Bioresource Technology 232 (2017) 126–132

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Optimization of simultaneous saccharification and fermentation conditions with amphipathic lignin derivatives for concentrated bioethanol production

Ningning Cheng [a], Keiichi Koda [b], Yutaka Tamai [b], Yoko Yamamoto [a], Taichi E. Takasuka [b,c,*], Yasumitsu Uraki [b,*]

[a] Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
[b] Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
[c] Global Institute for Collaborative Research and Education, Hokkaido University, Sapporo 060-8589, Japan

HIGHLIGHTS

- AL-Ds accelerate the bioethanol production on large scale fed-batch SSF.
- Various parameters for small and large scale fed-batch SSF were highly optimized.
- 30% high substrate loading was achieved by using a custom jar-fermenter.
- Lignocellulosic bioethanol fermentation reached high concentration of 87.9 g/L.

ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form 3 February 2017
Accepted 4 February 2017
Available online 8 February 2017

Keywords:
Amphipathic lignin derivative
Bioethanol
Enzymatic saccharification
Fed-batch simultaneous saccharification and fermentation (SSF)
Powerful mechanical stirrer

ABSTRACT

Amphipathic lignin derivatives (A-LDs) prepared from the black liquor of soda pulping of Japanese cedar are strong accelerators for bioethanol production under a fed-batch simultaneous enzymatic saccharification and fermentation (SSF) process. To improve the bioethanol production concentration, conditions such as reaction temperature, stirring program, and A-LDs loadings were optimized in both small scale and large scale fed-batch SSF. The fed-batch SSF in the presence of 3.0 g/L A-LDs at 38 °C gave the maximum ethanol production and a high enzyme recovery rate. Furthermore, a jar-fermenter equipped with a powerful mechanical stirrer was designed for 1.5 L-scale fed-batch SSF to achieve rigorous mixing during high substrate loading. Finally, the 1.5 L fed-batch SSF with a substrate loading of 30% (w/v) produced a high ethanol concentration of 87.9 g/L in the presence of A-LDs under optimized conditions.

1. Introduction

Bioethanol production is considered to be a clean and sustainable energy technology, which could reduce the current huge demand on crude oil and to alleviate various environmental damages (Sarkar et al., 2012). Lignocellulosic biomass is the most promising feedstock for bioethanol production because of its abundance in the terrestrial environment (Olofsson et al., 2008). The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin, which are very difficult to break down into the monomeric subunits necessary for biofuels production (Aysu and Durak, 2015). The conversion of lignocellulose to bioethanol can be achieved by the following four steps; a physico-chemical pretreatment (Alvira et al., 2010; Uppugundla et al., 2014), saccharification (Kumar and Murthy, 2013), fermentation, and distillation (Balat et al., 2008; Phillips et al., 2013). For the saccharification of polysaccharides from lignocellulosic biomass, enzymatic hydrolysis is more commonly used than acid hydrolysis due to the fact that enzymatic hydrolysis can be carried out under milder conditions, and does not require specialized reaction vessels (Sun and Cheng, 2002). A major drawback to enzymatic hydrolysis is the high cost of cellulolytic enzyme cocktails, which contain combinations of cellulases, hemicellulases and other enzymes with different substrate reactivities (Nguyen and Saddler, 1991). Thus, to achieve a cost effective enzymatic saccharification, it is necessary to improve

* Corresponding authors at: Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan (T.E. Takasuka and Y. Uraki).
E-mail addresses: takasuka@cen.agr.hokudai.ac.jp (T.E. Takasuka), uraki@for.agr.hokudai.ac.jp (Y. Uraki).

http://dx.doi.org/10.1016/j.biortech.2017.02.018
0960-8524/

stability of cellulolytic enzyme activities, and maximize activity over a prolonged reaction time in multiple rounds of saccharification (Deshpande and Eriksson, 1984). In conventional cocktails, activities of enzymes decrease during saccharification of lignocellulose due in part to non-productive binding between enzymes and cellulose, and non-specific hydrophobic interaction between enzymes and lignin in lignocellulose (Eriksson et al., 2002). Thus, for efficient enzyme hydrolysis of lignocellulosic biomass, it is essential to reduce both non-productive binding and non-specific hydrophobic interaction of enzymes to the biomass substrate. Toward this end, additives such as polymer compounds, nonionic surfactants, and non-catalytic proteins have been shown to improve enzymatic saccharification (Borjesson et al., 2007; Cai et al., 2016; Eriksson et al., 2002; Lin et al., 2015; Lou et al., 2013). Previously, we reported that a surfactant made from lignin and polyethylene glycol (PEG), called amphipathic lignin derivatives (A-LDs), when added to the reaction improved enzymatic saccharification (Uraki et al., 2001). A-LDs were shown to maintain high enzyme activity during the process and also significantly improved the recovery of enzymes after saccharification (Uraki et al., 2001; Winarni et al., 2014, 2013).

In addition to non-productive substrate and lignin binding, end-product inhibition is a considerable problem to efficient enzymatic saccharification. During saccharification, end products such as glucose, cellobiose, and other simple sugars can inhibit enzyme activity (Alfani et al., 2000). To overcome end-product inhibition, simultaneous saccharification and fermentation (SSF) methods can be employed, which is a combination of both enzymatic hydrolysis and fermentation in one reaction (Olofsson et al., 2008). In SSF, end product inhibition is averted during the process, since generated sugars are spontaneously utilized by the fermenter organism to produce ethanol.

