博士論文

Microbial Xylitol Production from Various Hemicellulose Hydrolyzates by the Yeast *Candida Magnoliae*

酵母 *Candida magnoliae* を用いた様々なヘミセルロース加水分解からの
キシリトール発酵生産

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CONTENS

CHAPTER I. GENERAL INTRODUCTION

Recently, the use of lignocellulosic wastes, such as whole small size trees, bamboos, straw, sugar cane bagasse, and corn cob, as a renewable source of biofuels and chemical feedstuffs, has received great interest. In the biosphere 27×10^{10} tons of carbon are bound in living organisms, more than 99% of which are plants [1].Cell walls of plant tissues are composed of three main components, *i.e*., cellulose, hemicelluloses, and lignin. Next to cellulose and lignin, hemicelluloses are the most plentiful organic substances in the biosphere.

In angiosperms, the hemicelluloses occur mainly as a variation of glucuronoxylans. Hardwood xylan contains about 8 to 17% acetyl groups in the xylose units [2]. Attachment of the sugar branches to the main chain of (1→4)-linked β-$_D$-xylopyranosyl residues is often by α-1,3-glycosidic bonds, while 4-*O*-methyl-$_D$-glucuronopyranosyl or $_D$-glucuronopyranosyl stubs are linked at the *O*-2 of the xylosyl residues. In gramineous plants, the main chain of (1→4)-linked β-$_D$-xylopyranosyl residues are attached α-$_L$-arabinofuranosyl or α-(2-*O*-β-$_D$-xylopyranosyl-$_L$-arabinofuranosyl) stubs at the *O*-3 and 4-*O*-methyl-$_D$-glucurono-pyranosyl or $_D$-glucuronopyranosyl stubs at the *O*-2 of the xylosyl residues. Bamboo xylan contains 6 to 7% acetyl groups in the xylose units [3].

It is known that acid hydrolytic cleavage of glycosidic bonds in polysaccharides proceeds in three steps (Figure 1) [4]. In the first step, the proton of the catalyzing acid interacts rapidly with the glycoside oxygen linking two sugar units (I), forming a conjugate acid (II). This step is followed by a slow cleavage of the C-O bond yielding an intermediate cyclic carbonium cation (III). The protonation may also occur at the pyranose ring oxygen (II'), resulting in a ring opening to form a non-cyclic carbonium cation (III'). It is uncertain which type of carbonium cation is most likely to be formed. However the protonation may occur mainly on the oxygen of glycosidic linkage to form conjugate acid II. Finally a water molecule adds to the carbonium cations to form the stable end product and to release a proton.

The hydrolysis rates of glycosidic bonds are significantly affected by both the steric and inductive effects of sugar substituents, as shown in Table 1 [5]. Furanoside rings are hydrolyzed more rapidly than those of pyranoside, due to higher structural angle strains in the conformation of furanose units compared to the strain-free pyranose ring. α-$_D$-Galactofuranosides are hydrolyzed about 100 times more quickly than α-$_D$-galactopyranosides [5]. Electrophilic substituents such as carboxyl or carbonyl groups reduce protonation and prevent C-O cleavage, thus having a stabilizing effect on the glycosidic bond. As shown in Table 1, the hydrolysis rate of glycosidic bond in β-$_D$-xylan is about 5 times more rapid than those of β-$_D$-glucan. Therefore hydrolysis of hardwoods or gramineous plants with dilute mineral acids under relatively mild conditions gives a sugar solution containing high concentration of $_D$-xylose. $_D$-Xylose can be converted to xylitol or useful chemicals such as furfural.

Interest has increased in the use of xylitol as a sweetener because of its high sweetening power to create sweetness, negative heat of dissolution, and anticariogenic properties. Xylitol is a normal metabolic intermediate in mammalian carbohydrate metabolism with an endogenous production. Adult human generally uses 5 to 15 g of xylitol daily. It absorbs slowly from human digestive tract and entries into metabolic pathway independently of insulin. The glycemic index of xylitol (GI = 13) compares favorably with that of sucrose (GI = 65) [6]. Xylitol is, therefore, used clinically as a sucrose substituent for diabetes.

