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# Chemical Characteristics and Ethanol Fermentation of the Cellulose Component in Autohydrolyzed Bagasse

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**Abstract** The chemical characteristics, enzymatic saccharification, and ethanol fermentation of autohydrolyzed lignocellulosic material that was exposed to steam explosion were investigated using bagasse as the sample. The effects of the steam explosion on the change in pH, organic acids production, degrees of polymerization and crystallinity of the cellulose component, and the amount of extractive components in the autohydrolyzated bagasse were examined. The steam explosion decreased the degree of polymerzation up to about 700 but increased the degree of crystallinity and the micelle width of the cellulose component in the bagasse. The steam explosion, at a pressure of 2.55 MPa for 3 mins, was the most effective for the delignification of bagasse. 40 g/L of glucose and 20 g/L of xylose were produced from 100 g/L of the autohydrolyzed bagasse by the enzymatic saccharification using mixed cellulases, acucelase and meicelase. The maximum ethanol concentration, 20 g/L, was obtained from the enzymatic hydrolyzate of 100 g/L of the autohydrolyzed bagasse by the ethanol fermentation using *Pichia stipitis* CBS 5773; the ethanol yield from sugars was 0.33 g/g sugars.

*Keywords*: ethanol fermentation, bagasse, cellulose component, degree of polymerization, enzymatic saccharification, X-ray diffraction

## INTRODUCTION

Lignocellulosic material such as wood, bark, and grass is a regenerable organic substance and an alternative source of energy [1,2]. Lignocellulosic material is abundant because about one-third of the world's land area is forestland [3]. However, since lignocellulosic material is composed of cellulose and hemicellulose that are incrusted in lignin, it is very difficult to decompose by either chemical or biological means and thereby convert into energy [4]. The resistance to degradation has led to research searching for a method of pretreatment to enhance the degradation and removal of lignin from lignocellulosic material. In recent years, a method of decomposition consisting of chemical and physical processes has been used for the autohydrolysis of lignocellulosic materials [5-8]. The main element of this method is steam hydrolysis at a high temperature and pressure, followed by the sudden reduction of the pressure which allows for a mechanical treatment of the hydrolyzed product which produces low-molecular weight substances from cellulose, hemicellulose, and lignin that are easier to extract.

This paper investigates the chemical characteristics and ethanol fermentation of the cellulose component in lingocellulosic material when treated by steam explosion. The effects of the steam explosion condition on the degree of polymerization and crystallinity of the cellulose component in the autohydrolyzed lignocellulosic material were examined. Furthermore, the enzymatic saccharification followed by ethanol fermentation of the autohydrolyzed lignocellulosic material was attempted by using mixed cellulases and *Pichia stipitis* that can convert not only glucose but also xylose into ethanol.

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## MATERIALS AND METHODS

### Sample

Bagasse derived from sugar cane was used as the lignocellulosic material. The samples were prepared by cutting the bagasse into pieces 10 cm in length and 2~3 cm in width.

### Autohydrolysis

Autohydrolysis of the bagasse was performed using a steam explosion apparatus (Japan Chemical Engineering and Machinery Co., Ltd., Osaka, Japan) [9]. The apparatus consists of a steam generator, pressurized reactor, receiver, and condenser with a silencing action. The reactor was insulated to maintain a constant temperature. The reactor has a capacity of 1.2 L, with a maximum pressure of 5.5 MPa, and a maximum temperature of 275°C. Approximately 100 g of bagasse was placed into