In our recent study, we performed fed-batch SSF with two different A-LDs derived from Japanese ceder, DOPEG-SL and EPEG-SL, which reduced enzyme adsorption and end-product inhibition, improving bioethanol production (Cheng et al., 2014). The maximum ethanol concentration was increased from 3% to 5% in 100 mL experimental vessels and the production efficiency based on the theoretical yield was increased from 40.7% to 64.1% with the addition of A-LDs. However, obtained bioethanol concentration of less than 5% is not high enough to achieve a cost effective SSF, since following distillation step requires high cost (Dias et al., 2011). Higher substrate loading is one logical way to improve bioethanol concentration. For example, it was reported that by using significantly high waste-paper loading (65% w/v) in the 5 L-scale fed-batch SSF, the ethanol yield was achieved to 11.6% (v/v) (Elliston et al., 2013).

In this study, the reaction conditions of the fed-batch SSF were optimized by varying A-LDs concentration, and reaction temperature to maximize the ethanol yield. To control agitation speed and pulp loading, a big jar fermenter (3 L) equipped with a strong mechanical stirrer was designed and manufactured. Finally, the optimized conditions gave 87.9 g/L ethanol concentration in the 1.5 L-scale fed-batch SSF, which was much higher than our previous result (49.4 g/L) (Cheng et al., 2014) using A-LDs.

2. Materials and methods

2.1. Materials

Softwood unbleached kraft pulp (NUKP) was provided by Nippon Paper Industries Co., Ltd. (Tokyo, Japan). The pulp is comprised of 87.8% cellulose, 3.4% mannan, 5.3% xylan, and 2.1% Klason lignin. Commercially available cellulases, Meicelase (power form; Meiji Seika Co. Ltd., Tokyo, Japan) and Genencor GC220 (liquid form; Genencor International Inc., USA; Lot # 4901121718) were used. Japanese Sake Yeast (Mauri Yeast Australia Pty Ltd., Queensland, Australia), *Saccharomyces cerevisiae*, was used as a fermenter microorganism for bioethanol fermentation. Soda lignin (SL) was obtained from the spent liquor of soda-anthraquinone pulping of Japanese cedar chips by acidic precipitation followed by filtration. Two kinds of A-LDs were prepared by the reaction of SL with dodecyloxy poly(ethylene glycol) glycidyl ether (DOPEG) and ethoxy-(2-hydroxy) propoxy poly(ethylene glycol) glycidyl ether (EPEG) to yield DOPEG-SL and EPEG-SL, respectively (Cheng et al., 2014).

2.2. NUKP saccharification with cellulase cocktails

10 filter paper units (FPU) of cellulase, GC220 and Meicelase were separately dissolved in 100 mL of citrate buffer (0.05 M, pH 4.8) with 0.1 g DOPEG-SL. Then, 1.0 g of substrate was added to the solutions. The suspension was shaken at 50 °C for 48 h. After the saccharification, the suspension was filtered through a G4 glass filter (Sansyo, Tokyo, Japan), and the precipitants were weighed after complete drying in an oven at 105 °C. All the experiments were performed in duplicate. The sugar yield (%) and residual enzyme activity (%) were measured according to the previous study (Cheng et al., 2014).

2.3. Glucose fermentation with Japanese Sake yeast

0.4 g of dry Japanese Sake Yeast powder was pre-incubated for 2 days at 38 °C in yeast extract-peptone (YP) medium [10 g/L of yeast extract and 20 g/L of peptone in 0.05 M of citrate buffer (pH 4.8)]. Cells were collected by centrifugation for 10 min at 2500 rpm. The pre-incubated yeast cells were mixed with 40 g of glucose in 100 mL of YP medium. The mixture in YP medium was gently shaken for 144 h at 20, 25, 28, 30, 35, 38 and 42 °C to determine the optimum temperature of fermentation. An aliquot was taken out every day, and ethanol concentration was measured by GC (Shimadzu-GC2010, Shimadzu, Kyoto, Japan) with a capillary column (0.25 mm i.d. ×30 m; TC-WAX, GL Science, Tokyo, Japan). For the 100 mL and 1.5 L batch-SSF, 0.4 g and 6.0 g equivalent of pre-incubated yeast was supplemented, respectively.

2.4. 100-mL scale fed-batch SSF

The reaction medium consisted of 60 FPU Meicelase mixed with DOPEG-SL at a concentration of 0–5.0 g/L in 90 mL of citrate buffer (0.05 M, pH 4.8) prior to pre-hydrolysis. In the pre-hydrolysis process, the initial substrate loading (air dried NUKP) was 3 g in the medium, and the mixture was horizontally shaken with an orbital shaker (OSI-50L, Co., Ltd., Tokyo, Japan) at 100 rpm for 12 h at 50 °C. After cooling, the medium to 25, 35, 36.5, 38 or 40 °C, and inoculated with 10 mL in 10X YP medium [100 g/L of yeast extract and 200 g/L of peptone in 0.05 M of citrate buffer (pH 4.8)]. Total volume of the reaction was 100 mL.

