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Optimization of enzymatic saccharification of palm oil mill effluent solid and oil palm fruit fibre to fermentable sugars

(Pengoptimuman pensakaridaan berenzim pepejal efluen kilang kelapa sawit dan serat kelapa sawit kepada gula boleh menapai)

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Key words: palm oil mill effluent, oil palm fruit fibre, cellulose, cellulose materials, enzymatic hydrolysis, fermentable sugar

Abstract

The effect of enzyme dosage and initial substrate concentration on the performance of enzymatic saccharification of palm oil mill effluent (POME) solid and oil palm fruit fibre (OPFF) was carried out in reactions using shake flask and 2-litre stirred tank reactor. The highest production of total reducing sugar and glucose from saccharification of POME and OPFF obtained using Novozyme/Celluclast (N/C ratio) were 0.4 and 0.25 at a dosage of 1 ml/g and 2 ml/g substrate, respectively. At this optimal condition, the highest production of reducing sugar (12.25 g/litre) and glucose (6.70 g/litre) was obtained when 150 g/litre POME solid was used, which gave the overall productivity and yield of 1.53 g/litre/h and 0.08 g/g, respectively. On the other hand, the saccharification of OPFF was optimal at 50 g/litre which produced 30.26 g/litre reducing sugar and 16.73 g/litre glucose, which corresponding to overall productivity of 0.28 g/litre/h and yield of 0.61 g/g.

Introduction

The bioconversion of plant biomass to liquid fuels gained global interest in recent years due to increase in the price of petroleum (a major source for liquid fuel production). The crucial step for conversion of biomass to liquid fuels is hydrolysis of lignocellulose to fermentable sugars. The hydrolysis can be carried out using either acid or cellulolytic enzymes. Acidic hydrolysis process has several disadvantages, such as: i) sugar products are further converted by acid to fermentation-inhibiting byproducts, ii) the process equipment needs to be resistant to corrosive action, and iii) the acid hydrolysis process uses high temperature and pressure, which demands more energy and high capital costs (Ladisch 1979).

On the other hand, enzymatic hydrolysis has the potential to produce fermentable sugar in large scale, since the process requires no special material in the equipment and can be performed under condition where energy consumption is comparatively low. However, the cost of enzymes normally constitutes about 80% of the total cost of enzymatic saccharification of lignocellulosic material to fermentable sugars (Zacchi et al. 1988).

The most important property of cellulase system in the saccharification of lignocellulosic material is the ability

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of cellubiohydrolase (FPase) to act synergistically with endoglucanase (CMCase) and β-glucosidase for the hydrolysis of crystalline cellulose to produce glucose. Since neither is effective on its own, therefore, the optimization of enzyme dosage for enzymatic saccharification process is necessary to obtain high yield of fermentable sugars at low enzyme consumption.

In addition, limitation of enzymatic saccharification is due to the presence of cellobiose in the reaction mixture, which accumulates during the reaction. The supplementation of cellulase with exogenously added β-glucosidase in order to increase the cellobiose hydrolysis has been used to overcome this problem (Bisset and Sternberg 1978).

The present study was carried out to determine the optimal enzymes and initial substrates concentrations for the enzymatic saccharification of palm oil mill effluent (POME) solid and oil palm fruit fibre (OPFF) to fermentable sugar in terms of yield and production rate.

Materials and methods

Enzymes

The cellulase from *Trichoderma reesei* (Celluclast 1.5L) and the cellobiose or β-glucosidase from *Aspergillus niger* (Novozyme 188) were used in the saccharification experiments. Both enzymes were obtained from NOVO Nordisk. The activity of these enzymes is shown in *Table 1*. Novozyme 188 has higher proportion of β-glucosidase than Celluclast 1.5L.

Substrate preparation

Palm oil mill effluent (POME) solid and oil palm fruit fibre (OPFF), obtained from Bukit Raja Palm Oil Mill in Klang, Selangor, Malaysia, were used as substrate for enzymatic saccharification experiment. POME was centrifuged at 3000 rpm for 20 min to obtain solid POME. The OPFF was reduced to an average of 2 mm fibre using Waring blender followed by grinding using hammer mill equipped with 2 mm round hole screens (Janke and Kunkel, IKA-Labortechnik, Staufen). The milled OPFF was collected by gravity drop after passing the particles through a 2 mm mesh.