Xylitol has been widely found in fruits and vegetables. However the small quantities present in nature make its extraction impractical. It is currently produced by a catalytic reduction of xylose present in the spent sulfite cooking liquors of hardwood chips or corn cob hemicellulose hydrolyzates. Because the hemicelluloses of hardwoods or gramineous plants contain other monosaccharides, such as $_{L}$-arabinose and $_{D}$-glucose, extensive separation and purification steps are necessary to remove these contaminants before chemical reduction. The inefficiency of current xylose preparation techniques seriously affects the recovery of xylose from lignocellulosic raw materials. The yields of xylitol correspond to only 50% to 60% of xylan present in the raw materials [7].

An alternative method for the xylitol production is microbial conversion of $_{D}$-xylose in the hemicellulose hydrolyzates [8]. Among the microorganisms that can assimilate xylose, the

yeasts belonging to the genus *Candida* are the best xylitol producers [9]. Although the microbial production of xylitol requires additional costs for the detoxification of hemicellulose hydrolyzates and purification of xylitol in the fermentation media, the additional costs for the environmentally friendly process may be accepted by ecology-minded consumers.

This study was performed to establish the microbial xylitol production from locally available lignocellulosic wastes by the yeaste, *Candida magnoliae*. Hemicellulose hydrolyzates were prepared from different lignocellulosic raw materials, *i.e*., the culms of bamboo (*Phyllostachy pubescens*) and bamboo grasses (*Sasa* genus), and Japanese white birch wood (*Betula platyphylla* var. *japonica*). These raw materials were subjected to hydrolysis with dilute sulfuric acid to give sugar solutions rich in $_{D}$-xylose. In the Chapter I, the effects of hydrolysis conditions on the sugar composition of the hydrolyzate were examined, in order to establish the optimal hydrolysis conditions for preparing the fermentation substrate. It is known that acid hydrolyzates of lignocelluloses usually contain not only fermentable sugars but also some undesirable byproducts, such as acetic acid, furan derivatives, and solubilized lignin fragments. They act as an inhibitor of the microbial metabolism [10]. Direct use of the hydrolyzates reduces the efficiency of the growth of microorganisms and the product formation [11]. Detoxification of the hydrolyzates before the fermentation is necessary for the successful bioconversion of the solubilized sugars. In the Chapter II, the detoxification of the hydrolyzates with carbonaceous

sorbents and an anion exchange resin was evaluated. It has been reported that yeast cultures adapted to the hemicellulose hydrolyzates by repeated recycling were used to overcome the inhibition of microbial metabolism caused by the toxic compounds present in the hydrolyzates [12-15]. In this study, adaptation of the parent strain of the yeast, *C. magnoliae* was performed by sequentially transferring and growing cells in media containing increasing concentrations of the hydrolyzate. The effect of adaptation on the fermentation performance is evaluated in the Chapter III. The microbial production of xylitol depends on the fermentation conditions employed [16]. Together with initial substrate concentration, the aeration is the most significant of all parameters that affect the microbial production of xylitol [17, 18]. The effects of aeration and initial concentration of $_{D}$-xylose on the bioconversion of the hydrolyzates is discussed in the Chapter IV.

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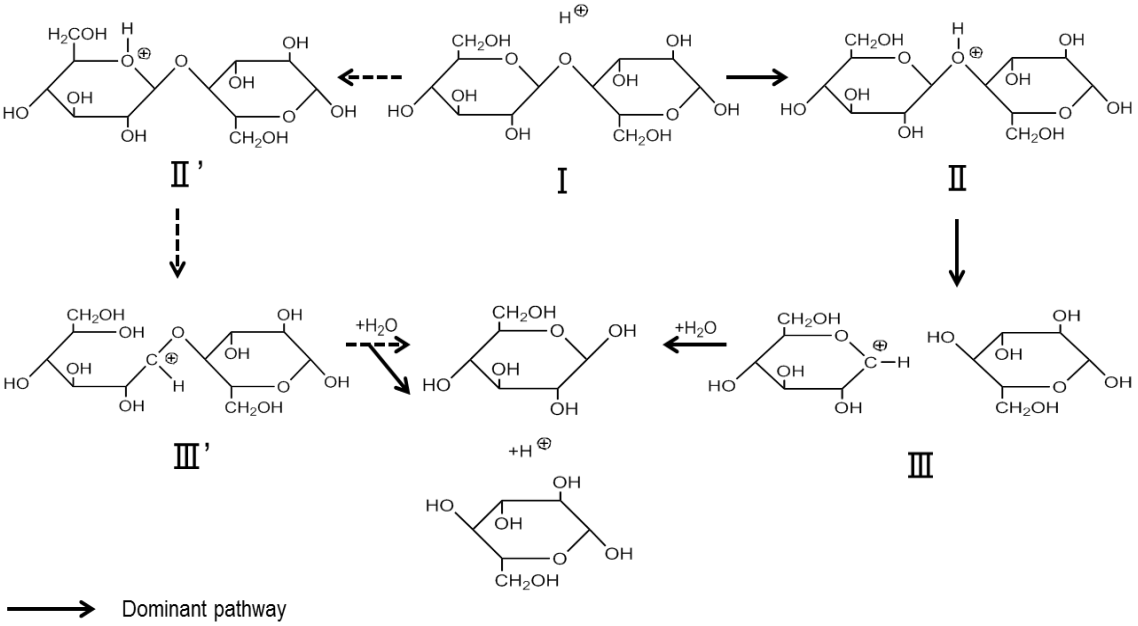

