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Direct saccharification and ethanol fermentation of cello-oligosaccharides with recombinant yeast

Xianxiang Liang[a], Takashi Yoshida[a,*], Toshiyuki Uryu[b]

[a] Department of Bio and Environmental Chemistry, Kitami Institute of Technology, 165 Koen-cho, Kitami 090-8507, Hokkaido, Japan
[b] Kochi University of Technology, Tosayamada-cho, Kochi 782-8502, Japan

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ABSTRACT

Ethanol was produced at good rates by direct saccharification and fermentation of cello-oligosaccharides with pYBGA1 yeast, a recombinant laboratory yeast expressing β-glucosidase. Cellobiose in the concentration of 50 g/L was directly fermented for 60 h with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C to give ethanol at an 80% theoretical conversion rate and a concentration of more than 20 g/L of concentration. Conversion to ethanol increased with increasing cellobiose concentration in the feed. When cellobiose was used at the concentration of 100 g/L, ethanol conversion and concentration increased to 85% and 45 g/L, respectively, in 96 h incubation. Other cello-oligosaccharides, cellotriose, cellotetraose, and cellopentaose at the concentration of 50 g/L, respectively, were also fermented directly for 72 h with 1×10^8 cells/mL of pYBGA1 yeast to produce ethanol in the conversion rates and concentrations of 71–73% and 18.0–18.5 g/L, respectively. The direct saccharification and fermentation mechanism of cello-oligosaccharides with pYBGA1 yeast, as revealed by HPLC measurements, suggesting that cellotetraose, for example, was saccharificated to cellotriose, cellobiose, and glucose and then fermented to give ethanol. These results suggest that the direct saccharification and fermentation of cello-oligosaccharides with pYBGA1 has several advantages as a simple procedure and for time, cost, and energy consumptions.

1. Introduction

Cellulosic bioethanol from renewable plant resources is an environment-friendly and alternative energy to replace petroleum-based fuels because cellulose is the most abundant non-food resource in nature (Cardona & Sánchez, 2007; Wyman, 1996). However, unlike starch, there are several problems in producing ethanol directly from a cellulosic biomass. The lignin that comprises 30–40%of the lignocellulosic biomass must be removed because lignin inhibits enzymatic saccharification and fermentation. In addition, cellulose crystal regions in the molecule resist enzymatic saccharification. Furthermore, the utilization of hemicellulose, which constitutes 20–30%of the lignocellulosic biomass, is necessary for the economical production of ethanol. Therefore, pretreatment of the lignocellulosic biomass before saccharification of cellulose and fermentation is necessary. In the current industrial production of cellulosic bioethanol (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010; Sun & Cheng, 2002), hydrolysis of cellulose including the crystalline region to glucose by sulfuric acid is the only method to give ethanol in good conversion rates (El-Zawawy, Ibrahim, Abdel-Fattah, Soliman, & Mahmoud, 2011; Su, Tzeng, & Shyu, 2011; Sun & Cheng, 2005). However, the sulfuric acid method has several problems: a large amount of sulfuric acid is necessary, it is difficult to recover acidic wastewater, and the manufacturing plant readily corrodes under acidic conditions. Therefore, there are many reports on the production of cellulosic bioethanol by alkaline pretreatment to remove lignin and decrease the crystallinity of cellulose molecules and then simultaneous saccharification and fermentation with a combination of cellulase and yeast (Linde, Galbe, & Zacchi, 2007; Wu, Arakane, et al. 2011a; Wu, Li, et al., 2011b).

