from hydrolysis.

In this research work, there were still some errors and uncertainties during the experiments. Firstly cellulose loss occurred when the primary filtration after pretreatment using Whattman filter paper. However, the dosage of enzyme as well as the percentage of solid loading in hydrolysis for each experiment was totally based on the initial quantity of microcrystalline cellulose before undergoing pretreatment. Secondly the measurement of sugar concentration after every hour was carried out by using DNS method in the reaction of sugar and DNS reagent to give a brown to black color intensity measured by spectrophotometer. The darker the color is, the higher sugar is obtained in hydrolysis. During the sugar measurement step, the method did not give a stable reading, whereas the reading will drop slightly. Hence double measurements and an average reading was taken to obtain concordant readings. This can probably give some softs of inaccuracy in preliminary data obtained. For the very high sugar concentration like in the case of 3% and 6% dosages, resulted back color samples were obtained, a notice in spectrophotometer showed "out of the range of 540 nm wavelength". Hence dilution of the sample with dilution factor of 10 was used in order to take readings. However the dilution by water might result in slight inaccurate compared to original one during the pipetting of water for the dilution task.

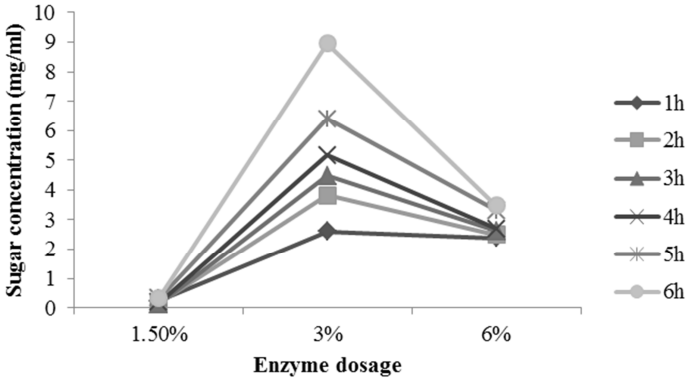

Fig. 10. Comparison of hydrolysis at dosages of 1.5%, 3% and 6% with UF.

Statistical analysis using T-test was applied to evaluate whether there is a significant difference in means, which is sugar concentration in both hydrolysis with and without ultrafiltration. From the resulted p values less than 0.05 or 5% shown in Table 2, it can be concluded that sugar concentration in hydrolysis with ultrafiltration is significantly different from that in hydrolysis without ultrafiltration.

Table 2. T-test for hydrolysis with and without ultrafiltration (UF).

Enzyme dosage	**1.5%**		**3%**		**6%**	
	Without UF	With UF	Without UF	With UF	Without UF	With UF
Mean	5.1323	9.5235	8.9058	157.0581	21.2918	86.2829
p value	0.007		0.003		0.000	

5. Conclusion

This work highlighted the suitability of applying ultrafiltration membrane in hydrolysis process to remove glucose product continuously, thus minimise the product inhibition on cellulases. The combination of industry-preferred alkaline 1M sodium hydroxide pretreated microcrystalline cellulose, hydrolysis using cellulases Cellic Ctec from Novozymes and the ultrafiltration membrane 10 kDa cutoff was effective to work together in a whole integrity. Based on the result, it is proved that concentration of sugar in hydrolysis with ultrafiltration was much higher than that in hydrolysis without filtration with insignificant reduction in sugar produced (1.5% dosage) and no sugar drop observed for 3% and 6% dosages. In addition, this method is able to handle low enzyme dosage (3% as optimum) to obtain the highest sugar concentration plus a high solid loading (10%) in hydrolysis. The significant difference of this research compared to others, is that the direct attachment of ultrafiltration membrane at the end of the tube connected with a pump to remove sugar produced in hydrolysis reactor, this method is potentially a new way of applying membranes to fractionate sugar in hydrolysis, and considered to be very effective to reduce product inhibition, increase conversion of cellulose to sugar, able to retain enzymes for reuse as a way of cost saving. A direct relationship between enzyme dosage and the rate of removing sugar was found. With a higher enzyme dosage, increasing sugar removal rate is necessary to ensure concentration of sugar inside reactor not sufficiently high to cause product inhibition on enzyme. Hence a further study on this relationship in order to clarify would be suggested for improvement.

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