**Table 1.** Pretreatment effect of pressure on the autohydrolyzed bagasse at a steaming time of 3 mins

| Pressure [MPa] | pH[–] | Organic acid produced in the liquid product of 1 g of autohydrolyzed bagasse [g] | | | | | Amount of extractive component in 1 g of autohydrolyzed bagasse [g] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | formic acid | acetic acid | levulinic acid | propionic acid | oxalic acid | holocellulose | water soluble material | methanol soluble lignin | Klason lignin |
| 1.57 | 4.6 | 0.008 | 0.007 | 0.001 | 0.001 | 0.001 | 0.61 | 0.10 | 0.02 | 0.27 |
| 2.06 | 4.1 | 0.018 | 0.017 | 0.003 | 0.001 | 0.002 | 0.53 | 0.20 | 0.08 | 0.17 |
| 2.55 | 3.9 | 0.036 | 0.035 | 0.014 | 0.001 | 0.002 | 0.51 | 0.21 | 0.12 | 0.16 |
| 3.04 | 3.8 | 0.038 | 0.041 | 0.017 | 0.001 | 0.002 | 0.49 | 0.22 | 0.11 | 0.17 |
| 3.53 | 3.8 | 0.038 | 0.042 | 0.017 | 0.001 | 0.002 | 0.49 | 0.22 | 0.09 | 0.19 |

the reactor at one time, and then steam-heated. A ball valve at the bottom of the reactor was then opened to bring the pressure of the reactor quickly down to atmospheric pressure. The solid and liquid products were recovered in a cyclone at the bottom of the receiver, and the gaseous products passed from the top of the receiver into the condenser. Steam explosions were conducted for 1~10 mins at various pressures and temperatures; 1.57 MPa (197°C), 2.06 MPa (211°C), 2.55 MPa (223°C), 3.04 MPa (235°C), and 3.53 MPa (243°C).

## Extraction

The amount of extractive components in the autohydrolyzed bagasse was measured using Wayman's method [10]. Five grams of dry autohydrolyzed bagasse was added to 300 mL of distilled water and extracted for 12 h at ambient temperature. The solids and liquid were separated by filtration, and extract 1 (water soluble material) was recovered from the liquid, then concentrated, dried, and weighed. Residue 1 (holocellulose and lignin) was extracted for 12 h in a Soxhlet extractor with 100 mL of methanol to dissolve the methanol soluble lignin (low-molecular weight lignin). After concentration and drying of extract 2 (methanol soluble lignin), the methanol soluble lignin was weighed. Residue 2 from the methanol extraction consisted of holocellulose and Klason lignin, the cellulose component. One gram of residue 2 was added to 15 mL of 72% (w/w) sulfuric acid and left to stand at room temperature for 4 h. The mixture was then placed in a 1-L conical flask, washed with 560 mL of distilled water, and then boiled for 4 h with reflux cooling. After allowing residue 3 (Klason lignin) to settle, the liquid was filtered. Residue 3 was then washed with hot water, dried at about 105°C to constant weight, and weighed. The weight of holocellulose was calculated by subtracting the weight of Klason lignin from 1 g of residue 2.

## Enzymatic Saccharification

The enzymatic saccharification of autohydrolyzed bagasse was performed in a reaction mixture containing 100 g/L of dry autohydrolyzed bagasse and 2 g/L of cellulase (1 g/L acucelase and 1 g/L meicelase) in a 0.3-L Erlenmeyer flask with 0.1 L of 0.5 M acetate buffer (pH 5). Acucelase, kindly supplied by Prof. Arai, University of Osaka Prefecture, Japan, and Meicelase, provided by Meiji Seika Co., Ltd., Tokyo, Japan, were used as a cellulase. Acucelase, which consists mainly of endo-type enzymes, is originated from *Aspergillus aculeatus* and has about 6,000 unit/g of filter paper decomposing activity. Meicelase, which consists mainly of exo-type enzymes, is originated *Tricoderma viride* and has about 8,000 unit/g of filter paper decomposing activity. One unit of filter paper decomposing activity is defined as the activity of an enzyme that makes 1 μmol glucose liberate from filter paper per minute. The reaction was performed at a shaker speed of 100 rpm and a temperature of 30°C for 120 h.