Substrate pretreatment

From the previous study (Khaw 2002), it was found that pretreatment with NaOH was the most suitable to modify the OPFF so that it is accessible for enzymatic saccharification. However, chemical pretreatment did not give any significant effect on the saccharification of POME solid. Therefore, OPFF that was pretreated with 2% NaOH, autoclaved at 121 °C, 15 psi for 5 min and chemically untreated POME solid were used in this study.

Saccharification experiment: *Effect of enzyme and initial substrate concentrations*

The saccharification of POME solid and OPFF was preliminary carried out in 200 ml shake flask, incubated at 40 °C and agitated at 200 rotations/min. An amount corresponding to 5 g of oven-dried substrate was added to 0.05 M sodium acetate buffer (pH 5.0). In the first stage, the enzyme Celluclast 1.5L was added at an amount

Table 1. Enzyme activities in Celluclast and Novozyme preparation

Enzymes	FPase (cellobiohydrolase, EC 3.2.1.91) (U/ml)	CMCase (endo-1,4-β-glucanase, EC 3.2.1.4) (U/ml)	β-glucosidase (β-1,4-glucanase, EC 3.2.1.21) (U/ml)
Celluclast 1.5L	47.4	66.0	51.1
Novozyme 188	2.79	10.0	168.0

corresponding to the desired enzyme concentration based on oven-dried substrate. The volume was made up to 100 ml using the same buffer. Sodium azide (0.02% w/v) was also added to the reaction mixture to avoid bacterial contamination.

In the second stage, the effect of using a combination of Novozyme 188 and Celluclast 1.5L on the performance of saccharification was investigated. In this set of experiment, the amount of Celluclast 1.5L was fixed at 1 and 2 ml/g substrate for saccharification of POME solid and OPFF, respectively. The amount of Novozyme 188 was varied according to the requirement of each experiment. The control experiments, saccharification using only Celluclast 1.5L at optimum concentration without the addition of Novozyme 188, were also conducted.

The effect of initial substrate concentration on saccharification of POME solid and OPFF was carried out in 2-litre stirred tank reactor with a working volume of 1.5 litres. The reactor was agitated at 200 rotations/min and temperature within the reactor was controlled at 40 °C. The substrate concentration of POME solid and OPFF was varied from 2–20% (w/v) and 1–6% (w/v), respectively.

Analytical procedures

The sample (2 ml) from the saccharification was withdrawn at time intervals of 15–20 h for analysis. The samples were centrifuged for 10 min at 13,000 rotations/min, and the supernatant was used for the determination of reducing sugar, glucose and cellobiose concentration.

Reducing sugar was determined using dinitrosalicylic acid (DNS) method (Miller 1959). Glucose concentration was determined using Sigma Diagnostics Glucose (Trinder) reagent. Cellobiose and glucose were determined using CostaMetric 3000, high-pressure liquid chromatography (HPLC) (LDC Analytical, Florida) with a refractive index detector according to method as described by Bonn and Bobleter (1984) with some modifications. In the HPLC method, the mobile phase was a mixture of acetonitrile and water in a ratio of 80:20, and the flow rate was 1.0 ml/min at room temperature. The stationary phase was a prepacked Merck NH-2 column.

Results and discussion

Effect of enzyme concentration on saccharification of POME solid

Basically, the hydrolysis of POME solid followed the typical trend of cellulose saccharification, which was characterized by an initial logarithmic phase, associated with the rapid release of soluble sugars and then followed by a declining rate of sugar production as the reaction proceeds (*Figure 1*).

