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Ethanol Production Using Concentrated Oak Wood Hydrolysates and Methods to Detoxify

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Abstract

Ethanol production from concentrated oak wood hydrolysate was carried out to obtain a high ethanol concentration and a high ethanol yield. The effect of added inhibitory compounds, which are typically produced in the pretreatment step of steam-explosion on ethanol fermentation, was also examined. *p*-Hydroxybenzoic aldehyde, a lignin-degradation product, was the most inhibitory compound tested in this study. Compounds with additional methyl groups had reduced toxicity and the aromatic acids were less toxic than the corresponding aldehydes. The lignin-degradation products were more inhibitory than the sugar-derived products, such as furfural and 5-hydroxymethylfurfural (HMF). Adaptation of yeast cells to the wood hydrolysate and detoxification methods, such as using charcoal and overlime, had some beneficial effects on ethanol production using the concentrated wood hydrolysate. After treatment with charcoal and low-temperature sterilization, the yeast cells could utilize the concentrated wood hydrolysate with 170 as well as 140 g/L glucose, and produce 69.9 and 74.2 g/L ethanol, respectively, with a yield of 0.46–0.48 g ethanol/g glucose. In contrast, the cells could not completely utilize untreated wood hydrolysate with 100 g/L glucose. Low-temperature sterilization, with or without charcoal treatment, was very effective for ethanol production when highly concentrated wood hydrolysates were used. Low-temperature sterilization has advantages over traditional detoxification methods, such as using overlime, ion exchange, and charcoal, because of the reduction in the total cost of ethanol production.

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Index Entries: Ethanol; concentrated wood hydrolysate; oak; detoxification; inhibitory compounds; low-temperature sterilization.

Introduction

Recently fuel ethanol production from biomass, especially cellulosic materials, has been drawing increasing attention because of its low cost *(1–4)*. The cellulosic material can be enzymatically hydrolyzed to glucose, which is fermented to ethanol. Prior to hydrolysis, the lignocellulosic material must be subjected to pretreatment to make the structure more amenable for hydrolysis. The usual method of pretreatment is to use high-temperature steam and acidic treatment *(5–8)*. The presence of compounds inhibitory to microorganisms in acid-pretreated or steam-pretreated wood has long been acknowledged *(9–13)*. During pretreatment, water-soluble inhibitors, such as sugar-derived byproducts, lignin degradation products, and extractives, are formed. The nature and concentrations of final inhibiting compounds vary greatly with the pretreatment conditions and the raw material used. The sugar-derived byproducts are primarily formed from furans, e.g., furfural, HMF, and levulinic acid. The lignin degradation products include a wide range of aromatic and polyaromatic compounds with a variety of substituents. It is likely that some of these aromatic substances inhibit both the hydrolysis and fermentation steps. However, little progress has been made on the effect of inhibitors in ethanol production because of the complexity of the cellulosic material despite the need for using cellulosic materials for ethanol fermentation *(14–17)*.

Considering the high cost of ethanol separation, it is necessary to have a high ethanol concentration in the fermentation broth. However, a high ethanol concentration in the fermentor is usually limited by the presence of various inhibitory compounds, as mentioned above. A number of studies were carried out to overcome the toxicity of the hydrolysates using high cell density *(18)* and detoxification processes *(19–21)*.

In this study, the effects of the sugar-derived products and the lignin degradation products produced by a steam explosion using oak wood, which is abundant in Korea, on ethanol fermentation were investigated. We also attempted to find an effective method for the utilization of oak wood hydrolysate so it could be fermented to ethanol, especially in highly concentrated wood hydrolysate, to get a higher ethanol concentration while considering the economics of ethanol production.

Materials and Methods

Microorganism

The yeast strain used in this study was an industrial *Saccharomyces cerevisiae* kindly provided by Seoyoung Ethanol Industry, Kunsan, Korea. This strain was maintained on agar slants containing 0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, 2% glucose, and 2% agar at 4°C.