During the SSF process, additional substrate and Meicelase were intermittently added to the SSF media according to the charging program indicated as a dashed line and an arrow, respectively, in Fig. 1. The ratio of enzyme to substrate was maintained at 10 FPU/g of substrate throughout the 144 h process. The final substrate loading after the whole process was 18 g (3 g × 6 times), and the total enzyme loading was 180 FPU (60 FPU × 3 times). An aliquot of incubated media was sampled out every day, and the ethanol concentration was measured by GC. These fed-batch SSF experiments in flasks were carried out in duplicate and the resulting ethanol concentration is shown as the average value. The ethanol conversion (%) is a produced total ethanol divided by the theoretical maximum ethanol production estimated using the following equation:

$$[EtOH]_{produced} = [EtOH]_t - [EtOH]_o$$

$$\text{Bioethanol conversion } (\%) = \frac{[EtOH]_{produced}}{0.51([W] \times f \times 1.11)} \times 100\%$$

where $[EtOH]_{produced}$ is the ethanol concentration generated by the fermentation, $[EtOH]_t$ is ethanol concentration at the time (t) of the fermentation (g/L), $[EtOH]_o$ is ethanol concentration at the beginning of the fermentation (g/L), [W] is the weight consistency of total dry pulp in SSF, f is a proportion of cellulose fraction of dry biomass (g/g; 0.878 in this study) and 0.51 is a conversion factor for glucose to ethanol based on stoichiometric fermentation of yeast (Hayward et al., 1995).

2.5. 1.5 L-scale of fed-batch type SSF

Large scale SSF was performed in a custom-made jar fermenter, equipped with strong mechanical stirrer and two impellers. The speed of the stirrer was controlled by the combination with a gear head motor (GFV4G15, Oriental Motor Co., Ltd., Japan) and a controller (BMUD60-A2, Oriental Motor Co., Ltd., Tokyo, Japan). The jar fermenter was sterilized with 75% (v/v) ethanol before use.

Cellulase (Meicelase or GC220; 1200 or 1500 FPU) in 1.35 L of citrate buffer mentioned above was poured into the jar fermenter together with A-LDs at concentrations of 0–5.0 g/L, and the jar fermenter was heated to 50 °C. Forty grams of substrate was added to this medium for pre-hydrolysis process. After 12 h, the jar fermenter was cooled to 38 °C, and pre-incubated yeast (6 g dry yeast) in 150 mL of 10XYP medium was poured into the fermenter medium. Total volume of the media was 1.5 L.

During SSF process, substrate and cellulase (Mecelase or GC220) were intermittently added to the SSF media according to the charging program indicated as a dashed line and an arrow, respectively, in Figs. 2, 3, 4 and 5. The ratio of the enzyme to substrate was maintained at 10 FPU/g throughout the process. The final substrate loading was 360 or 450 g (40 or 50 g × 9 times), and the total enzyme loading was 3600 or 4500 FPU (1200 or 1500 FPU × 3 times). The stirring conditions in the whole process are listed in Table 1. Ethanol concentration during the SSF process was monitored by GC as mentioned above.

3. Results and discussion

3.1. Determination of optimum temperature for the 100 mL fed-batch SSF with DOPEG-SL

Because optimum temperatures of enzymatic saccharification and fermentation are different, we looked at ethanol concentration after Japanese Sake yeast fermentation using glucose as a substrate at temperature ranging from 20 to 42 °C. The maximum ethanol concentration at 20, 25, 28, 30, 35, 38 and 42 °C was 56, 102, 111, 115, 151, 134 and 2 g/L, respectively. Thus, higher ethanol concentrations were obtained at 35 and 38 °C. These temperatures are slightly higher than the optimum yeast growth temperature of 30 °C (Aldiguier et al., 2004) and lower than the optimum temperature for cellulase activity of 50–60 °C (Zambare et al., 2011).

Table 1
Stirring programs used in this study.

Time	Stirring speed (rpm)		
	−12–0 h	0–72 h	72–144 h
Program 1	10	10	10
Program 2	40	40	40
Program 3	40	20	10
Program 4	40	20	The following program was repeated: 40 rpm for initial 5 min, and then 0 rpm for the remaining 55 min.

Table 2
Optimum temperature test for the 100 mL fed-batch SSF with DOPEG-SL after 6 days.

Temp. (°C)		Maximum Ethanol conc. (g/L)	Bioethanol conversion (%)	Residual enzyme activity (%)
25.0	Control	35.0	39.1	28.8
	DOPEG-SL	40.1	44.8	63.8
35.0	Control	37.0	41.3	30.9
	DOPEG-SL	49.6	55.4	65.5
36.5	Control	40.0	44.7	25.1
	DOPEG-SL	51.7	57.8	55.6
38.0	Control	44.3	49.5	20.4
	DOPEG-SL	56.2	62.8	42.7
40.0	Control	40.0	44.7	20.6
	DOPEG-SL	43.3	48.4	38.2

Next, the optimum temperature of SSF was investigated at different temperatures in a small-scale (100 mL) fed-batch SSF in the presence and absence of DOPEG-SL, where pre-hydrolysis was conducted at 50 °C. The highest ethanol concentration (g/L) during each SSF condition for 144 h, ethanol conversion based on the theoretical maximum yield (%), and residual enzyme activity (%) are shown in Table 2. The maximum ethanol concentration (56.2 g/L) and ethanol conversion (62.8%) were observed at 38 °C in the presence of DOPEG-SL. Whereas, the highest recovery of enzyme activity (65.5%) was observed at 35 °C in the presence of DOPEG-SL. Overall, an addition of DOPEG-SL improved both ethanol production and recovery of enzyme, and is consistent with the previous studies (Cheng et al., 2014; Winarni et al., 2014, 2013). The results indicate that the observed maximum ethanol concentration at 38 °C might be driven by several different factors including optimum temperature for fermentation, optimum enzyme activity, temperature-dependent enzyme recovery rate and enzyme stability.

3.2. Optimum amount of A-LDs in the 100 mL scale fed-batch SSF

Like most surfactants, both DOPEG-SL and EPEG-SL possess hydrophilic PEG and hydrophobic lignin domains (Cheng et al., 2014; Homma et al., 2010, 2008). The function of above two A-LDs was shown to decrease the non-productive adsorption of enzyme to the substrate, and the improvement of ethanol production in the 100 mL fed-batch SSF in the presence of 2.5 g/L A-LDs was described (Cheng et al., 2014; Winarni et al., 2013).