Several reports on the direct saccharification and fermentation of cellulose to produce ethanol have appeared. Ingram reported the conversion of waste office paper to ethanol by a recombinant strain *Klebsiella oxytoca*, in which the paper was pretreated with 1% sulfuric acid at 140 °C, cellobiose and cellotriose were fermented by eliminating β-glucosidase, and then cellulase was added to promote saccharification to ethanol (Ingram, Conway, Clark, Sewell, & Preston, 1987). Ingram also reported the development of a recombinant *Escherichia coli* that expressed alcohol dehydrogenase and pyruvate decarboxylase from *Zymomonas mobilis* (Brooks & Ingram, 1995). The *E. coli* converted 00 g/L of glucose to 45 g/L of ethanol. It was reported that a recombinant sake yeast, *Saccharomyces cerevisiae* GRI-117-UK was prepared from *Aspergillus oryzae* by cloning β-glucosidase- and endoglucanase-encoding genes. The

* Corresponding author. Tel.: +81 157 26 9388; fax: +81 157 26 9388.
E-mail address: yoshida@chem.kitami-it.ac.jp (T. Yoshida).

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recombinant yeast produced ethanol from cellobiose and cellulose materials (Kotaka, Bando, et al., 2008a). Glucoamylase-displaying yeast, *S. cerevisiae*, was also constructed and it produced ethanol from liquefied starch (Kotaka, Sahara, et al., 2008b). The recombinant pYBGA1yeast was produced by encoding both the *bgl*A gene of *Aspergillus kawachii* IFO4308 and an uracil-encoding gene *Ura* 3, in *S. cerevisiae* YPH499, and it expressed β-glucosidase in both the extracellular fluid and cell wall (Iwashita, Todoroki, Kimura, Shimoi, & Ito, 1998). Therefore, the pYBGA1 yeast directly fermented cellobiose with a 1, 4-β-glucopyranosidic linkage to ethanol. Previously, we reported a method for a two-step acid hydrolysis of cellulose materials such as tissue paper, cotton, and sawdust to give a mixture of cello-oligosaccharides containing glucose, cellobiose, and higher cello-oligosaccharides, a 40 g/L solution of which was fermented with pYBGA1 yeast to give ethanol as high as 70% conversion and 19 g/L concentration (Uryu et al., 2006).

Cello-oligosaccharides are one of the key intermediates in the production of ethanol because cello-oligosaccharides are readily produced from cellulose under mild enzymatic saccharification compared to glucose production and fermentation by pYBGA1 yeast. In this study, we describe the direct saccharification and fermentation of cello-oligosaccharides, cellobiose, cellotriose, cellotetraose, and cellopentaose, to ethanol by using pYBGA1 yeast. We found that the direct saccharification and fermentation of cello-oligosaccharides proceeded smoothly to give ethanol in relatively good conversions and concentrations, and the process was monitored by high performance liquid chromatography (HPLC). This method using cello-oligosaccharides is a simple procedure to produce fuel ethanol.

2. Experimental

2.1. Measurement

Cell-oligosaccharides in the medium were identified by aqueous phase HPLC (column; Tosoh TSK-gel Amide-80, 7.6 mm × 250 mm eluted with acetonitrile aqueous solution at a flow rate of 0.5 mL/min) with a Tosoh RI detector. The ethanol concentration was recorded by gas chromatography (model; Shimadzu GC-8A; column: SE-30, 3.2 mm × 3.0 m, Shimadzu) fitted with a flame ionization detector and operated at column and injector temperatures of 60 and 130 °C, respectively. Nitrogen carrier gas was used at the flow rate of 25 mL/min.

2.2. Material

Recombinant pYBGA1 yeast was provided by the National Research Institute of Brewing. Commercially available cello-oligosaccharides, yeast extract, and peptone were purchased from Seikagaku Biobusiness Co. Ltd., Merck Chemicals, Japan, and Kyokuto Pharmaceutical Industrial Co. Ltd., Japan, respectively.

Preincubation and increase of pYBGA1 yeast on agarose medium at pH 5.0 with cellobiose as a carbon source were carried out for 5 days at 28.3 °C. Before use, the number of pYBGA1 yeast cells was counted using a cell counter.