Preparation of Wood Hydrolysate

Chips of oak wood (2 × 4 mm) were made as the initial feed material by a chipper designed and built in our laboratory. The composition of oak wood is as follows, 49.3% cellulose; 25.9% hemicellulose; 21.7% Klasson lignin. Steam explosion of the oak-wood chips was conducted for 3 min at 215°C in an 8-L exploder designed in our laboratory. The yields of sugars after steam explosion are as follows; glucan 0.88; xylose-maltose-galactose (XMG), 0.2. However the concentration of individual sugar (xylose, maltose, galactose) was not detected. Five kilograms (dry weight) of residue after explosion and washing was enzymatically hydrolysed with Celluclast (Novo Co., Bagsvaerd, Denmark) and Novozym (Novo) in a reactor with 30 L working volume (Korea Fermentor Co., Inchon, Korea) for 3 d at 50°C. The enzyme loading was 20 and 30 IU/g residue for filter paper and cellobiase activities, respectively. Cellulase activities were measured according to the method suggested by IUPAC *(22)*. The hydrolysate was centrifuged and the supernatant contained about 40 g/L glucose. Other sugars existed and the concentration of reducing sugar included is about 5% (w/v). However, there are no contained fermentable sugars in the hydrolysates, except for glucose. The concentration of lignin-degradation products and sugar-derived inhibitors in the hydrolysates are as follows, mg/L; 84 mg/L vanillin; 5.5 mg/L 3,4 dimethoxy bezoic acid, and 220 mg/L furfural. Other toxic compounds, such as syringaldehyde, *p*-hydroxybenzoic acid, and others, existed but were not measured exactly because of the complexity of the hydrolysates. By concentrating the supernatant using a vacuum evaporator (Korea Fermentor Co.), high glucose concentrations up to 170 g/L were obtained.

Culture Media

The seed culture medium consisted of 0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, and 4% glucose. Two fermentation media were used in this study. For testing the toxicity effect, the fermentation medium consisted of 0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, 9% glucose, and the inhibitory compounds. Only the concentrated wood hydrolysate (the glucose concentrations are specified in the experimental results) was used as the fermentation medium to obtain high ethanol concentrations.

Cultivation

The seed culture was prepared by inoculating a loopful of cells from a stock plate into 100 mL of seed culture medium. The seed culture was grown in a shaking incubator overnight at 30°C before transfer to the flask. The inoculum volume was 10–20% (v/v) of the working volume in the flask according to the culture conditions.

Ethanol fermentation was carried out in 250-mL flasks containing 50 mL of control medium at an initial pH of 5.0. The inhibitory compounds

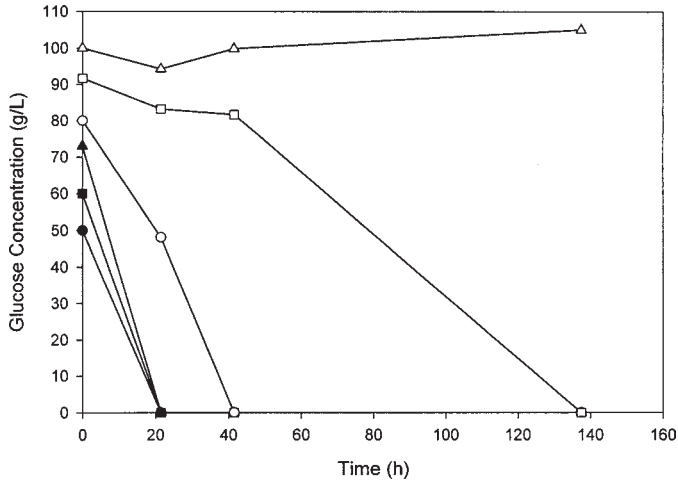

Fig. 1. Effect of glucose concentration in wood hydrolysate (sterilization at 121°C for 15 min). —●—, 10% (v/v) inoculum; —■—, 10% (v/v) inoculum; —▲—, 20% (v/v) inoculum; —○—, 10% (v/v) inoculum; —□—, 20% (v/v) inoculum; —△—, 10% (v/v) inoculum.

were added to the flasks for testing their toxicity. Some detoxification methods were carried out before sterilization as needed and sterilization was carried out at 121°C for 15 min or 60°C for 120 min. Each flask was incubated at 150 rpm and 30°C in a shaker incubator.

Assays

Ethanol concentration was measured by gas chromatography equipped with a flame ionization detector (HP5890A, Hewlett-Packard Inc., USA) using *n*-propanol as an internal standard. Glucose concentration was measured by an enzymatic glucose oxidase/peroxidase method (Glucose E-kit, Yeoungdong Pharm. Inc., Korea). Cell concentrations were determined spectrophotometrically by absorbance measurement at 570 nm (HP8452, Hewlett-Packard Inc.). Dry cell weights were determined after two rounds of centrifugation and washing with distilled water and drying at 105°C for 1 d.