## Ethanol Fermentation

Five grams of yeast extract, 10 g of polypeptone, and 1 g of $MgSO_4 \cdot 7H_2O$ were added to 1 L of enzymatic hydrolyzate of the autohydrolyzed bagasse at a pressure of 2.55 MPa for 3 mins, and then fermented by *Pichia stipitis* CBS 5773 in a 3-L fermentor (Sakura TBR-2-1, Chiyoda Seisakujyo Co., Ltd., Tokyo, Japan) at pH 5 and a temperature of 30°C. The pH was adjusted to maintain a constant value with 1 N NaOH using a digital pH controller (MD-2C, Mituwa Co., Ltd., Osaka, Japan). The air flow rate was 1 vvm and the dissolved oxygen concentration was adjusted to a value of more than 1 ppm (about 13% of saturated oxygen) by altering the agitation speed.

## Analysis

The pH of the autohydrolyzed bagasse was measured using a pH meter (HM-26S, Thoa Denpa Kogyo Co., Ltd., Japan). The organic acids produced in the liquid product of the autohydrolyzed bagasse were measured using high performance liquid chromatography (LC-9A, Shimadzu Co., Ltd., Kyoto, Japan) with Shodex Ionpack C-811 column. The degree of polymerization of the cellulose component extracted from the autohydrolyzed bagasse was determined by Brown's method [11]. The degree of crystallinity and the micelle width of the cellulose component were measured using an X-ray diffraction apparatus (Ultrax 18HF, Rigaku Co., Ltd., Tokyo, Japan). Optical den-

sity of cultures was used as an indicator of growth and was measured using a spectrophotometer (UV-120-02, Shimadzu Co., Ltd., Kyoto, Japan) at 660 nm. Glucose concentration was measured by the mutarotase GOD method (Glucose C-Test, Wako Pure Chemicals Co., Ltd., Osaka, Japan). Xylose concentration was measured using high performance liquid chromatography (LC-9A, Shimadzu Co., Ltd., Kyoto, Japan) with Bio-Rad HPX-87 column. Ethanol was measured by gas chromatography (GC-8A, Shimadzu Co., Ltd., Kyoto, Japan) with Porapack Q column.

## RESULTS AND DISCUSSION

### Pretreatment Effect of Steam Explosion Condition on Autohydrolyzed Bagasse

Table 1 shows the pretreatment effect of pressure on the autohydrolyzed bagasse at a steaming time of 3 mins. At a pressure of 1.57 MPa, the pH was 4.6, and the pH fell to as low as 3.8 at a pressure of 3.04 MPa. Conversely, the following production of organic acids was observed: the amounts of formic acid, acetic acid, and levulinic acid increased significantly as the pressure increased, but the amounts of propionic acid and oxalic acid stayed relatively constant regardless of pressure. This suggests that the pH decreased as the pressure increased, presumably as a result of the formation of the organic acids, formic acid, acetic acid, and levulinic acid. Furthermore, acetic acid seems to be produced from the hemicellulose acetate in the bagasse by the autohydrolysis of steam explosion [10]. The amounts of extractive components varied considerably with changes in the pressure. The amount of holocellulose decreased as the pressure increased and then reached a constant value of 0.49 g, at a pressure of 3.04 MPa. In contrast, the amount of water soluble material, monosaccharides, oligosaccharides, and organic acids, increased as the pressure increased and then reached a constant value of 0.22 g, at a pressure of 3.04 MPa. The reason the amount of holocellulose decreased seems to be that some part of the holocellulose was converted into a water soluble material by the autohydrolysis of the steam explosion. The amount of methanol soluble lignin (low-molecular weight lignin) increased up to a pressure of 2.55 MPa and then decreased. Conversely, the amount of Klason lignin (high-molecular weight lignin) decreased up to a pressure of 2.55 MPa and then increased. Since in the autohydrolysis of steam explosion with a comparatively low pressure the high-molecular weight lignin was hydrolyzed into low-molecular weight lignin, the amount of Klason lignin decreased below a pressure of 2.55 MPa. The increase of Klason lignin at a comparatively high pressure depended on the recondensation reaction of the low-molecular weight lignin and the combination reaction of the water soluble material and the high-molecular weight lignin. The autohydrolyzed sample treated at a comparatively low pressure of 2.55 MPa with a small amount of high-molecular weight lignin seems to be suitable for the microbial conversion into sugars because the high-molecular weight lignin inhibited the hydrolysis reaction of the holocellulose by the amylolytic enzyme or microorganism.