2.3. Ethanol fermentation of cello-oligosaccharides

A typical procedure for the fermentation of cello-oligosaccharides is as follows. Yeast peptone cellobiose (YPC) medium was prepared from 10 g/L of yeast extract, 20 g/L of peptone and 50 g/L of cellobiose in 1000 mL of deionized water at pH 5.0, and then the medium was autoclaved for 20 min at 121 °C before use. Cellobiose was fermented of performed in 30 mL of the YPC medium with 1 × 108 cells/mL of pYBGA1 yeast for 120 h at 30 °C. A small amount of the fermentation solution was sampled every 12 h after the start of fermentation to measure quantitatively the ethanol produced by gas chromatography (GC) and the cellobiose remaining was determined by HPLC. Ethanol conversion was calculated from the theoretical concentration of ethanol from cellobiose in the feed. The results are demonstrated in Fig. 4B.

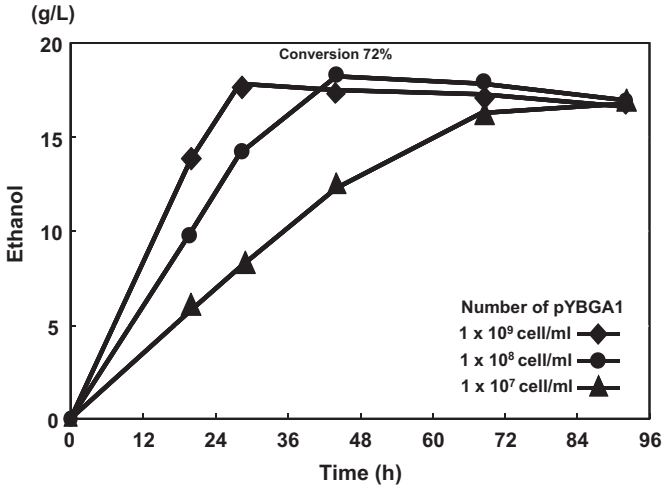

Fig. 1. Effect of number of pYBGA1 yeast cells on ethanol concentration at 28 °C in the fermentation of 50 g/L of glucose in the feed.

3. Results and discussion

3.1. Effect of pYBGA1 yeast concentration on ethanol fermentation

The recombinant pYBGA1 yeast expressed β-glucosidase in both the extracellular fluid and cell wall. Therefore, pYBGA1 yeast has ability to cause both saccharification of cello-oligosaccharides and ethanol fermentation. Before fermentation of cello-oligosaccharides, the suitable concentration of pYBGA1 yeast cells for fermentation of glucose was investigated, and the results are shown in Fig. 1. When 50 g/L of glucose in the feed was fermented with 1×10^9 cells/mL of pYBGA1 yeast at 28 °C, ethanol was produced at a 70% conversion rate and 17.8 g/L in 28 h. It was found that the highest conversion of glucose to ethanol was obtained with 1×10^8 cells/mL of pYBGA1 yeast to produce 72% conversion and 18 g/L after 44 h at 28 °C. Decreasing the number of yeast cells to 1×10^7 cells/mL decreased both the conversion of glucose and concentration of ethanol to 70% and 17 g/L, respectively, in 94 h. Accordingly, the concentration of pYBGA1 yeast cells 1×10^8 cells/mL, was selected for the direct saccharification of cello-oligosaccharides and fermentation to ethanol.

3.2. Effect of different temperatures

Based on the result of the optimal number of pYBGA1 yeast cells for the fermentation of glucose, direct saccharification and ethanol fermentation of cellobiose were performed. Fig. 2 shows the effect of the fermentation temperature on 50 g/L of cellobiose in the feed. The temperature varied from 26 °C to 33 °C. At 28 °C, cellobiose was converted to ethanol at a 76% conversion rate and 19 g/L of concentration after 96 h. A long fermentation time was necessary because pYBGA1 yeast first saccharified cellobiose to glucose and then the resulting glucose was fermented to ethanol. It was found that the conversion and concentration of ethanol increased with increasing temperature. At 30 °C, ethanol was produced at 80% conversion rate and 21 g/L after 72 h incubation. However, higher and lower