Figure 1. Proposed mechanism for acid hydrolysis of glycosidic bonds

Table 1. Relative hydrolysis rate of methylglycosides with 0.5 N hydrochloric acid at 75°C*[1]

	Relative hydrolysis rate
Methyl-α-$_D$-glycoside	0.4
Methyl-β-$_D$-glycoside	0.8
Methyl-α-$_D$-mannoside	1.0
Methyl-β-$_D$-mannoside	2.4
Methyl-α-$_D$-galactoside	2.2
Methyl-β-$_D$-galactoside	3.9
Methyl-α-$_D$-xyloside	1.9
Methyl-β-$_D$-xyloside	3.8
Methyl-α-$_L$-arabinoside	5.5
Methyl-β-$_L$-arabinoside	3.8

*[1]After F. Shafizadeh [5].

CHAPTER II. ACID HYDROLYSIS OF LIGNOCELLULOSES

Introduction

Bamboos and bamboo grasses belong to the gramineous plant family. Bamboos occur in natural vegetation of tropical, subtropical, and temperate regions except Europe. They are the fastest growing plants and reach their full height of 15 m to 35 m within a few months by diurnal growth rates of about 20 cm up to 100 cm [1]. Bamboo grasses are only distributed in a limited area of the Far East, and also characterized by their rapid growth [2]. Because the hemicelluloses of gramineous plants are mainly composed of arabinoglucuronoxylans, bamboos and bamboo grasses are considered to be a potential source of $_{D}$-xylose [3, 4].

It has been reported that $_{D}$-xylose was obtained in good yields from several species of hardwoods [5-9], sugarcane bagasse [10], corn cob [11], sorghum straw [12], rice straw [13], and oil palm empty fruit bunch [14] by hydrolysis with dilute sulfuric acid. In this study, the culms of *Sasa kurilensis* (common name: Chishimazasa) and *Phyllostachy pubescens* (common name: Mosochiku) were hydrolyzed with dilute sulfuric acid to prepare the substrates for the microbial xylitol production. To establish the optimal hydrolysis conditions, the effects of sulfuric acid concentration, residence time, and liquor-to-solid ratio on the sugar composition of the hydrolyzates were examined.

Materials and Methods

The air-dried plant materials were ground in an Ultra Centrifugal Mill ZM1 (Retsch, Haan, Germany). The ground samples (P42-R80 mesh) were extracted in a Soxhlet apparatus with a benzene-ethanol mixture (2:1, v v^{-1}) for 48 h. The defatted samples were further refluxed with water for 3 h. The resulting residues were air-dried, homogenized, and used in the hydrolysis experiments. The extracted residues were hydrolyzed with dilute sulfuric acid at 121°C for 1 h using an autoclave. Neutral sugars in the hydrolyzates were determined by HPLC equipped with RI detection and an Aminex HPX-87P column (300 × 7.8 mm, Bio-Rad, Richmond, VA) eluted with water as eluent at a flaw rate of 0.6 ml min^{-1} and at 85°C. The overall content of furan derivatives and lignin degradation products was estimated by the absorbance at 280 nm (A_{280}) [15].