Results and Discussion

Ethanol Fermentation with Concentrated Wood Hydrolysate

Considering the high cost of ethanol separation, it is essential to have a high ethanol concentration in the fermentation broth. Thus, the possibility of ethanol fermentation with concentrated wood hydrolysate was studied (Fig. 1). Concentrated wood hydrolysate was obtained using a vacuum evaporator at 50°C and 700 mmHg to avoid production of additional inhibitory compounds. The rate of fermentation was slower as the glucose

concentration was increased. When an initial glucose concentration of 90 g/L was used, all glucose was consumed with a longer lag and a lower ethanol yield of 0.29 g ethanol/g glucose compared to when an initial glucose concentration of 80 g/L was used with a yield of 0.46 g ethanol/g glucose. *S. cerevisiae* used in this study did not grow and did not produce ethanol when the initial glucose concentration was above 100 g/L because of the presence of toxic chemicals in the wood hydrolysate produced during the pretreatment step *(14)*.

Generally, both the sugar-derived products, like furfural and HMF, and the lignin-degradation products, like *p*-hydroxybenzoic aldehyde, syringaldehyde, and vanillin, have a great effect on cell growth and ethanol production *(11,14)*. However, the lignin-degradation products might have had more influence on ethanol production in this study because the concentration of the sugar-derived products was not higher in the concentrated wood hydrolysate obtained by evaporation and sterilization at 121°C. It is known that furfural is volatile to an extent and is polymerized at high temperatures *(21)*. Some inhibitors may have been removed during the concentration step.

Effect of Added Inhibitory Compounds on Ethanol Fermentation

Generally, the concentrations of inhibitory compounds decrease in the following order: furfural, HMF (sugar-derived products) > *p*-hydroxybenzoic acid, vanillin, syringaldehyde, vanillic acid, syringic acid (lignin degradation products) > (extractives) *(14)*. The lignin degradation products (*p*-hydroxybenzoic acid, vanillin, syringaldehyde, vanillic acid, syringic acid, and so on) were assumed to be produced in the pretreatment step and were toxic to cells. Most of the lignin-degradation products in steam-exploded oak hindered the growth of yeast and ethanol production. However, the degree of inhibition differed from one compound to another and was clearly related to the functional groups of the lignin-degradation products *(11,12)*.

Figure 2 shows the effect of vanillin on ethanol fermentation by *S. cerevisiae*. The initial cell concentration in the flask was 0.57 g/L and the initial glucose concentration was controlled at 90 g/L in a rich medium. Vanillin below concentrations of 2.5 g/L affected the fermentation in the early stage, but the fermentation recovered and the final cell and ethanol concentrations were the same as in the control experiment. The lag phases observed when 1–2.5 g/L of vanillin was added to the medium indicate that the yeast required an adaptation period resulting from the vanillin added to the fermentation medium. However, the yeast cells used in this study could not overcome the toxicity and did not grow when the vanillin concentration was above 3 g/L.

The toxicity of lignin-degradation products and sugar-derived products as the inhibitory compounds on ethanol fermentation were investigated in similar experiments and are summarized in Figs. 3 and 4. The mean fermentation rate was calculated as the change in glucose concentration