To further optimize the amount of A-LDs for the fed-batch SSF in this study, the ethanol production (g/L) was monitored in the 100 mL fed-batch SSF in the presence of either DOPEG-SL or EPEG-SL with various amounts (2.5 g/L, 3.0 g/L, 5.0 g/L) for up to 144 h at 38 °C (Fig. 1). The highest ethanol concentration was observed in the presence of 3.0 g/L DOPEG-SL (Fig. 1B) or 3.0 g/L EPEG-SL (Fig. 1E) at 144 h. Thus, we decided to carry out further optimization using 3.0 g/L A-LDs in the following experiments.

It is noteworthy that the ethanol production was increased linearly until 72 h in the presence of 2.5 g/L and 3.0 g/L A-LDs, then leveled off. Several phenomena might have occurred after 72 h, such as, end product inhibition of enzymes, increased adsorption and inactivation of enzymes to biomass, or ethanol-inhibition of yeast fermentation (Fig. 1A, B, D, and E). Similar phenomenon can also be seen in other studies (Buruiana et al., 2014; Liu et al., 2010). Because the ethanol concentration increased linearly for 96 h in the presence of 5.0 g/L A-LDs (Fig. 1C and F), it is unlikely that produced ethanol inhibited yeast fermentation. Instead, results suggest that higher amounts of A-LDs results in a lesser end product formation, or better turnover of enzymes in the presence of 5.0 g/L A-LDs. In fact, the residual enzyme activities based

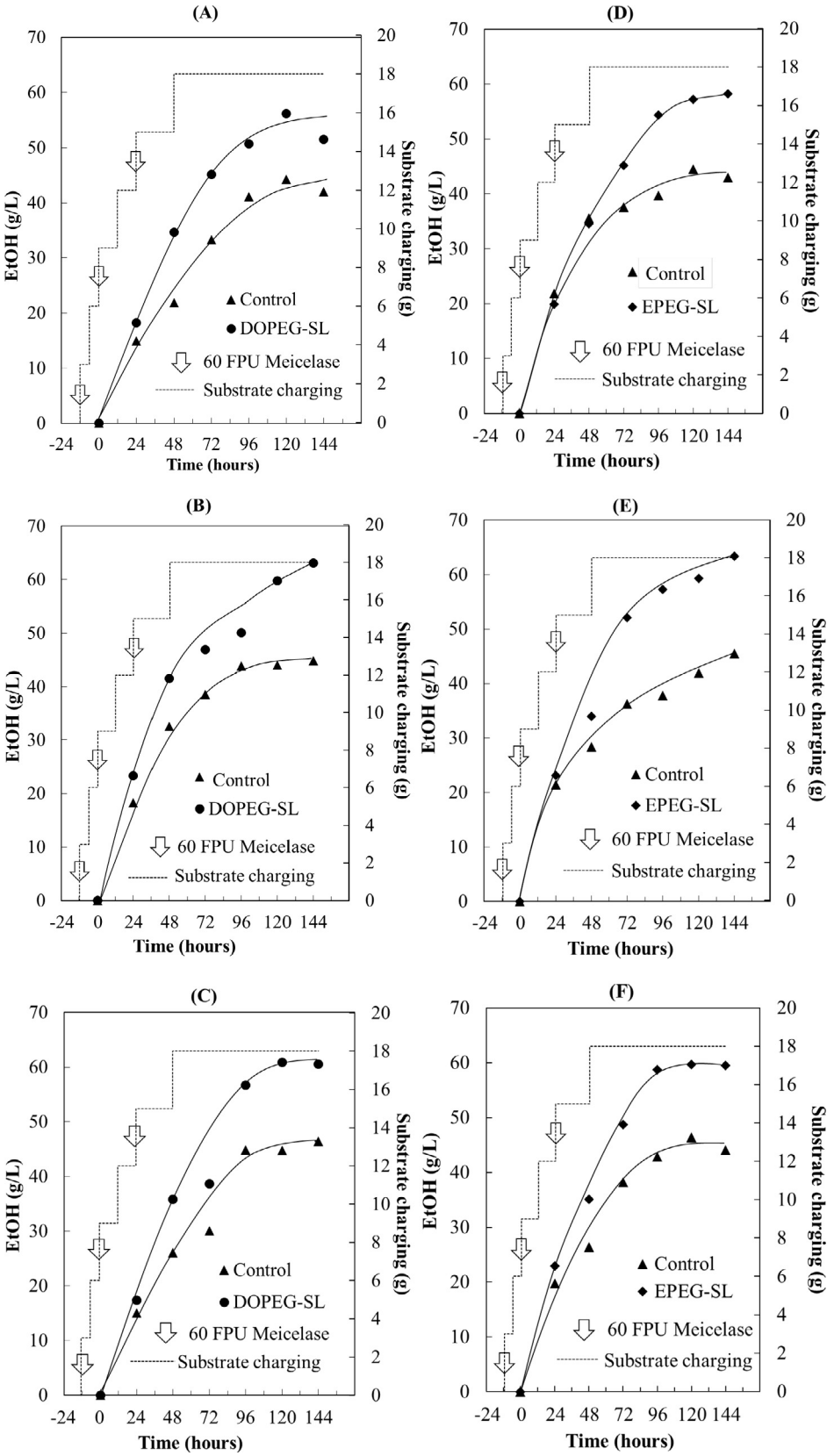

Fig. 1. Ethanol production (g/L) with a commercial cellulase (Meicelase) in the 100 mL fed-batch SSF with different DOPEG-SL loading: 2.5 g/L (A), 3.0 g/L (B) and 5.0 g/L (C); EPEG-SL of 2.5 g/L (D), 3.0 g/L (E) and 5.0 g/L (F). Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow.