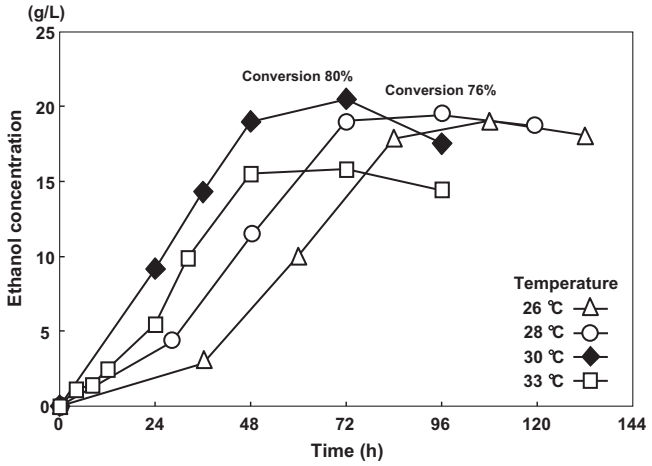

Fig. 2. Effect of saccharification and fermentation temperatures of cellobiose with 1×10^8 cells/mL of pYBGA1 yeast. The concentration of cellobiose in the feed was 50 g/L. The highest conversion to ethanol was 80% at 30 °C.

temperatures of 33 °C and 26 °C gave the lower conversion rates and concentrations of ethanol than incubation at 30 °C and 28 °C, respectively. The temperature of 33 °C gave the lowest activity of pYBGA1 yeast. Therefore, we concluded that the optimal temperature was between 30 °C and 28 °C.

3.3. Relationship between concentration of cellobiose and ethanol conversion

The effect of the fermentation concentration of cellobiose in the feed is shown in Fig. 3, in which the fermentation was carried out in 75 g/L, 100 g/L, and 150 g/L of cellobiose with 1×10^8 cells/mL of pYBGA1 yeast at 28 °C. Cellobiose at a high concentration was readily fermented with pYBGA1 yeast, and the fermentation time increased with an increase in the concentration of cellobiose. When the fermentation was performed with 75 g/L of cellobiose in the feed, the resulting conversion of cellobiose and ethanol concentration were 77% and 29 g/L, respectively. At 100 g/L of cellobiose in the feed, ethanol was obtained at the highest conversion rate of 85% after 96 h and the ethanol concentration was 45 g/L. With increasing concentration of cellobiose to 150 g/L in the feed, the ethanol concentration

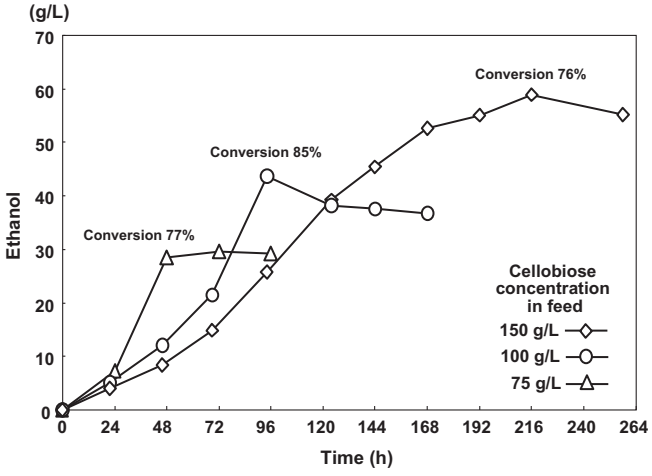

Fig. 3. Effect of saccharification and fermentation concentrations of cellobiose at 28 °C with 1×10^8 cells/mL of pYBGA1 yeast. The highest rates of conversion to ethanol were 77%, 85%, and 76% with the concentrations of 75, 100, and 150 g/L of cellobiose in the feed, respectively.