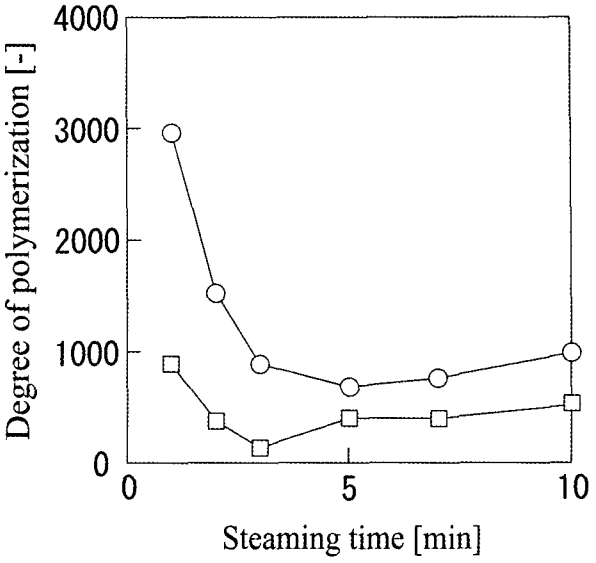


**Fig. 1.** Degree of polymerization of residue 2 (holocellulose and Klason lignin) extracted from autohydrolyzed bagasse and that of the autohydrolyzed cellulose native at a pressure of 2.55 MPa for various steaming times. Symbols: ○, bagasse; □, cellulose.

### Chemical Characteristics of the Cellulose Component in Autohydrolyzed Bagasse

Fig. 1 shows the degree of polymerization of residue 2 (holocellulose and Klason lignin), the cellulose component, extracted from the autohydrolyzed bagasse and that of the autohydrolyzed cellulose at a pressure of 2.55 MPa for various steaming times. In this experiment the cellulose provided by Merck Co. Ltd. (degree of polymerization: approximately 1,500) was used and autohydrolyzed as a standard pure cellulose material and compared with the result of the autohydrolyzed bagasse. The degree of polymerization of residue 2 decreased significantly as the steaming time increased and reached a minimum value of about 700 at a steaming time of 5 mins, and then increased slightly. In addition, the tendency of the autohydrolyzed cellulose to a change in the degree of polymerization was almost the same as that of residue 2. This means that the cellulose component was depolymerized at a comparatively short steaming time but repolymerized at a comparatively high steaming time. The reason the degree of polymerization of residue 2 was higher than that of the autohydrolyzed cellulose seems to be that the degree of polymerization of the cellulose in the bagasse, about 10,000, was larger than that in present in the cellulose, and the lignin covered cellulose inhibited the depolymerization of the cellulose in the bagasse. As a result, it was found that a steam explosion at a pressure of 2.55 MPa and a steaming time of 5 mins was the most effective method for the depolymarization of the cellulose