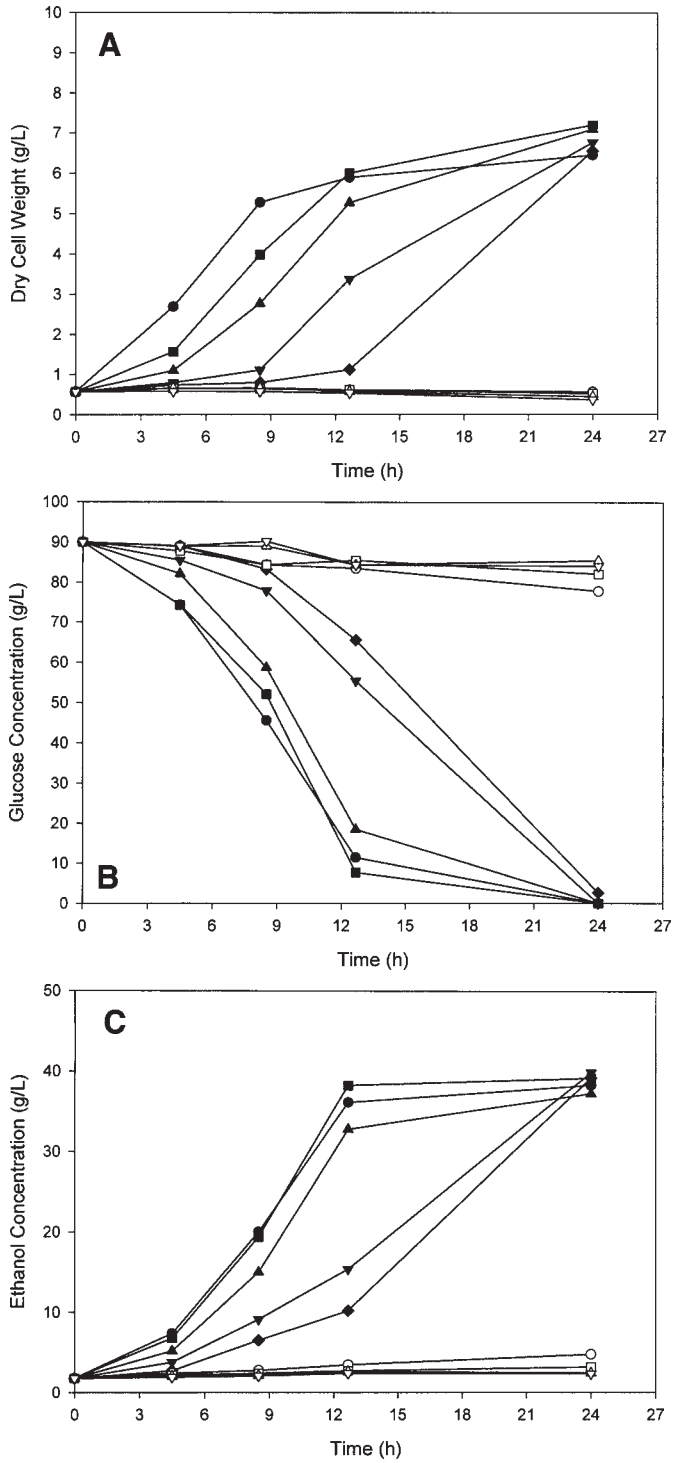

Fig. 2. Effect of vanillin concentration on cell growth **(A)**, glucose consumption **(B)**, and ethanol production **(C)**. —●—, control; —■—, 1.0 g/L; —▲—, 1.5 g/L; —▼—, 2.0 g/L; —◆—, 2.5 g/L; —○—, 3.0 g/L; —□—, 3.5 g/L; —△—, 4.0 g/L; —▽—, 5.0 g/L.

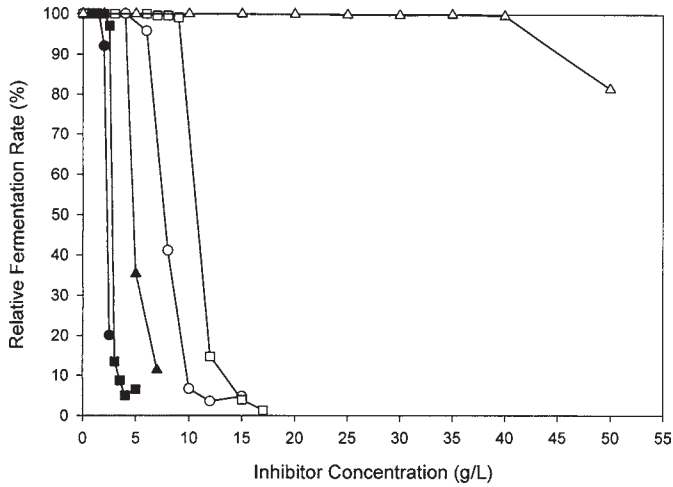

Fig. 3. Effect of lignin degradation products on ethanol fermentation. —●—, *p*-Hydroxybenzoic aldehyde; —■—, vanillin; —▲—, syringaldehyde; —○—, *p*-hydroxybenzoic acid; —□—, vanillin acid; —△—, syringic acid.

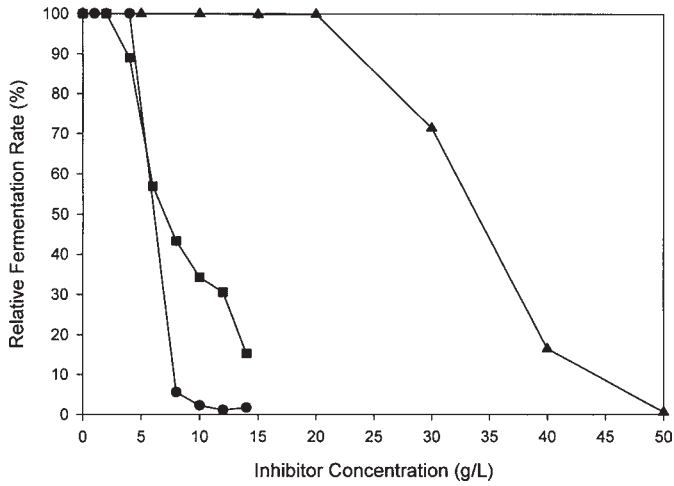

Fig. 4. Effect of sugar-derived products on ethanol fermentation. —●—, Furfural; —■—, 5-hydroxymethylfurfural; —▲—, levulinic acid.