Table 1
Direct saccharification and fermentation of cello-oligosaccharides.[a,b]

	Concentration (g/L)	Time (h)	Ethanol (g/L)	Conversion (%)
C1	50	24	17.5	70
C2	50	60	18.7	75
C3	50	72	18.0	71
C4	50	72	18.5	73
C5	50	72	18.0	71

[a] C1, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; C5, cellopentaose.
[b] pYBGA1 yeast (1×10^8 cells/mL) was used at 30 °C.

increased to 60 g/L, however, the conversion decreased to 76% and the long fermentation time of 216 h was needed.

The effect of the length of cello-oligosaccharides on the conversion to ethanol was examined in the concentration of 50 g/L in the feed with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C as shown in Figs. 4 and 5. As shown in Fig. 4A, the concentration of ethanol increased rapidly after starting fermentation with a decreasing amount of glucose. In 24 h incubation, glucose was converted to ethanol at 70% and in the concentration of 17.5 g/L, respectively. For cellotriose and cellotetraose, ethanol was produced at 73% and 71% conversions and in the concentrations of 40 g/L and 45 g/L for 72 h, respectively. These cello-oligosaccharides had been consumed at this time. pYBGA1 yeast used here was preincubated and increased in the cellobiose medium to optimize the saccharification and fermentation of cello-oligosaccharides. Therefore, the conversion rate of glucose to ethanol was the lowest, however, the difference of the rate was not so much.

Fig. 5 shows the relationship between the ethanol concentration and fermentation time up to 120 h at 30 °C. For cellotriose, cellotetraose, and cellopentaose, the maximum ethanol conversion and concentration were obtained in 72 h fermentation with pYBGA1 yeast, and then the ethanol concentration decreased rapidly, suggesting that the ethanol produced was being consumed by pYBGA1 yeast as a carbon source.

3.4. Direct saccharification and fermentation mechanism of cello-oligosaccharides with pYBGA1 yeast

As mentioned above, pYBGA1 yeast has a β-glucosidase gene expressing both extracellular and cell wall-bound β-glucosidases. Therefore, cello-oligosaccharides with a (1 → 4)-β-D-glucopyranosidic linkage are hydrolyzed to glucose by pYBGA1 yeast and then fermented to produce ethanol. Fig. 6 shows the HPLC profiles of the saccharification and fermentation of cellotetraose (50 g/L) with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C. Fig. 6A shows the profile of standard cello-oligosaccharides and ethanol. After 24 h incubation (Fig. 6B), it was found that cellotetraose was saccharified into cellotriose, cellobiose, and glucose, and then fermented to ethanol. After 32 h, the proportion of cellotetraose decreased and then the absorption due to glucose only appeared after 48 incubation (Fig. 6C and D). After 72 h, saccharificated cello-oligosaccharides and glucose disappeared and ethanol was obtained at 73% conversion. The absorptions due to the medium used here also appeared between 4 and 11 min in the HPLC profiles. These results reveal that pYBGA1 yeast was saccharified cellotetraose to the lower cello-oligosaccharides and glucose, and then fermented them to produce ethanol. The optimal results are summarized in Table 1. Cello-oligosaccharides consisting of more than three glucose units required a longer fermentation time than cellobiose and glucose because the fermentation occurred after the saccharification to glucose. The conversion of cello-oligosaccharides at the concentration of 50 g/L into ethanol was 70–75% of the theoretically possible, probably because ethanol produced was also consumed by pYBGA1 yeast as a carbon source.