on the initial activity in the presence of DOPEG-SL at 2.5, 3.0 and 5.0 g/L were determined to be 42.7, 49.5 and 91.6%, respectively, and those in the presence of EPEG-SL were 45.2, 56.5 and 88.7%, respectively. Apparently the A-LDs improved the enzyme activity over the reaction time compared to the residual activities measured in the absence of A-LDs (20.4%). These results strongly suggest that A-LDs suppress the inhibition of cellulase activity. However, at the 5.0 g/L A-LDs, slight reduction of ethanol production was observed at 144 h, and it might be due to the negative influence of ethanol fermentation by yeast in the presence of DOPEG-SL (Fig. 1A to C) or EPEG-SL (Fig. 1D to F) (Adeboye et al., 2014).

3.3. Effect of stirring program on the 1.5-L fed-batch SSF bioethanol production

Agitation of the medium during SSF is one of the key conditions to produce bioethanol production (Khongsay et al., 2012). Four different stirring programs were tested in SSF experiments in the absence of A-LDs (Table 1). The constant mechanical stirring at 10 rpm and 40 rpm were carried out in programs 1 and 2, respectively. In program 3, the stirring rate in pre-hydrolysis, 0–72 h and 72–144 h of the SSF were at 40, 20 and 10 rpm, respectively. In program 4, the stirring rate was similar to that of program 3 until 72 h, then stirring was increased to 40 rpm for 5 min followed by no agitation for 55 min. Fig. 2 shows ethanol production after 144 h in the 1.5-L fed-batch SSF with the 4 aforementioned stirring programs. The jar fermenter was equipped with a powerful custom-made mechanical stirrer. To achieve rigorous agitation with high substrate loading, the 4 stirring programs were tested in the presence of 360 g of NUKP, 6 g dry yeast and 3600 FPU Meicelase in the absence of A-LDs. Programs 3 and 4 generated higher ethanol production compared to programs 1 and 2, suggesting that continuous stirring at the same speed might reduce yeast fermentation efficacy (van Maris et al., 2006). Comparing programs 1 and 2, the higher stirring speed was shown to be required to facilitate glycoside hydrolases to physically interact with substrate, consistent with the previous work (Sakata et al., 1985). Interestingly, programs 3 and 4 were almost identical until 120 h, though the ethanol production in program 3 was significantly reduced at 144 h. This observation might be explained by aeration that is unfavorable for yeast fermentation. Overall, program 4 was the most effective stirring program because it provided an optimum enzyme to substrate interaction as well as potentially a lower oxygen environment favorable for yeast fermentation at high substrate loading.

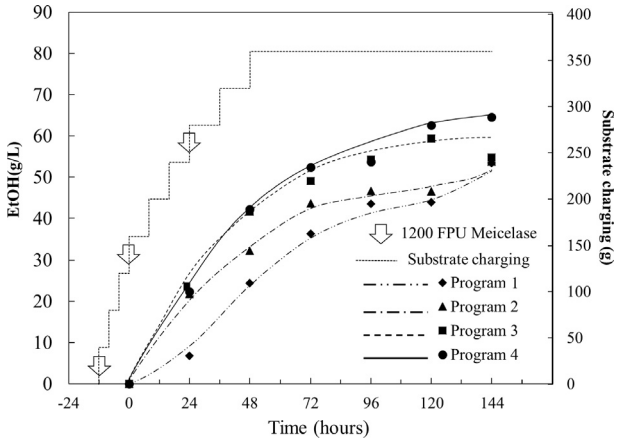

Fig. 2. Ethanol production with a commercial cellulase (Meicelase) of the 1.5 L fed-batch SSF under 4 different stirring programs. Four different stirring programs are shown in Table 1. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow.

3.4. Effect of A-LDs charging on 1.5 L fed-batch SSF on bioethanol production

A series of different loadings of A-LDs, 0, 2, 3, and 5 g/L, was added to the 1.5 L fed-batch SSF (Fig. 3). Ethanol conversion and enzyme recovery (%) were measured every 24 h for 144 h at 38 °C in the presence of 360 g NUKP, 6 g dry yeast and 3600 FPU Meicelase under the stirring program 4 (Fig. 3). Reactions in the presence of DOPEG-SL showed higher ethanol production and enzyme recovery compared to the reaction without DOPEG-SL. The highest ethanol production of 80.5 g/L with ethanol conversion of 67.4% was determined, when 3.0 g/L DOPEG-SL was added to the 1.5 L fed-batch SSF. Similarly the optimum amount of EPEG-SL for the highest ethanol production of 81.0 g/L with ethanol conversion of 67.8% was determined to be 3.0 g/L in the 1.5 L fed-batch SSF reaction (Fig. 3B). Interestingly, the enzyme recovery rate during SSF was higher in the 3.0 g/L DOPEG-SL (15.5%) compared to the