divided by the fermentation time (24 h). By comparison with a control, relative fermentation rates are reported. Almost complete inhibition for *p*-hydroxybenzoic aldehyde occurred at approx 2.5 g/L, whereas almost complete inhibition for vanillin corresponded to about 3.5 g/L (Fig. 3). Syringaldehyde was less toxic than vanillin. *p*-Hydroxybenzoic acid was somewhat less inhibitory, with almost complete inhibition at about 8 g/L as compared to that for *p*-hydroxybenzoic aldehyde as its corresponding aldehyde. Vanillic acid was less toxic than *p*-hydroxybenzoic acid, as was the case between their corresponding aldehydes. Syringic acid had no effect

in concentrations up to 40 g/L. Thus, additional methyl groups reduced the toxicity, and the aromatic acids were less toxic than the corresponding aldehydes *(11,14)*.

By comparison, the sugar-derived products were much less inhibitory. Under the standard assay conditions, almost complete inhibition occurred when the concentrations of furfural, HMF, and levulinic acid were about 8 g/L, above 14 g/L, and about 50 g/L, respectively (Fig. 4). The inhibitory effects of furfural and HMF on growth and fermentation were caused by their action on glucolysis *(23,24)*, and furfural was more inhibitory to fermentation *(15,16)*, as shown in Fig. 4.

It is known that there were no significant interactions among any of the inhibitory compounds and that the inhibitory effects in wood hydrolysate are cumulatively caused by the toxicity of sugar-derived products and lignin-degradation products *(11)*. From these results, it is clear that a method to overcome the inhibitory effects of these compounds present in the wood hydrolysate is necessary.

Adaptation of S. cerevisiae *to Wood Hydrolysate*

Yeast cells can be very easily adapted to the presence of furfural and HMF, and such cells lose their activity when transferred to a medium without furfural and HMF *(23)*. Figure 5 shows the adaptability of the yeast cells to wood hydrolysate containing toxic materials with respect to their cell growth and ethanol-producing efficiencies. Adaptation of yeast was carried out through preculture in the wood hydrolysate containing a lower concentration of glucose (55 g/L), which allowed cell growth in that medium instead of a synthetic medium for seed culture.

Adaptation of yeast cells to inhibitory compounds occurred at short exposure. Growth of the cells in the wood hydrolysate medium for 24 h only enabled them to withstand its inhibitory effect much better in the initial stage of cultivation. The cells utilized about 60 and 40% of glucose, respectively, when using initial glucose concentrations of 90 and 100 g/L. However, the yeast cells did not grow after 2 d because of long exposure to high toxic levels and low cell density. In this study, we obtained similar results as those reported by Banerjee et al. *(23)*, and knew that adaptation of yeast cells to the wood hydrolysate medium had partially influenced ethanol production in the medium containing a higher concentration of toxic materials.

Detoxification of Inhibitory Compounds Present in Wood Hydrolysate

Some methods using charcoal, overlime, ion exchange, and microorganisms were proposed to solve the effect of inhibitory compounds produced during the pretreatment of wood *(11,14,21)*. Figure 6 shows the effect of charcoal treatment on ethanol fermentation in the range of 0.05–0.20 g charcoal/g glucose for initial glucose concentrations of 80 and 100 g/L.