Increase in the rate of saccharification and sugar was obtained with increasing Celluclast concentration from 0.2–1 ml/g substrate. However, the use of enzyme

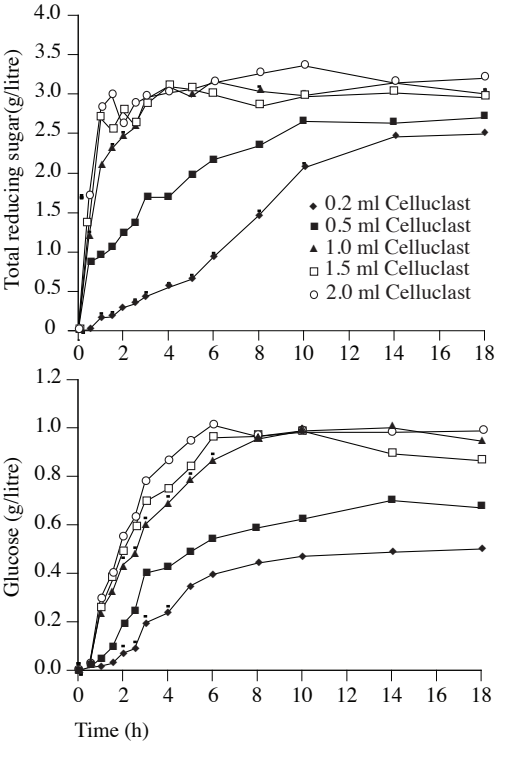

Figure 1. Effect of different cellulase (Celluclast) concentration on the performance of saccharification of POME solid

Table 2. Reducing sugar and glucose production from saccharification of POME solid and OPFF carried out in shake flask using different enzyme (Celluclast 1.5L) concentrations

Substrate	Enzyme concentration		Reducing sugar (g/litre)	Glucose (g/litre)	Productivity (g/litre/h)	Enzymatic consumption (FPU/g sugars)
	(ml/g substrate)	(FPU/litre)				
POME solid	0.2	474	2.49a	0.50a	0.14	190.36
	0.5	1185	2.70a	0.70b	0.15	438.89
	1.0	2370	3.16b	1.00c	0.53	750.00
	1.5	3555	3.10b	0.99c	0.52	1146.77
	2.0	4740	3.36b	1.01c	0.34	1410.71
OPFF	0.5	1185	15.91a	6.45a	0.20	74.48
	1.0	2370	17.15b	6.88a	0.22	138.19
	2.0	4740	19.13c	8.13b	0.29	247.78
	3.0	7111	19.53c	8.11b	0.25	364.11
	4.0	9480	20.14c	8.14b	0.26	470.71

FPU = Filter Paper Unit
Mean values in the same row not followed by the same letter are significantly different (p <0.05)

concentration above 1 ml/g substrate did not improve the rate of saccharification or sugar production. In addition, the enzyme consumption for saccharification of POME solid increased considerably when the enzyme concentration was high (*Table 2*). Among the enzyme concentration used for saccharification of POME solid, the highest productivity was obtained at 1 ml/g substrate; indicating that this was the optimal in terms of productivity and enzyme consumption for enzymatic saccharification of POME solid.

Effect of enzyme concentration on saccharification of OPFF

Similar pattern to saccharification of POME solid was also observed for OPFF, where the sugar production increased rapidly in the begining of the saccharification process and the rate of production decreased slowly towards the end of the process (*Figure 2*). Generally, increase in saccharification rate and sugar production was found proportional with increasing enzyme concentration up to 2.0 ml/g substrate. When the enzyme concentration was further increased to above 2 ml/g substrate, saturation of enzyme loading was observed.

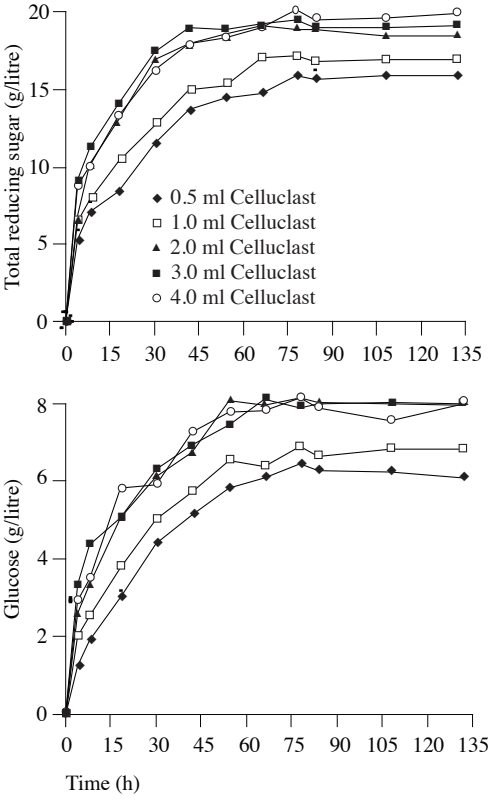

Figure 2. Effect of different cellulase (Celluclast) concentration on the performance of saccharification of OPFF