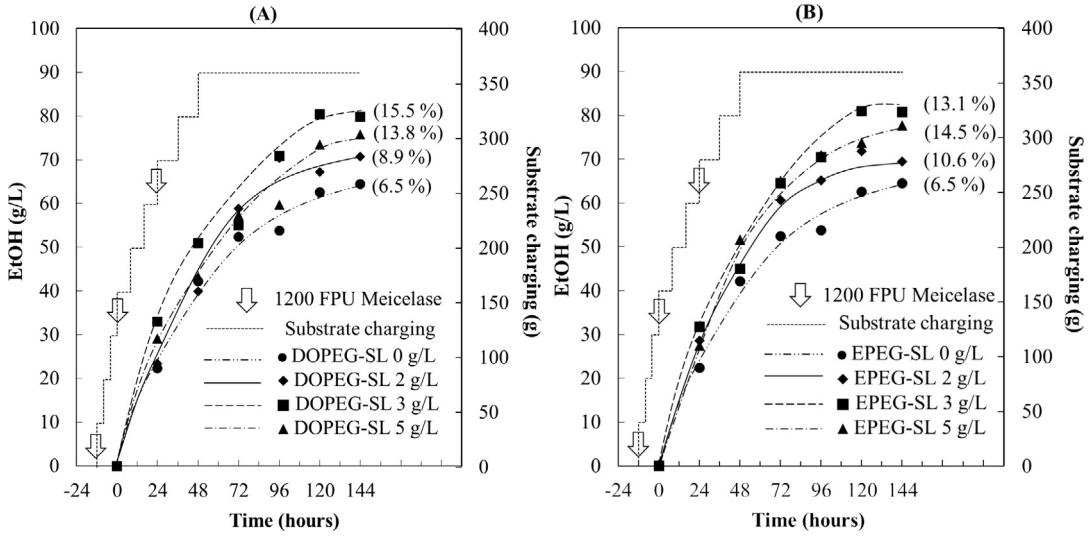

Fig. 3. Ethanol production with a commercial cellulase (Meicelase) at different loadings of DOPEG-SL (A) and EPEG-SL (B) in the 1.5 L fed-batch SSF. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

5.0 g/L DOPEG-SL (13.8%), whereas higher enzyme recovery was seen with 5.0 g/L EPEG-SL (14.5%) compared to the 3.0 g/L EPEG-SL (13.1%). This result indicated that the observed effect of A-LDs to ethanol production is not likely due to the enzyme recovery, but is likely because of fermentation by yeast. Perhaps, too much A-LDs negatively affects yeast cells to produce ethanol, and is consistent with the 100 mL scale fed-batch SSF in Fig. 1. Among the tested various amount of A-LDs, we found the optimum amount of A-LDs for ethanol production is 3.0 g/L in the 1.5 L fed-batch SSF, consistent with the results from the 100-mL scale of SSF (Fig. 1B and E).

3.5. High substrate loading bioethanol production in the maximally optimized condition

Under the optimal temperature, A-LDs loading and stirring program, the ethanol conversion was 67.8% (81.0 g/L) based on the theoretical maximum yield, suggesting that 32.2% of the added cellulose has not been converted to ethanol. Two different commercial enzyme cocktails were compared in order to improve saccarification in the presence of A-LDs. 10 FPU/g of Meicelase or GC220 were tested for saccharification of NUKP substrate for

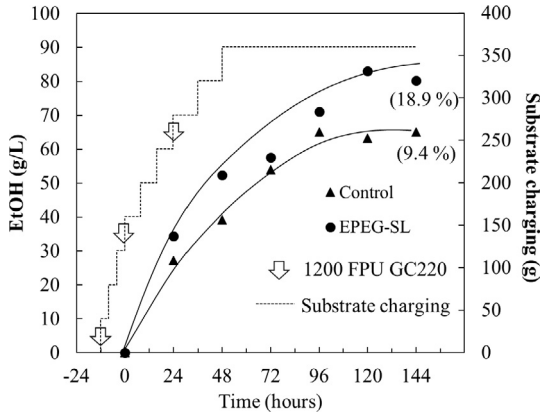

Fig. 4. Ethanol production with a commercial cellulase (GC220) in the 1.5 L fed-batch SSF in the presence and absence of 3.0 g/L EPEG-SL under the optimum condition. Total substrate loading was 360 g and total cellulase charge was 3600 FPU. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

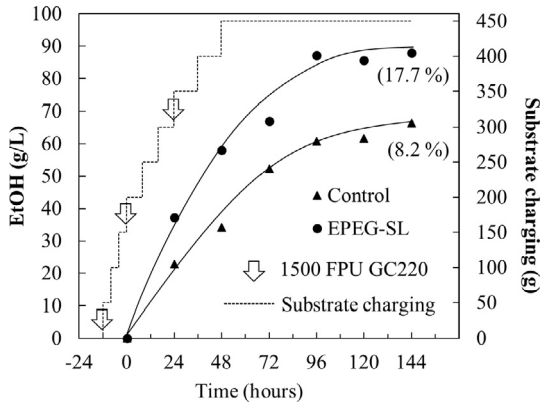

Fig. 5. Ethanol production with a commercial cellulase (GC220) in the 1.5 L fed-batch SSF in the presence and absence of 3.0 g/L EPEG-SL under the optimum condition. Total substrate loading was 450 g and total cellulase charge was 4500 FPU. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

Table 3
Comparison of two different commercial enzyme cocktails on saccharification in the presence of DOPEG-SL.