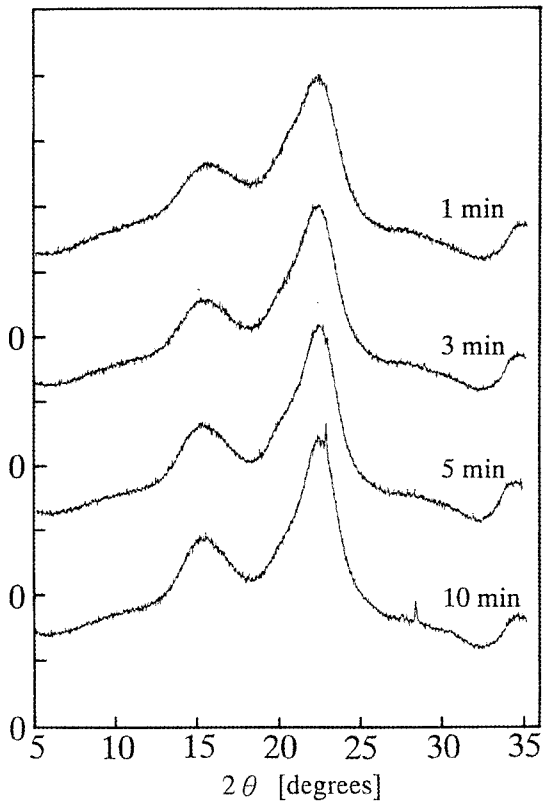


**Fig. 2.** X-ray diffractometry of residue 2 (holocellulose and Klason lignin) extracted from autohydrolyzed bagasse at a pressure of 2.55 MPa for various steaming times.

component in the bagasse.

The degree of crystallinity and the micelle width of residue 2 extracted from the autohydrolyzed bagasse were estimated using X-ray diffraction analysis. Fig. 2 shows the X-ray diffractometry of residue 2 extracted from the autohydrolyzed bagasse at a pressure of 2.55 MPa for various steaming times. The diffractometry patterns indicated that residue 2 was composed of cellulose I. Considering the shape of the peak in the X-ray diffractometry varied significantly with the steaming time, it appears as though the steam explosion affected the crystallinity of the cellulose component in the autohydrolyzed bagasse.

Fig. 3 shows the degree of crystallinity and the micelle width of residue 2 extracted from the autohydrolyzed bagasse at a pressure of 2.55 MPa for various steaming times. The degree of crystallinity increased as the steaming time increased, reaching its maximum value of 0.45 at a steaming time of 5 mins and then decreased slowly. Furthermore, the micelle width increased slightly as the steaming time increased. These results show that the steam explosion crystallized some part of the amorphous region of the cellulose but a longer steaming time converted the crystalline area back to a noncrystalline area again. Tanahashi *et al.* [12] have also reported that X-ray diffractometry analysis of steam exploded birch and larch showed an increase in the degree of crystallinity and the

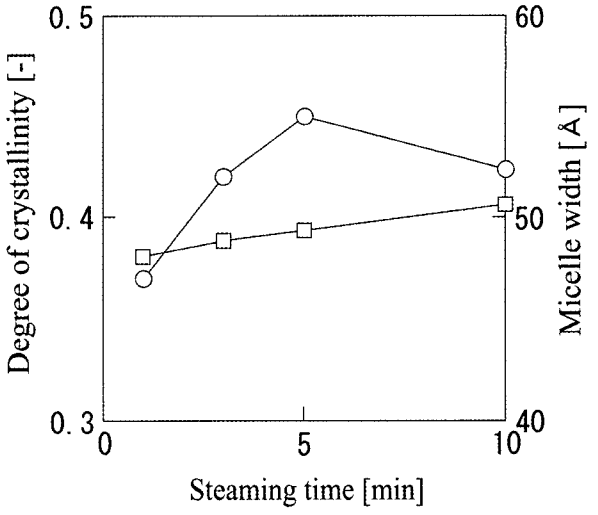


**Fig. 3.** Degree of crystallinity and micelle width of residue 2 (holocellulose and Klason lignin) extracted from autohydrolyzed bagasse at a pressure of 2.55 MPa for various steaming times. Symbols: ○, degree of crystallinity; □, micelle width.

micelle width of cellulose components. These results indicates that some amorphous region of cellulose was transformed to crystalline region by the steam explosion, resulting in the increase in the degree of crystallinity and the micelle width of the cellulose component of the autohydrolyzed lignocellulosic materials such as grass and wood. Furthermore, it was proven that the steam explosion caused the property change of the cellulose component in the bagasse because the tensile strength, Young's modulus, hardness, density, among other indicators increased as the level of crystallinity increased, and the elongation, toughness, flexibility, water adsorption, chemical reactivity, among other indicators decreased as the level of crystalinity increased [13].