The highest productivity of reducing sugar was obtained from the saccharification of OPFF using 2 ml/g substrate (*Table 2*). The profile of enzyme consumption for saccharification of OPFF was similar to POME solid, where the enzyme consumption increased with increasing enzyme concentration. However, the activity of enzyme (FPU/litre) needed to produce 1 g of reducing sugar for OPFF was lower compared to POME solid. The high crystallinity and low surface area in POME solid might be the explanation for the requirement of higher enzyme concentration for complete hydrolysis.

This result suggests that the structural features for POME solid and OPFF was significantly different even though both are from the same sources. Study on enzymatic saccharification of oil palm trunk by Ishihara et al. (1991) showed that different portions of the trunk would have different susceptibility to enzymatic attack. Ability of the substrate to adsorp enzyme is another important parameters that determined the rate of cellulose hydrolysis (Gregg and Saddler 1996; Sun and Cheng 2002).

One of the limiting factors restricting the effective and efficient saccharification of lignocellulosic materials is the recalcitrance of the substrate following pretreatment. Consequently, the enzymatic process requires relatively high enzyme loadings to produce fermentable sugars. Mais et al. (2002) proposed the use of a simultaneous physical and enzymatic treatment to circumvent the need for larger enzyme loading. Almost 100% hydrolysis of lignocellulosic substrate could be achieved with minimum enzyme loading in reaction using a ball-mill reactor.

Effect of the addition of β*-glucosidase on saccharification of POME solid*

From the previous study, the optimum concentration of Celluclast, used as a sole cellulase enzyme, for the saccharification of POME solid and OPFF were 1 and 2 ml/g substrate, respectively (Khaw 2002). This cellulase preparation contained low level of β-glucosidase. The effect of the addition of different amount of Novozyme, which contains high level of β-glucosidase, to Celluclast (at optimal concentration) on the performance of the saccharification of POME is shown in *Figure 3*.

In all cases, the production of reducing sugar and glucose levelled off at approximately the same time (after 1 h), but at different levels based on the Novozyme concentration. The maximum concentration of glucose was achieved after about 6 h. Generally, glucose concentration increased with increasing Novozyme concentration

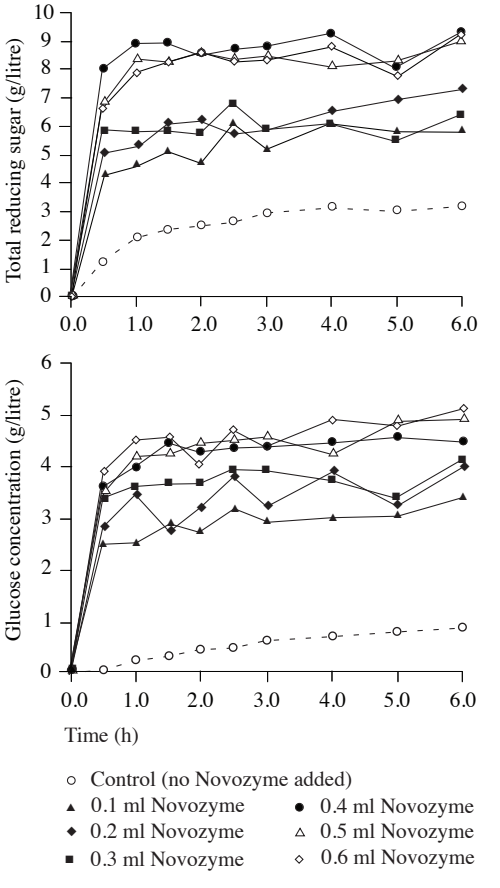

Figure 3. Effect of increasing amount of β*-glucosidase (Novozyme) into cellulase enzyme preparation with low level of* β*-glucosidase (Celluclast) on the performance of saccharification of POME solid*

from 0.1 to 0.3 ml/g substrate. However, further increase in the enzyme concentration (0.4–0.6 ml/g substrate) did not significantly improve the glucose yield or glucose production rate.