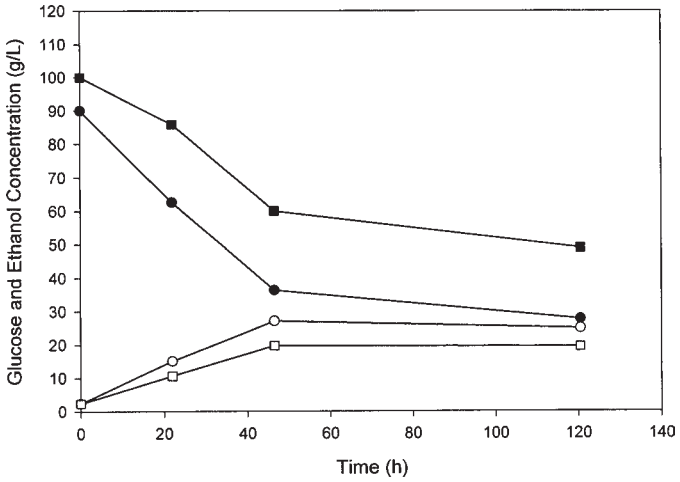

Fig. 5. Adaptation of *S. cerevisiae* to wood hydrolysate with 55 g glucose/L (sterilization at 121°C for 15 min). —●—, Glucose concentration for 90 g glucose/L; —■—, glucose concentration for 100 g glucose/L; —○—, ethanol concentration for 90 g glucose/L; —□—, ethanol concentration for 100 g glucose/L.

After charcoal treatment, glucose used at a concentration of 100 g/L was fermented to ethanol, which was not the case without charcoal treatment (*see* Fig. 1). Moreover, the fermentation rate improved with charcoal treatment when the glucose concentration was 80 g/L. The fermentation rate also increased as the amount of charcoal used for treating increased. However, when glucose concentrations above 100 g/L were used, the charcoal treatment had no or little effect on ethanol fermentation. We also examined the detoxification of inhibitory compounds using overlime and silicalite. The charcoal treatment was more effective than any other methods tested (data not shown). From these results, it is evident that charcoal treatment could reduce inhibitory compounds present in the wood hydrolysate to some extent, but not completely eliminate them.

Effect of Sterilization Temperature on Ethanol Production

It was known that the inhibitory compounds were produced by a high-temperature reaction during steam explosion pretreatment of cellulosic materials. Generally, sterilization was carried out at a high temperature of 121°C, which probably influenced the production of inhibitory compounds in addition to the pretreatment step. We investigated the effects of temperature during sterilization and amount of inoculum on ethanol production (Fig. 7). Sterilization was carried out at a low temperature of 60°C for 120 min. The yeast used in this study grew and consumed almost all the glucose when the initial glucose concentration was 140 as well as 100 g/L. *S. cerevisiae*, however, could not use the wood hydrolysate containing an initial glucose concentration of 100 g/L that was sterilized at 121°C for 15 min, as shown in Fig. 1. The fermentation for ethanol production with