Results and Discussion

Table 2 shows the effect of sulfuric acid concentration on the neutral sugar composition of the culm hydrolyzate of bamboo grass (*S. kurilensis*). Both the yields of $_{D}$-xylose and $_{L}$-arabinose increased with increasing sulfuric acid concentration in the range of 1-3%. An additional increase in the sulfuric acid concentration resulted in thermal decomposition of the solubilized pentoses. In contrast, the concentration of $_{D}$-glucose in the hydrolyzate stayed almost constant, indicating that $_{D}$-glucose originated from storage carbohydrates in the culm [2]. In this study, the generation of furan derivatives and lignin degradation products from cell wall components was evaluated by the absorbance of the hydrolyzate at 280 nm (A_{280}) [15]. These compounds act as an inhibitor of microbial metabolism [16]. The A_{280} increased in proportion to the sulfuric acid concentration. Although a maximum yield of $_{D}$-xylose was obtained by hydrolysis with 3% sulfuric acid, taking into account the selectivity of xylan saccharification and the generation of the inhibitors, 2% sulfuric acid is considered to be an optimal sulfuric acid concentration for the bamboo grass xylan.

Table 3 shows the effect of sulfuric acid concentration on the neutral sugar composition of the culm hydrolyzate of bamboo (*P. pubescens*). The yield of $_{D}$-xylose increased with increasing sulfuric acid concentration in the range of 1-3%. An additional increase in the sulfuric acid concentration resulted in thermal decomposition of $_{D}$-xylose. The yield of $_{L}$-arabinose stayed

almost constant at sulfuric acid concentration in the range of 1-4%, whereas D-glucose yield increased slightly with increasing the sulfuric acid concentration. The bamboo culm hydrolyzate is characterized by relatively low content of D-glucose, compared with those of bamboo grasses [17, 18]. The A_{280} of the hydrolyzate increased in proportion to the sulfuric acid concentration. From Table 3, 3% sulfuric acid is considered to be an optimal sulfuric acid concentration for the saccharification of bamboo xylan. Yamaguchi and Aoyama [9] reported that, when the hydrolysis condition was fixed at 121°C and for 1 h, a maximum yield of D-xylose from Japanese linden wood (*Tilia japonica*) was obtained by treatment with 3% sulfuric acid. Bamboo and hardwoods xylans seem to be less susceptible to acid hydrolysis, compared with those of bamboo grasses. The difference in the susceptibility among these xylans can be explained by their chemical structures. It has been reported that, when the culm of *S. senanensis* was steamed at 183ºC for 20 min, a sugar solution containing D-xylose and 4-*O*-methyl-D-glucuronic acid in a molar ratio of 65:1 was obtained [19]. On the other hand, most of xylans isolated from various hardwood species have a D-xylose and 4-*O*-methyl-D-glucuronic acid ratio of 10:1 [20]. The bamboo xylan contains D-xylose, arabinose, and 4-*O*-methyl-D-glucuronic acid in a molar ratio of 24-25:1.0-1.3:1.0 [3]. Hardwood and bamboo xylans are characterized by high content of acidic sugar units, compared with those of bamboo grasses. Electrophilic substituents such as carboxyl group on the

neighboring sugar unit reduce protonation on the glycosidic oxygen bonding two sugar units to stabilize the glycosidic bond, as shown in Figure 2. Xylans of hardwoods and bamboo culm are, therefore, less susceptible to acid hydrolysis than those of bamboo grasses under mild hydrolysis conditions.

Tables 4 and 5 show the effect of residence time on the neutral sugar compositions of the *S. kurilensis* and *P. pubescens* hydrolyzates, respectively. The yield of D-xylose in the culm hydrolyzate of *S. kurilensis* increased with increasing the residence time up to 90 min. However, for the residence time ranging 40-90 min, there is no significant difference in the total yield of solubilized sugars (26.3-26.8%). A prolonged residence time (120 min) resulted in a decrease in the D-xylose yield. For the *P. pubescens* culm hydrolyzate, the D-xylose yield increased with increasing the residence time up to 60 min. A prolonged residence time (90 min and beyond) resulted in a decrease in the D-xylose yield. The A_{280} of these hydrolyzates increased steadily with increasing residence time. As shown in Table 6, the concentration of D-xylose in the *P. pubescens* culm hydrolyzate increased with decreasing the liquid-to-solid ratio. However, the A_{280} value of the hydrolyzate also increased with a decrease in the ratio. It is noted that, when the substrate is not completely soaked in dilute sulfuric acid, an incomplete xylan hydrolysis as well as thermal decomposition of solubilized sugars may take place.