This result indicates that Novozyme concentration at 0.4 ml/g substrate was optimal for the saccharification of POME solid. Among the enzyme concentration investigated, the addition of 0.4 ml Novozyme/g substrate gave the highest glucose production (*Table 3*). Thus, the Novozyme/Celluclast (N/C) ratio of 0.4 was chosen as an optimal concentration for subsequent experiment in saccharification of POME solid.

Effect of the addition of ß-glucosidase on saccharification of OPFF

The effect of the addition of Novozyme (contain high level β-glucosidase) to Celluclast (containing low level of β-glucosidase) on the saccharification of OPFF is shown in *Figure 4.* The profile of sugar production during saccharification of OPFF was almost similar to the trend of POME solid saccharification, where the glucose was produced rapidly in the first 15 h and declined gradually towards the end of the process. The highest glucose production rate and optimal glucose concentration was obtained in experiment OPFF 2 where N/C ratio of 0.25 was used (*Table 3*). As shown in *Figure 4,* glucose production during saccharification process with the addition of Novozyme was nearly two times higher than the reaction without Novozyme (control).

The trend of cellobiose production during the saccharification of POME solid and OPFF is shown in *Figures 5* and *6*. The cellobiose concentration for control (no Novozyme added) was higher than the saccharification with the addition of Novozyme. The improvement of saccharification for POME solid and OPFF using Novozyme can be explained by the hydrolysis of cellobiose, which is known to be an inhibitor for cellulase complex.

Celluclast contains only small amounts of β-glucosidase, which are not sufficient to convert the cellobiose at the same rate as it is produced. Therefore, exogenously added β-glucosidase is necesssary to circumvent the inhibitory effect of cellobiose accumulation by enhancing the conversion of cellobiose to glucose. Similar results

Table 3. Reducing sugar and glucose production from saccharification of POME solid and OPFF in shake flask fermentation using different combinations of Novozyme 188 and Celluclast 1.5L

Experiment	Novozyme (ml/g substrate)	Celluclast (ml/g substrate)	Novozyme/ Celluclast (N/C) Ratio	Total reducing sugar (g/l)	Glucose (g/l)	Glucose production rate (g/l/h)
POME 1	0.1	1	0.10	6.17b	3.42b	0.57
POME 2	0.2	1	0.20	7.33c	3.98b	0.66
POME 3	0.3	1	0.30	6.80c	4.10b	0.68
POME 4	0.4	1	0.40	9.24d	4.54c	0.91
POME 5	0.5	1	0.50	9.10d	4.64c	0.82
POME 6	0.6	1	0.60	9.26c	5.11d	0.85
POME Control	–	1	–	3.16a	0.867a	0.14
OPFF 1	0.1	2	0.05	30.69b	13.84b	0.10
OPFF 2	0.5	2	0.25	32.47b	16.78c	0.16
OPFF 3	1.0	2	0.50	32.14b	17.34c	0.13
OPFF 4	1.5	2	0.75	34.30b	17.24c	015
OPFF 5	2.0	2	1.00	34.75b	19.31d	0.15
OPFF Control	–	2	–	19.13a	8.13a	0.10

Mean values in the same row not followed by the same letter are significantly different (p <0.05)