## Enzymatic Saccharification of Autohydrolyzed Bagasse

It is well known that mixed enzymes having different hydrolysis reactions display a higher reactivity in enzymatic saccharification than a single enzyme; this is referred to as a synergy effect [14]. Meicelase and acucelase are two cellulase having different reactions with cellulose. Meicelase is produced from *T. viride* and consists mainly of exo-type enzymes that cut the cellobiose units from the outside of a cellulose fiber. Acucelase is produced from *A. aculeatus* and consists mainly of endo-type enzymes that randomly cut the cellobiose units from the inside of the cellulose fiber. The enzymatic saccharification of autohydrolyzed bagasse using mixed enzymes such as meicelase and acucelase was investigated in an attempt to produce an efficient process for converting bagasse into sugars. Fig. 4 shows the enzymatic saccharification of 100 g/L of autohydrolyzed bagasse at a steaming time of 3 mins at various pressures using meicelase and acucelase. The concentrations of glucose and xylose increased as the incubation time increased, reaching their

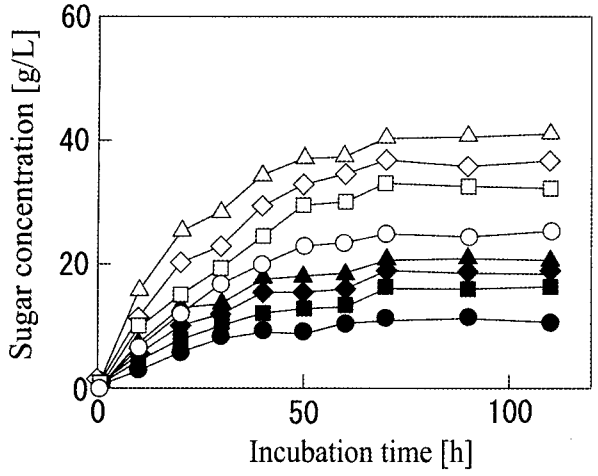


**Fig. 4.** Enzymatic saccharification of 100 g/L of autohydrolyzed bagasse at a steaming time of 3 mins at various pressures. Symbols: ○, glucose at 1.57 MPa; ●, xylose at 1.57 MPa; □, glucose at 2.06 MPa; ■, xylose at 2.06 MPa; △, glucose at 2.55 MPa; ▲, xylose at 2.55 MPa; ◇, glucose at 3.04 MPa; ◆, xylose at 3.04 MPa.

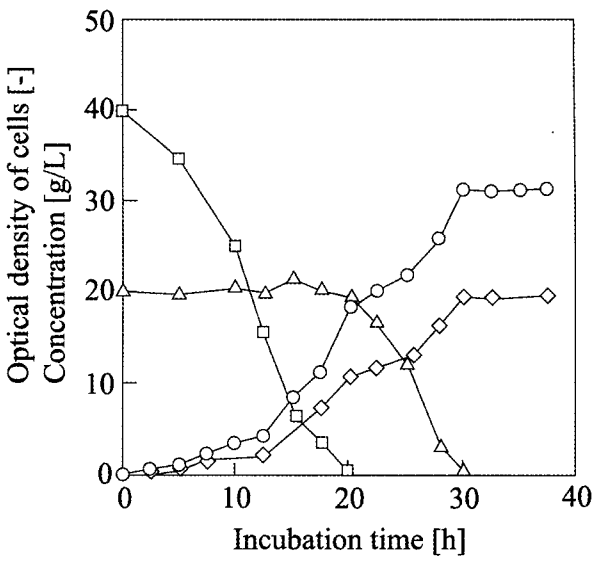


**Fig. 5.** Ethanol fermentation of enzymatic hydrolyzate of autohydrolyzed bagasse at a pressure of 2.55 MPa and a steaming time of 3 mins. The cells incubated in the glucose medium were transferred into the enzymatic hydrolyzate medium. Symbols: ○, optical density of cells; □, glucose; △, xylose; ◇, ethanol.