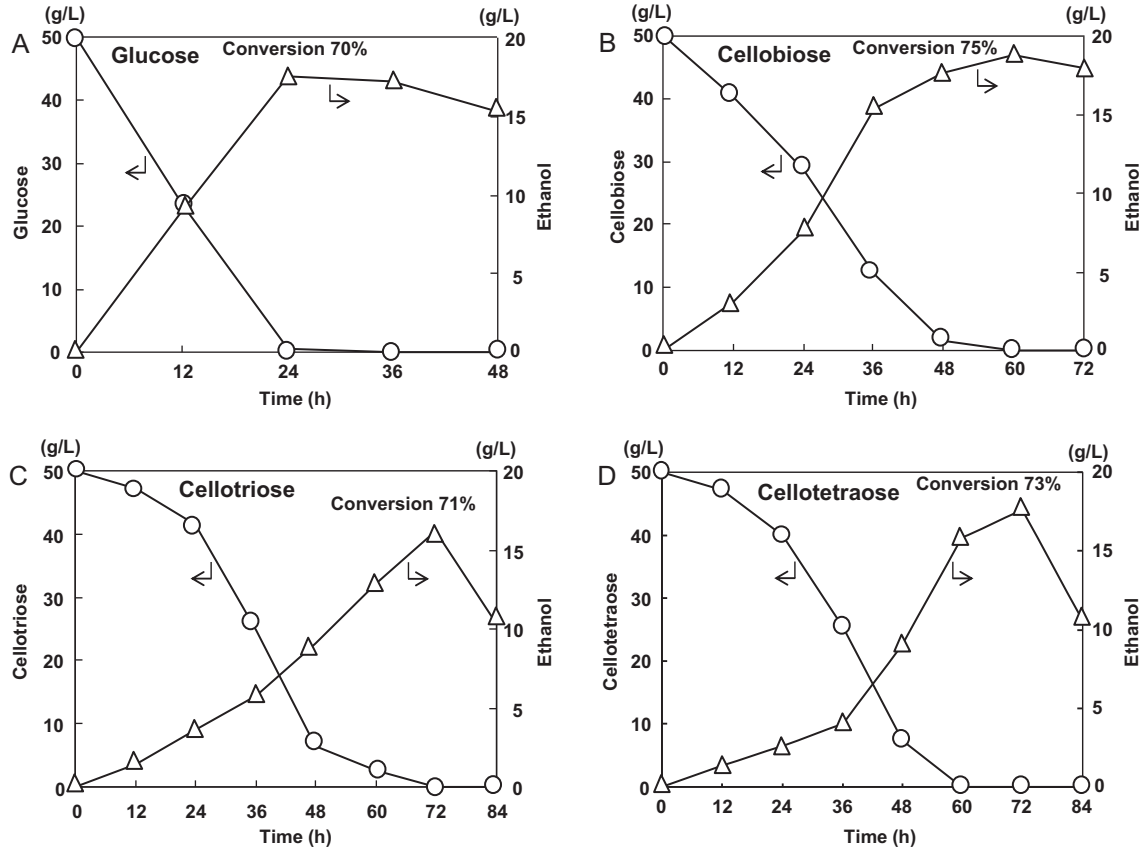

Fig. 4. Time course of changes in ethanol and cello-oligosaccharide concentrations during fermentation with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C. (A) Glucose, (B) cellobiose, (C) cellotriose, and (D) cellotetraose. The concentration of cello-oligosaccharides in the feed was 50 g/L.

In conclusion, the direct saccharification and fermentation of cello-oligosaccharides to ethanol was carried out using recombinant pYBGA1 yeast to give ethanol in relatively good conversions and concentrations. At 30 °C, cellobiose in the concentration of 100 g/L in the feed gave ethanol at an 85% conversion rate and 45 g/L concentration with 1×10^8 cells/mL of pYBGA1 yeast. Cello-oligosaccharides such as cellotriose, cellotetraose, and cellopentaose at the concentration of 50 g/L, respectively, were