	Enzyme	Sugar yield (%)	Recovered enzyme activity (%)
Control	GC220	62.9	32.4
DOPEG-SL		73.7	86.4
Control	Meicelase	51.4	17.4
DOPEG-SL		63.9	78.7

48 h at 50 °C in the presence and absence of DOPEG-SL (Table 3). The results show that DOPEG-SL improved both hydrolysis and enzyme recovery rate for both enzyme cocktails. More importantly, the sugar yields from the GC220 (73.7%) was significantly higher than that of the Meicellase (63.9%). Thus, GC220 is better enzyme cocktails for the experimental context that we used, and the 1.5 L fed-batch SSF with optimum reaction conditions obtained was used to carry out further experiments. The 3600 FPU GC220 was used in the 1.5 L fed-batch SSF in the presence of 3.0 g/L EPEG-SL and 360 g NUKP at 38 °C for 144 h under the program 4, and ethanol production was monitored (Fig. 4). The ethanol concentration was increased to 83.0 g/L and the theoretical ethanol production of 69.5% was achieved, which are slightly higher than the reaction with Meicelase (81.0 g/L, 67.8% and Fig. 3B). Additionally, the residual enzyme activity in GC220 (18.9%) was higher than that of the Meicelase (14.5%). In an attempt to achieve even higher ethanol concentration, NUKP loading was increased to 450 g/SSF reaction (30% w/w) (Fig. 5). The resulting ethanol concentration of 87.9 g/L and theoretical ethanol conversion of 58.9% were achieved in the presence of 3.0 g/L EPEG-SL. In contrast, significantly less ethanol concentration (66.3 g/L) and ethanol conversion (44.5%) were determined in the absence of EPEG-SL.

In order to envision how the optimized fed-batch SSF performed compared to other studies in terms of ethanol yield (g/L) and ethanol conversion (% on maximum theoretical yield), we looked closely at previously reported methods.

As far as we have investigated, Maeda et al. (2013) produced the highest ethanol concentration (100 g/L) with a relatively high conversion rate of 78% from pre-hydrolyzed sugar cane bagasse as the lignocellulosic substrate in SSF (Maeda et al., 2013). They used a special enzyme that they produced themselves, which may contribute to a higher ethanol concentration and conversion rate than our methods (87.9 g/L and 59%, respectively).

Elliston et al. (2013) achieved an ethanol concentration of 11.6% (v/v), which was calculated to be 91.5 g/L (specific gravity of ethanol: 0.789 at 25 °C), at an ethanol conversion rate of 54%. These values are comparable to our results, although the enzyme loading (3.7 FPU/g substrate) is smaller than ours (10 FPU/g). The reason for their improved ethanol conversion is probably explained by the fact that they used special mixing equipment that enabled substrate loading at a very high consistency (65% w/v). In addition, the cellulase cocktail they used was supplemented with β-glucosidases. In our results, A-LDs may have helped to achieve similarly high ethanol concentration (87.9 g/L) even at a lower substrate loading (30% w/v), without modification of enzyme compositions in the cocktail.

A paper by Zhang et al. (2010) showed a higher ethanol conversion rate (79.6%) with a slightly lower ethanol concentration (84.7 g/L) at lower substrate loading (19% w/v) (Zhang et al., 2010). Their results were likely obtained by higher enzyme loading (22.8 FPU/g substrate) than ours (10 FPU/g).

4. Conclusions

In this study, we improved fed-batch SSF by optimizing a series of reaction parameters. The optimum temperature for fed-batch

SSF was determined to be around 38 °C. Maximum ethanol yields were obtained when 3.0 g/L A-LDs were added in fed-batch SSF with better enzyme recovery using EPEG-SL. The stirring program 4 with reduced agitation during the final 72 h of SSF was shown to be the most effective. In the end, we achieved ethanol concentration of 87.9 g/L in the presence of GC220 commercial enzyme cocktail and high NUKP loading (30% w/w) under optimized 1.5 L fed-batch SSF.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.

Acknowledgements

We thank Mr. Ogawa from Research Faculty of Science, Media laboratory, Hokkaido University, to design and build the 3 L jar fermenter used in the fed-batch SSF in this study. We also thank Dr. Emily Beebe for her help to improve manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.02.018.

References

Adeboye, P.T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. AMB Express 4.

Aldiguier, A.S., Alfenore, S., Cameleyre, X., Goma, G., Uribelarrea, J.L., Guillouet, S.E., Molina-Jouve, C., 2004. Synergistic temperature and ethanol effect on Saccharomyces cerevisiae dynamic behaviour in ethanol bio-fuel production. Bioprocess Biosyst. Eng. 26 (4), 217–222.

Alfani, F., Gallifuoco, A., Saporosi, A., Spera, A., Cantarella, M., 2000. Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. J. Ind. Microbiol. Biotechnol. 25 (4), 184–192.

Alvira, P., Tomas-Pejo, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. 101 (13), 4851–4861.

Aysu, T., Durak, H., 2015. Thermochemical conversion of Datura stramonium L. by supercritical liquefaction and pyrolysis processes. J. Supercrit. Fluids 102, 98–114.

Balat, M., Balat, H., Oz, C., 2008. Progress in bioethanol processing. Prog. Energy Combust. Sci. 34 (5), 551–573.

Borjesson, J., Peterson, R., Tjerneld, F., 2007. Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition. Enzyme Microb. Technol. 40 (4), 754–762.

Buruiana, C.-T., Vizireanu, C., Garrote, G., Parajó, J.C., 2014. Bioethanol production from hydrothermally pretreated and delignified corn stover by fed-batch simultaneous saccharification and fermentation. Energy Fuels 28 (2), 1158–1165.

Cai, C., Qiu, X.Q., Lin, X.L., Lou, H.M., Pang, Y.X., Yang, D.J., Chen, S.W., Cai, K.F., 2016. Improving enzymatic hydrolysis of lignocellulosic substrates with pre-hydrolysates by adding cetyltrimethylammonium bromide to neutralize lignosulfonate. Bioresour. Technol. 216, 968–975.