maximum values at an incubation time of about 70 h. Monosaccharides such as galactose, arabinose, and mannose were also produced, but in very small amounts compared to the amounts of glucose and xylose (data not shown). The maximum concentrations of glucose and xylose in the enzymatic saccharification of the autohydrolyzed bagasse at a pressure of 2.55 MPa were larger than those at a pressure of 1.57, 2.06, and 3.04 MPa with values of approximately 40 and 20 g/L, respectively. The reason the maximum concentrations decreased at a higher pressure of 3.04 MPa, was as a result of the fact that the Klason lignin undergoes a condensation reaction with methanol soluble lignin, causing a lower susceptibility of holocellulose to the enzymes as shown in Table 1. The ratio of the dry weight of both glucose and xylose produced to the dry weight of the autohydrolyzed bagasse at a pressure of 2.55 MPa was about 0.6; this means that the holocellulose and the water soluble material were both converted into monosaccharides at a comparatively high yield. Furthermore, both glucose and xylose were produced in the enzyme saccharification of the autohydrolyzed bagasse, and the ratio of glucose to xylose was almost 2:1. This means the ratio of cellulose, composed of glucose subunits, and hemicellulose, composed of xylose subunits, is about 2:1 in bagasse.

### Ethanol Fermentation of Enzymatic Hydrolyzate of Autohydrolyzed Bagasse

Fig. 5 shows the time courses of optical density of cells, glucose concentration, xylose concentration, and ethanol concentration in the ethanol fermentation of enzymatic hydrolyzate of 100 g/L of autohydrolyzed bagasse using *P. stipitis* precultured in the glucose medium. This enzymatic hydrolyzate medium contained about 40 g/L of glucose and 20 g/L of xylose. When the cells that were precultured in the glucose medium were transferred to the enzymatic hydrolyzate medium, they increased with the degradation of only the glucose without the consumption of xylose and then grew with the degradation of xylose after showing a lag time, presumably for synthesizing an inducible enzyme necessary to degrade xylose; this is a typical diauxic growth [15,16]. The ethanol was produced with the growth of cells and the tendency in the time course of the ethanol production was almost similar to that of the cell growth. This means that ethanol is a primary metabolite of this microorganism. The maximum optical density of cells and the maximum amount of ethanol produced were obtained at an incubation time of about 30 h; their values were about 32 and 20 g/L, respectively. As a result, it was found that *P. stipitis* was an effective yeast for the ethanol fermentation of enzymatic hydrolyzate of autohydrolyzed bagasse containing not only glucose but also xylose. In this experiment, the ethanol yield from sugars was 0.33 g/g sugars. Ferrari *et al.* [17] studied the ethanol production from eucalyptus wood hemicellulose hydrolysate by *P. stipitis* and reported that the ethanol yield of 0.35 g/g sugars was obtained from the wood treated using a Wiley mill till to pass through a 2 mm opening sieve followed by a hydrolysis with 0.5% $H_2SO_4$ at a temperature of 120°C for 180 mins. Since the acid or alkaline treatment requires the post-treatment, it seems that the steam explosion is not only a rapid pretreatment for ethanol production from lignocellulosic material but also an environmentally friendly method.