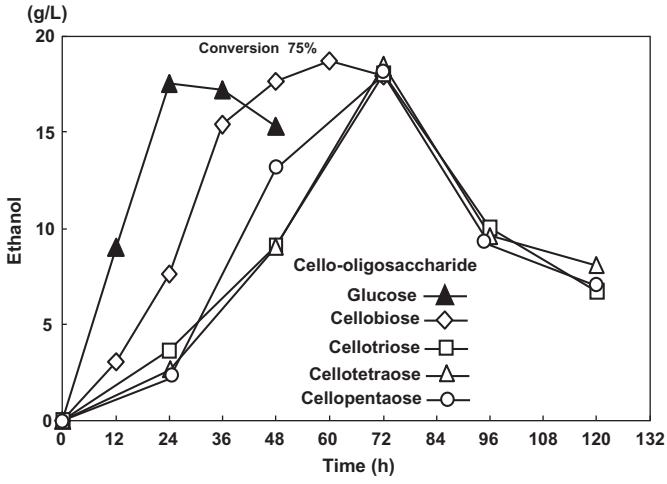

Fig. 5. Ethanol fermentation of glucose and cello-oligosaccharides with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C. The conversion to ethanol was 71–75% under the conditions. The concentration of cello-oligosaccharides in the feed was 50 g/L.

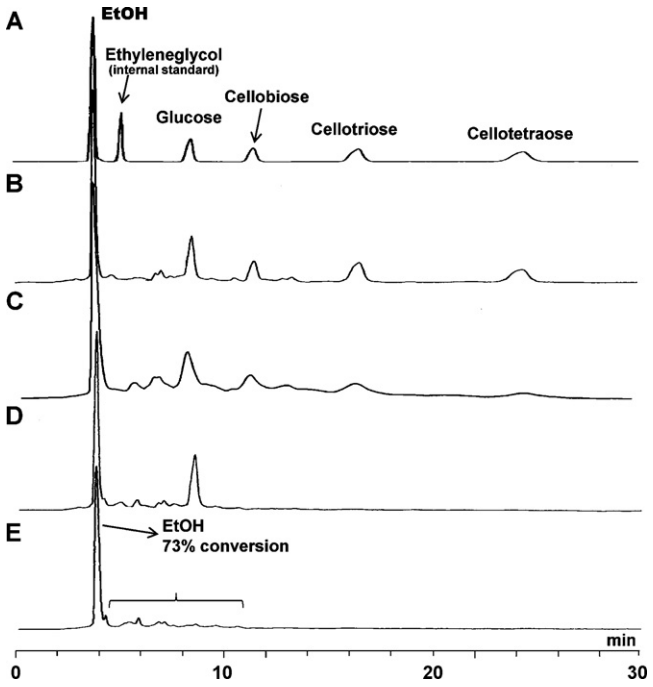

Fig. 6. HPLC profiles of saccharification and fermentation of cellotetraose with 1×10^8 cells/mL of pYBGA1 yeast at 30 °C. (A) Standard cello-oligosaccharides, (B) after 24 h, (C) 32 h, (D) 48 h, and (E) 72 h incubation, respectively. Cellotetraose was saccharified into cellotriose, cellobiose, and glucose with elapse of saccharification time. HPLC condition: column: amide-80; solvent: CH_3CN: H_2O = 3:2, speed: 0.8 mL/min.

fermented with 1×10^8 cells/mL of pYGBA1 yeast at 30 °C to produce ethanol in the concentrations as high as 18.0–18.5% (71–73 conversions), respectively. The direct saccharification and fermentation mechanism of cello-oligosaccharides with pYBGA1 yeast were revealed by HPLC measurements, suggesting that cello-oligosaccharides were saccharified into the lower cello-oligosaccharides and glucose by pYBGA1 yeast, and then fermented to produce ethanol. The direct saccharification and fermentation of cello-oligosaccharides was found to have advantages in the simplicity of the procedure, and in time, cost, and energy consumptions. Further experiments to determine the best conditions for the direct saccharification and fermentation of cello-oligosaccharides are in progress. In addition, enzymatic hydrolysis of naturally occurring cellulose resources such as wheat straw and corn stover into cello-oligosaccharides and then the direct saccharification and fermentation are also being investigated.

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