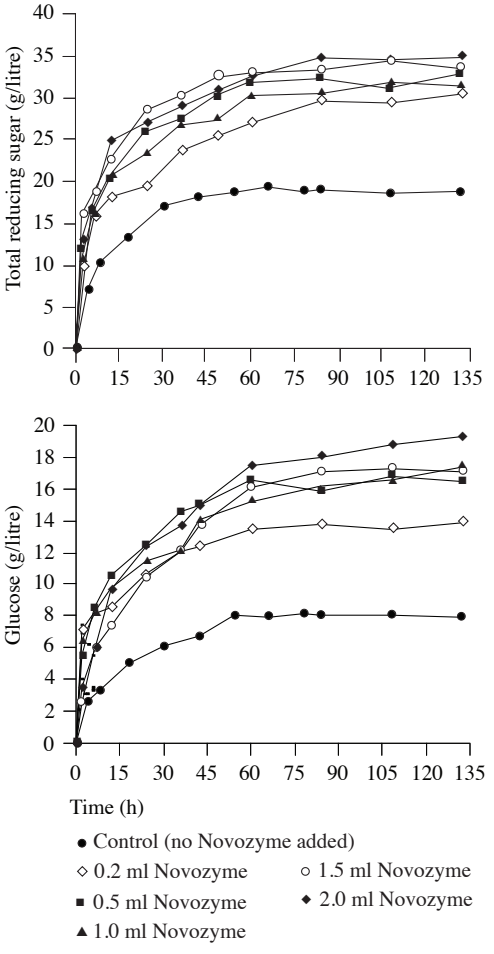

Figure 4. Effect of increasing amount of β-glucosidase (Novozyme) into cellulase enzyme preparation with low level of β-glucosidase (Celluclast) on the performance of saccharification of OPFF

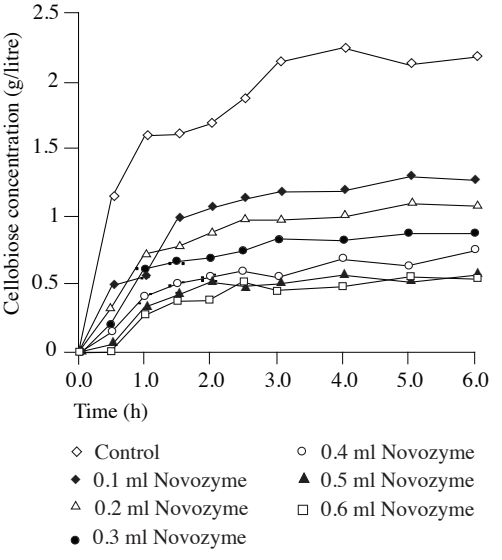

Figure 5. Profile of cellobiose production during the saccharification of POME solid using different Novozyme concentrations

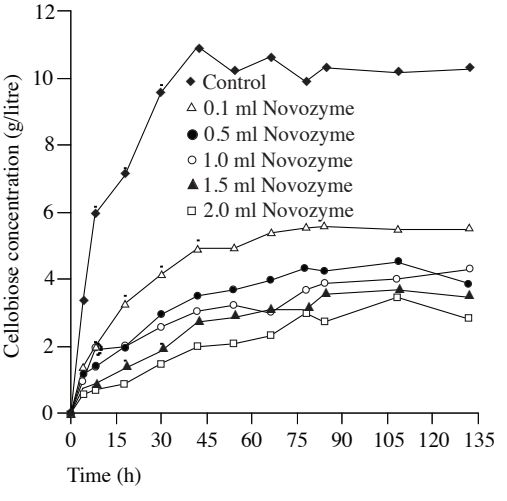

Figure 6. Profile of cellobiose production during the saccharification of OPFF using different Novozyme concentrations

have also been reported for saccharification of steam-pretreated willow (Robert et al. 1990). Different rates of hydrolysis during degradation of lignocellulosic materials was also dependent on the individual enzyme performance from different microbial sources as well as their synergistic actions (van Wyk 1999).

Effect of initial substrate concentration on saccharification of POME solid

Different initial concentrations of POME solid (2, 5, 10, 15 and 20% w/v) and OPFF (2, 3, 4, 5 and 6% w/v) on saccharification process were used. In all cases, reducing sugar production reached a maximum concentration after about 1 h and 60 h of saccharification time for POME solid and OPFF, respectively. On the other hand, accumulation of glucose in saccharification of POME solid and OPFF only achieved maximum after about 6–8 h and 40–60 h, respectively.

The amounts of reducing sugar and glucose produced increased almost linearly with increasing initial concentration of OPFF. However, this was not the case for POME solid, where the concentrations of reducing sugar and glucose increased linearly only at concentration below 15% w/v. The production of reducing sugar and glucose remained rather constant for the POME solid at concentration above 15% w/v.