In conclusion, fermentable sugar solutions with relatively high xylose concentration can be prepared from the culms of bamboo and bamboo grasses by hydrolysis with dilute sulfuric acid. The concentration of $_D$-xylose in the hydrolyzates increased with decreasing the liquid-to-solid ratio. When lower liquid-to-solid ratio (less than 5 g g^{-1}) is employed, the A_{280} is too high to perform the microbial conversion of $_D$-xylose in the hydrolyzate. Tada *et al*. [15] reported that successful xylitol production from diluted corn cob hydrolyzates required an A_{280} value below 0.02. Therefore, detoxification of the hydrolyzates before fermentation is necessary for the successful bioconversion of sugar solutions to xylitol or to ethanol.

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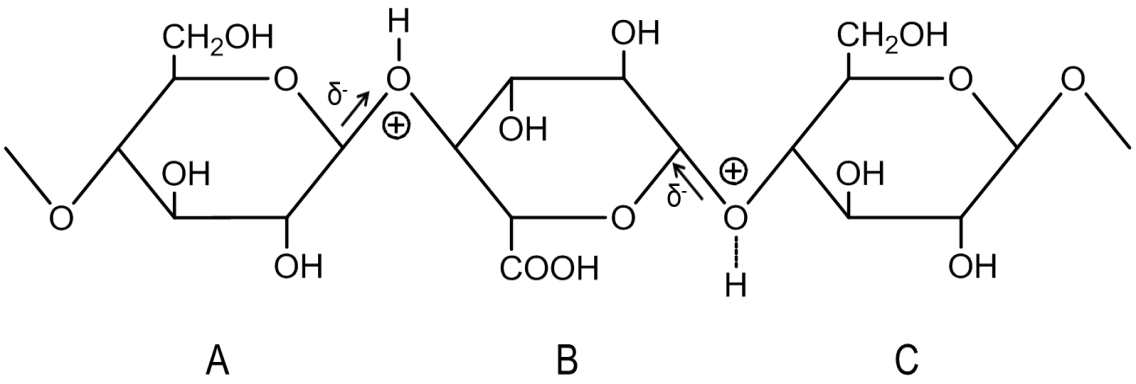

Figure 2. Inductive effect of carboxyl group on mild acid hydrolysis of polysaccharides

Table 2. Effect of sulfuric acid concentration on the chemical composition of *Sasa kurilensis* hemicelluloses hydrolyzates[1]

Sulfuric acid concentration (%)	Yield (%, dry material basis)				A_{280}[2]
	D-Xylose	L-Arabinose	D-Glucose	D-Mannose	
4.0	17.3	1.3	4.5	0.1	11
3.0	21.4	1.8	5.3	0.8	7
2.0	20.1	1.6	5.1	nd[3]	6
1.0	18.3	1.1	5.1	Nd	3

[1] The pre-extracted culm (0.3 g) was hydrolyzed with dilute sulfuric acid (87 g) at 121°C for 1 h.

[2] Absorbance of the hydrolyzate at 280 nm.

[3] Less than detection limit (< 0.01%).

Table 3. Effect of sulfuric acid concentration on the chemical composition of *Phyllostachy pubescens* hemicelluloses hydrolyzates[1]

Sulfuric acid concentration (%)	Yield (%, dry material basis)				A_{280}[2]
	D-Xylose	L-Arabinose	D-Glucose	D-Mannose	
4.0	19.7	1.0	0.9	0.1	10
3.0	20.5	0.9	0.7	0.1	7
2.0	19.3	0.9	0.5	nd[3]	5
1.0	17.0	0.8	0.2	Nd	3

[1-3]See Table 2.

Table 4. Effect of residence time on the chemical composition of *S. kurilensis* hemicelluloses hydrolyzates[1]

Residence time (min)	Yield (%, dry material basis)				A_{280}[2]
	D-Xylose	L-Arabinose	D-Glucose	D-Mannose	
40	19.6	1.1	4.8	0.8	6
60	20.1	1.6	5.1	nd[3]	6
90	20.6	1.0	4.5	0.2	8
120	18.9	1.1	4.8	0.3	10

[1] The pre-extracted culm (0.3 g) was hydrolyzed with 2% sulfuric acid (87 g) at 121°C.