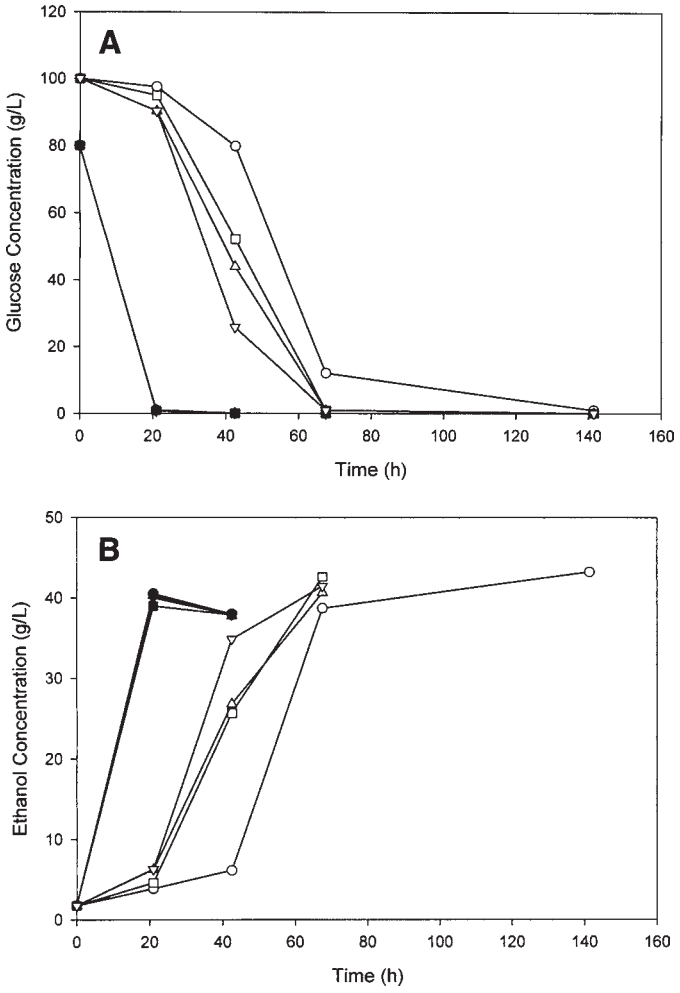

Fig. 6. Effect of charcoal treatment on glucose consumption **(A)** and ethanol production **(B)** (sterilization at 121°C for 15 min). —●—, 0.05 g charcoal/g glucose (80 g glucose/L); —■—, 0.10 g charcoal/g glucose (80 g glucose/L); —▲—, 0.15 g charcoal/g glucose (80 g glucose/L); —▼—, 0.20 g charcoal/g glucose (80 g glucose/L); —○—, 0.05 g charcoal/g glucose (100 g glucose/L); —□—, 0.10 g charcoal/g glucose (100 g glucose/L); —△—, 0.15 g charcoal/g glucose (100 g glucose/L); —▽—, 0.20 g charcoal/g glucose (100 g glucose/L).

an initial glucose concentration of 140 g/L was done after 2 d under the condition of 20% (v/v) inoculum. However, an initial glucose concentration of 170 g /L caused a lag of over 100 h and was only 53.1% and 79.6% utilized, respectively, by *S. cerevisiae* after 210 h when the yeast cells were inoculated with 10 and 20% (v/v) glucose. The ethanol yield using an initial glucose concentration of 170 g/L also decreased to about 0.40 g ethanol/g glucose in comparison with the yield of 0.43–0.47 g ethanol/g glucose when using an initial glucose concentration of 140 g/L. We concluded that during sterilization a reaction producing inhibitory compounds took place.

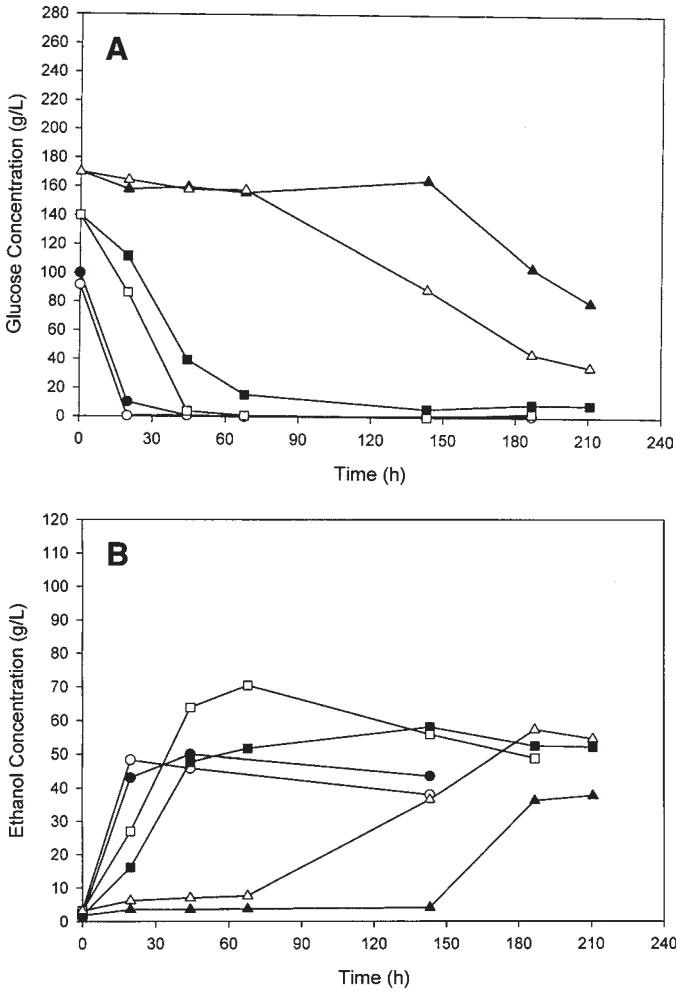

Fig. 7. Effect of sterilization temperature and amount of inoculum on glucose consumption **(A)** and ethanol production **(B)** without charcoal treatment (sterilization at 60°C for 120 min). —●—, 10% (v/v) inoculum (100 g glucose/L); —○—, 20% (v/v) inoculum (92 g glucose/L); —■—, 10% (v/v) inoculum (140 g glucose/L); —□—, 20% (v/v) inoculum (140 g glucose/L); —▲—, 10% (v/v) inoculum (170 g glucose/L); —△—, 20% (v/v) inoculum (170 g glucose/L).