The data of reducing sugar and glucose produced from the saccharification of POME solid and OPFF are shown in *Table 4*. The production of reducing sugar and glucose increased with increasing POME solid concentration. This result indicates that the POME solid concentration at 200 g/litre seemed to be optimal for saccharification process. However, the use of POME solid concentration at 200 g/litre was not possible for the enzymatic saccharifications in the stirred tank reactor due to its high viscosity, where perfect mixing could not be obtained and sampling was difficult. Thus, POME solid at the concentration of 150 g/litre which gave comparable sugar productivity and yield was suggested as optimal concentration for the process.

Effect of initial substrate concentration on saccharification of OPFF

In the case of OPFF saccharification, the reducing sugar and glucose were also found to increase linearly with increasing substrate concentration. However, this was not true for sugar productivity and yield, where the highest sugar productivity and yield were obtained from saccharification at 50 g/litre OPFF, rather than 60 g/litre. Therefore, the OPFF concentration at 50 g/litre was

Table 4. Reducing sugar and glucose production from saccharifictaion of POME soild and OPFF carried out in 2-litre stirred tank reactor using different substrate concentrations

Substrate	Substrate concentration (g/litre)	Reducing sugar (g/litre)	Glucose (g/litre)	Overall productivity (g/litre/h)	Sugars yield of sugars (g/g)
POME	20	2.14a	1.73a	0.43	0.11
Solid	50	3.84b	2.83b	0.55	0.08
	100	5.37c	4.47c	0.67	0.05
	150	12.25d	6.70d	1.53	0.08
	200	13.31d	7.10d	1.66	0.07
OPFF	20	7.90a	6.52a	0.07	0.40
	30	11.60b	8.08b	0.4	0.39
	40	20.20c	11.50c	0.17	0.51
	50	30.26d	16.73d	0.28	0.61
	60	32.50d	16.74d	0.27	0.54

Mean values in the same row not followed by the same letter are significantly different (p <0.05)

chosen as an optimal concentration for the enzymatic hydrolysis of OPFF, which gave the glucose production rate of 0.28 g/litre/h.

Further improvement of the hydrolysis may be achieved by the pretreatment of lignocellulosic materials with high temperature steam and different modes of hydrolysis. For example, glucose production rate from the fed-batch hydrolysis of SO_2-impregnated steam exploded *Eucalyptus viminalis* was 1.08 g/litre/h (Ramos and Saddler 1994). Significant improvement of Salix, lignocellulosic materials, by steam pretreatment using H_2SO_4 has also been reported (Sassner et al. 2008).

Conclusion

Enzyme dosage and substrate concentration were identified as important factors that affect the performance of saccharification of POME solid and OPFF. Enhancement of reducing sugar and glucose production from saccharification of POME and OPEFB can be achieved using a combination of cellulase enzymes with high proportion of β-glucosidase. Very high POME solid concentration (150 g/litre) was found optimal for the saccharification process as compared to OPFF (50 g/litre OPFF). However, the yield obtained from saccharification of POME was very low (0.08 g sugar/g substrate) as compared to OPFF, where the yield was 0.61 g sugar/g substrate.

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Abstrak

Kesan dos enzim dan kepekaan substrat permulaan terhadap prestasi pensakaridaan berenzim pepejal efluen kilang kelapa sawit (POME) dan serat buah kelapa sawit (OPFF) dijalankan menggunakan kelalang dan tangki reaktor berkacau 2 liter. Pengeluaran tertinggi bagi jumlah gula penurun dan glukosa daripada pensakaridaan POME dan OPFF diperoleh dengan mengggunakan Novozyme/Celluclast (nisbah N/C ialah 0.4/0.25) masing-masing pada dos substrat 1 ml/g dan 2 ml/g. Pada keadaan optimum ini, hasil tertinggi penurunan gula (12.25 g/liter) dan glukosa (6.70 g/liter) diperoleh apabila 150 g/liter pepejal POME digunakan, memberi produktiviti dan hasil keseluruhan masing-masing 1.53 g/jam/liter dan 0.08 g/g. Manakala, pensakaridaan OPFF adalah optimum pada 50 g/liter yang menghasilkan 30.26 g/liter gula penurun dan 16.73 g/liter glukosa yang sepadan dengan produktiviti keseluruhan 0.28 g/liter dan hasil 0.61 g/g.

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