Cheng, N.N., Yamamoto, Y., Koda, K., Tamai, Y., Uraki, Y., 2014. Amphipathic lignin derivatives to accelerate simultaneous saccharification and fermentation of unbleached softwood pulp for bioethanol production. Bioresour. Technol. 173, 104–109.

Deshpande, M.V., Eriksson, K.E., 1984. Reutilization of enzymes for saccharification of lignocellulosic materials. Enzyme Microb. Technol. 6 (8), 338–340.

Dias, M.O.S., Modesto, M., Ensinas, A.V., Nebra, S.A., Maciel, R., Rossell, C.E.V., 2011. Improving bioethanol production from sugarcane: evaluation of distillation, thermal integration and cogeneration systems. Energy 36 (6), 3691–3703.

Elliston, A., Collins, S.R.A., Wilson, D.R., Roberts, I.N., Waldron, K.W., 2013. High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. Bioresour. Technol. 134, 117–126.

Eriksson, T., Borjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb. Technol. 31 (3), 353–364.

Hayward, T., Combs, N., Schmidt, S., Philippidis, G., 1995. Chemical analysis and testing task: LAP-008 (SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation) National Renewable Energy Laboratory.

Homma, H., Kubo, S., Yamada, T., Koda, K., Matsushita, Y., Uraki, Y., 2010. Conversion of technical lignins to amphiphilic derivatives with high surface activity. J. Wood Chem. Technol. 30 (2), 164–174.

Homma, H., Kubo, S., Yamada, T., Matsushita, Y., Uraki, Y., 2008. Preparation and characterization of amphiphilic lignin derivatives as surfactants. J. Wood Chem. Technol. 28 (4), 270–282.

Khongsay, N., Laopaiboon, L., Jaisil, P., Laopaiboon, P., 2012. Optimization of agitation and aeration for very high gravity ethanol fermentation from sweet sorghum juice by Saccharomyces cerevisiae using an orthogonal array design. Energies 5 (3), 561–576.

Kumar, D., Murthy, G.S., 2013. Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. Biotechnol. Biofuels 6 (1), 63.

Lin, X., Qiu, X., Yuan, L., Li, Z., Lou, H., Zhou, M., Yang, D., 2015. Lignin-based polyoxyethylene ether enhanced enzymatic hydrolysis of lignocelluloses by dispersing cellulase aggregates. Bioresour. Technol. 185, 165–170.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qu, Y., Xiao, L., 2010. High concentration ethanol production from corncob residues by fed-batch strategy. Bioresour. Technol. 101 (13), 4952–4958.

Lou, H., Wang, M., Lai, H., Lin, X., Zhou, M., Yang, D., Qiu, X., 2013. Reducing non-productive adsorption of cellulase and enhancing enzymatic hydrolysis of lignocelluloses by noncovalent modification of lignin with lignosulfonate. Bioresour. Technol. 146, 478–484.

Maeda, R.N., Barcelos, C.A., Santa Anna, L.M., Pereira Jr., N., 2013. Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. J. Biotechnol. 163 (1), 38–44.

Nguyen, Q.A., Saddler, J.N., 1991. An integrated model for the technical and economic-evaluation of an enzymatic biomass conversion process. Bioresour. Technol. 35 (3), 275–282.

Olofsson, K., Bertilsson, M., Liden, G., 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 1.

Phillips, R.B., Jameel, H., Chang, H.M., 2013. Integration of pulp and paper technology with bioethanol production. Biotechnol. Biofuels 6 (1), 13.

Sakata, M., Ooshima, H., Harano, Y., 1985. Effects of agitation on enzymatic saccharification of cellulose. Biotechnol. Lett. 7 (9), 689–694.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. Renewable Energy 37 (1), 19–27.

Sun, Y., Cheng, J.Y., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83 (1), 1–11.

Uppugundla, N., Sousa, L.D., Chundawat, S.P.S., Yu, X.R., Simmons, B., Singh, S., Gao, X.D., Kumar, R., Wyman, C.E., Dale, B.E., Balan, V., 2014. A comparative study of ethanol production using dilute acid, ionic liquid and AFEX (TM) pretreated corn stover. Biotechnol. Biofuels 7.

Uraki, Y., Ishikawa, N., Nishida, M., Sano, Y., 2001. Preparation of amphiphilic lignin derivative as a cellulase stabilizer. J. Wood Sci. 47 (4), 301–307.

van Maris, A.J., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A., Wisselink, H.W., Scheffers, W.A., van Dijken, J.P., Pronk, J.T., 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. Antonie Van Leeuwenhoek 90 (4), 391–418.

Winarni, I., Koda, K., Waluyo, T.K., Pari, G., Uraki, Y., 2014. Enzymatic saccharification of soda pulp from sago starch waste using sago lignin-based amphipathic derivatives. J. Wood Chem. Technol. 34 (3), 157–168.

Winarni, I., Oikawa, C., Yamada, T., Igarashi, K., Koda, K., Uraki, Y., 2013. Improvement of enzymatic saccharification of unbleached cedar pulp with amphipathic lignin derivatives. Bioresources 8 (2), 2195–2208.

Zambare, V., Zambare, A., Muthukumarappan, K., Christopher, W.P., 2011. Biochemical characterization of thermophilic lignocellulose degrading enzymes and their potential for biomass bioprocessing. Int. J. Energy Environ. (IJEE) 2 (1), 99–112.

Zhang, M., Wang, F., Su, R., Qi, W., He, Z., 2010. Ethanol production from high dry matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment. Bioresour. Technol. 101 (13), 4959–4964.