The effects of sterilization temperature in combination with treatment of charcoal on ethanol production using concentrated oak wood hydrolysate were also studied (Fig. 8). We compared the fermentability of the yeast cells among the sterilization methods at 121°C for 15 min and at 60°C for 120 min after charcoal treatment with 0.2 g charcoal/g glucose. We also considered the effect of the amount of inoculum on ethanol fermentation under such conditions as mentioned above. Even after charcoal treatment, there was not much cell growth and ethanol production for initial glucose concentrations >140 g/L when the sterilization was done at 121°C for

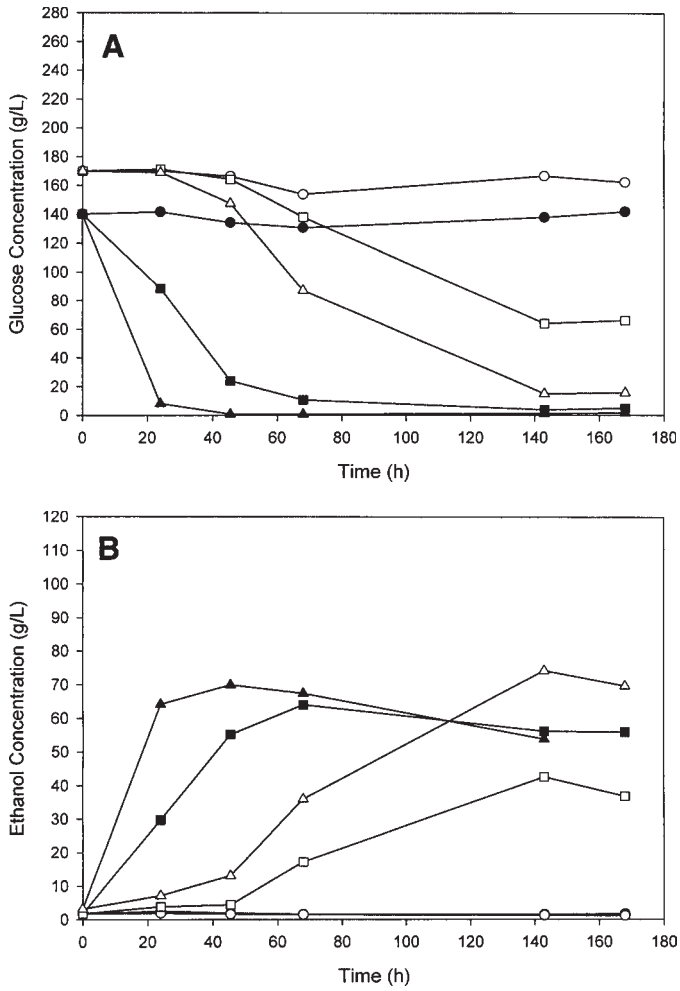

Fig. 8. Effect of sterilization temperature on glucose consumption **(A)** and ethanol production **(B)** after charcoal treatment with 0.2 g charcoal/g glucose. —●—, at 121°C for 15 min (10% (v/v) inoculum, 140 g glucose/L); —■—, at 60°C for 120 min (10% (v/v) inoculum, 140 g glucose/L); —▲—, at 60°C for 120 min (20% (v/v) inoculum, 140 g glucose/L); —○—, at 121°C for 15 min (10% (v/v) inoculum, 170 g glucose/L); —□—, at 60°C for 120 min (10% (v/v) inoculum, 170 g glucose/L); —△—, at 60°C for 120 min (20% (v/v) inoculum, 170 g glucose/L).

15 min. When the sterilization was carried out at a lower temperature of 60°C for 120 min, an initial glucose concentration of 140 g/L was almost utilized for cell growth and ethanol production, with a yield of 0.46–0.48 g ethanol/g glucose. Over 90% of the wood hydrolysate containing 170 g glucose/L could be fermented by a 20% (v/v) inoculum at low-temperature sterilization after charcoal treatment. These results show that low-temperature sterilization in combination with charcoal treatment was more effective for ethanol production from concentrated wood hydrolysate.

Contamination might have been the problem in sterilization at lower temperatures, but contamination did not occur during several trials, probably because of the high toxicity in the wood hydrolysates. From these results, it is evident that sterilization was carried out at a lower temperature, with or without charcoal treatment, which prevented cell contamination and did not produce more inhibitory compounds in the sterilization step. Low-temperature sterilization has advantages over other detoxification methods, such as using overlime, ion exchange, or charcoal, and may lower the overall cost of ethanol production. However, a complete cost comparison is needed.

Acknowledgment

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