[2] The absorbance of the hydrolyzate at 280 nm.

[3] Less than detection limit (< 0.01%).

Table 5. Effect of residence time on the chemical composition of *P. pubescens* hemicelluloses hydrolyzates[1]

Residence time (min)	Yield (%, dry material basis)				A_{280}[2]
	D-Xylose	L-Arabinose	D-Glucose	D-Mannose	
30	18.0	0.7	0.5	nd[3]	5
60	20.5	0.9	0.7	0.1	7
90	19.8	0.9	0.8	0.1	9
120	19.7	1.0	1.2	0.1	12

[1] The pre-extracted culm (0.3 g) was hydrolyzed with 3% sulfuric acid (87 g) at 121°C.

[2] The absorbance of the hydrolyzate at 280 nm.

[3] Less than detection limit (< 0.01%).

Table 6. Effect of liquid-to–solid ratio on the concentration of neutral sugars in *P. pubescens* hemicelluloses hydrolyzates[1]

Liquid-to-solid ratio (g g^{-1})	Concentration (g L^{-1})			A_{280}[2]
	$_D$-Xylose	$_L$-Arabinose	$_D$-Glucose	
290	0.065	0.03	0.02	7
10	18.9	0.8	0.8	119
5	40.0	2.0	2.0	188
3	61.1	2.6	2.2	262

[1-2] See Table 5.

CHAPTER III. DETOXIFICATION OF HYDROLYZATES

Introduction

Hydrolysis with dilute mineral acids under relatively mild conditions is the most conventional method employed for preparing D-xylose from hardwoods and gramineous plant wastes. The resulting sugars can be converted to xylitol or to ethanol by fermentation. However, besides solubilized sugars, hemicellulose hydrolyzates usually contain undesirable byproducts, such as organic acids, furan derivatives, and lignin degradation products. They act as an inhibitor of the microbial metabolism. Detoxification of the hydrolyzates before the fermentation is necessary for the successful bioconversion of the solubilized sugars.

Various detoxification methods including biological, physical and chemical ones have been proposed to transform inhibitors to inactive compounds or to reduce their concentration [1, 2]. Among these, charcoal sorption is considered to be a suitable method for removing furan derivatives and low molecular weight phenols [3, 4]. It has been reported that charcoal treatment improved the conversion of sugars in the wood hydrolyzates into xylitol [5] or into ethanol [4, 6] by yeasts. Ion exchange resins also improved the ethanol production from sugar cane bagasse hydrolyzates [7, 8]. Canilha *et al*. [9] reported that the xylitol yield factor provided by the ion exchange resins treatment of eucalyptus hydrolyzate (0.76 g g^{-1}) was higher than the one

provided by the activated charcoal treatment (0.66 g g^{-1}). In this study, the effect of the sorbents on removal of the inhibitors in the hydrolyzates of Japanese white birch (*Betula platyphylla* var. *japonica*) wood was examined. Sorbents include three different carbonaceous materials and a commercially available anion exchange resin.

Materials and Methods

Acid hydrolysis and analytical methods

The ground wood of Japanese white birch (P42 - R80 mesh) was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120 °C for 1 h. Neutral sugars in the hydrolyzate were determined by HPLC as described previously. Furfural and acetic acid were determined by HPLC with RI detection using a Shodex SH column (300 × 8 mm, Showa Denko, Tokyo, Japan) eluted with 0.01 M sulfuric acid at a flaw rate of 0.7 ml min^{-1} and at 50°C. For the determination of lignin degradation products, the pH of the hydrolyzates was adjusted to 12.0 before diluted 1:1000 with distilled water. The content of lignin degradation products in the diluted hydrolyzate was estimated according to the method of Mussatto *et al*. [10].

Preparation of carbonaceous sorbent from hydrolysis residue

The hydrolysis solid residue of Japanese white birch wood was neutralized with dilute ammonia solution, washed thoroughly with hot water and dried in an electric oven at 105 °C. The residue was treated with concentrated sulfuric acid according to the method of Namasivayam and Kadirvelu [11]. The sorbent was prepared by mixing 1 part of the hydrolysis solid residue, 1.8 parts of concentrated sulfuric acid and 0.1 part of ammonium persulfate and keeping the mixture in an electric oven at 80°C for 12 h. The reaction mixture was soaked in water, and the supernatant was neutralized with dilute ammonia solution to remove any residual acid. The solid residue was washed thoroughly with hot water, dried in an electric oven at 105°C, and pulverized to pass a 100 mesh screen. The yield of the sorbent was 68.9 ± 0.6% **(**mean ± SD, n = 5, based on dry hydrolysis solid residue).