Fig. 6 shows the time courses of optical density of cells, glucose concentration, xylose concentration, and ethanol concentration in the ethanol fermentation of enzymatic

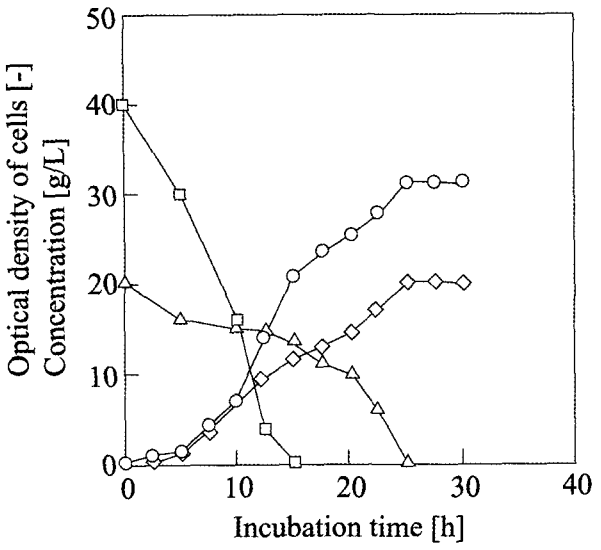


**Fig. 6.** Ethanol fermentation of enzymatic hydrolyzate of autohydrolyzed bagasse at a pressure of 2.55 MPa and a steaming time of 3 mins. The cells incubated in the xylose medium were transferred into the enzymatic hydrolyzate medium. Symbols: ○, optical density of cells; □, glucose; △, xylose; ◇, ethanol.

hydrolyzate of 100 g/L of autohydrolyzed bagasse using *P. stipitis* precultured in the xylose medium. When the cells that were precultured in the xylose medium were transferred into the enzymatic hydrolyzate medium, both glucose and xylose were simulateously consumed in the early stage of incubation; this means that the reactivity of the inducible enzyme responsible for xylose metabolism was not affected by the glucose. The initial growth rate was almost equal to the sum of the growth rates of glucose consumption and xylose consumption, but the growth rate gradually decreased as the incubation time increased, approaching the growth rate of only glucose consumption. Since the synthesis of the inducible enzyme for xylose metabolism is strongly repressed in the presence of glucose, this finding can be explained by the decrease in the inducible enzyme content per cell during cell growth. In this incubation, the maximum optical density of cells, 32, and the maximum amount of ethanol produced, 20 g/L, were obtained at a shorter incubation time of about 25 h. If the glucose concentration was kept lower than the limit below which the inducible enzyme for xylose metabolism could be synthesized, both glucose and xylose may be consumed simultaneously. Therefore, a fed batch culture or a continuous culture maintaining the glucose concentration below the limit glucose concentration appears to be an effective incubation method for the ethanol fermentation of enzymatic hydrolyzate containing both glucose and xylose. This point requires subsequent future research.

## CONCLUSION

Bagasse, a lignocellulosic material, was treated under various steam explosion conditions, and the chemical characteristics, enzymatic saccharification, and ethanol fermentation of the autohydrolyzed bagasse were examined. The pH of autohydrolyzed bagasse decreased as the pressure increased due to the formation of the organic acids such as formic acid, acetic acid, and levulinic acid. The amounts of extractive components, holocellulose, water soluble material, methanol soluble lignin, and Klason lignin, varied with changing the steam explosion conditions. Steam explosion at pressure of 2.55 MPa and a steaming time of 3 mins was the most effective for the delignification of bagasse. The degree of polymerization of the cellulose component in bagasse decreased up to about 700 by steam explosion. The degree of crystallinity and the micelle width of the cellulose component in bagasse were found to be increased by steam explosion. Autohydrolyzed bagasse was converted into ethanol with a comparatively high yield by enzymatic saccharification using mixed cellulases, acucelase and meicelase, followed by ethanol fermentation using *P. stipitis*. About 20 g of ethanol was produced from 100 g of autohydrolyzed bagasse.

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