Detoxification

Three different carbonaceous sorbents, *i.e*., a dehydration product prepared from the birch wood hydrolysis residue, steam- and $ZnCl_2$-activated charcoals, and an anion exchange resin (Amberlite IRA 67, Organo Corp., Tokyo, Japan) were used for the detoxification experiments.

Sorption experiments were conducted by agitating 20 ml of the hydrolysate with desired amounts of sorbents in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h.

Results and Discussion

When Japanese white birch wood was hydrolyzed with 3% sulfuric acid at 120ºC for 1 h, a sugar solution containing 42.9 g l^{-1} $_D$-xylose could be obtained. However the hydrolyzate also contained significant amounts of undesirable compounds, such as acetic acid, furfural and low molecular weight phenols originated mainly from lignin. Watson *et al*. [7] have reported that the maximum specific growth rate of *Pachysolen tannophilus* was reduced by 63% at a furfural concentration of 0.35 g l^{-1}. Delgenes *et al*. [12] found that the cell growth of *C. shehatae* was reduced by 38% at furfural concentration of 1 g l^{-1}. Detoxification before the fermentation run is, therefore, necessary for the successful bioconversion of the birch wood hydrolyzate.

Tables 7 and 8 show the chemical composition of the birch wood hydrolyzate treated with three different carbonaceous sorbents. Sorption properties of carbonaceous sorbents are due to factors such as surface area, porous structure and degree of surface reactivity [13]. Chemical composition and the BET surface area of the sorbents are listed in Table 9.

When the hydrolyzate treated with these sorbents, the amounts of furfural and lignin degradation products decreased with increasing the amount of sorbents added, whereas the concentrations of neutral sugars and acetic acid stayed almost constant. The results indicate that furfural and lignin degradation products are selectively removed from the hydrolyzate by sorption onto the carbonaceous sorbents. Among these inhibitors, lignin degradation products, such as Hibbert's ketones, vanillin and syringaldehyde, are more toxic than furan derivatives [3, 5, 12, 14]. In this study, interest was focused on removal of lignin degradation products monitored by the absorbance at 280 nm (A_{280}). Sorption capacities of the steam-activated charcoal were superior to those of the other sorbents. Although the $ZnCl_2$-activated char has the largest surface volume (Table 9), its sorption capacities to the inhibitors was inferior to those of the steam-activated char. The $ZnCl_2$-activated char is probably rather suitable for relatively large molecular size of the sorbate because of its well-developed mesoporous structure [13]. It has been reported that 20 g l^{-1} of the steam-activated char was necessary for detoxifying bamboo hydrolyzate containing 19 g $l^{-1}$of D-xylose [15]. D-Xylose in the detoxified hydrolyzate was converted into xylitol in about 60% yield by *C. magnoliae*. The circulating price of the activated charcoal required corresponds to more over half of the product price. Activated charcoals are generally too expensive to employ as a sorbent for detoxifying lignocellulose hydrolyzates. On the other hand, mild hydrolysis of lignocelluloses produces waste fiber in large quantities. In

this study, we prepared an alternative sorbent from the hydrolysis residue of Japanese white birch wood by dehydration with concentrated sulfuric acid. The use of the hydrolysis residue fits into the biorefinery concept.

Although the pore structure of the dehydration product prepared from the acid hydrolysis residue was not sufficiently developed, when the dose increased up to 125 g l^{-1}, the concentrations of furfural and lignin degradation products reduced greatly. Undesirable effects caused by these inhibitors on the microbial metabolism can be eliminated. On the other hand, acetic acid, which releases from acetyl groups of the birch wood xylan, could not be removed by treatment with the carbonaceous sorbents used (Table 8). At the optimal pH range of fermentation (pH 4-5), acetic acid occurs largely in an undissociated form. After its diffusion into cell cytoplasm, it dissociates, uncouples the energy production and impairs transport of nutrients. van Zyl *et al*. [8] reported that, under oxygen limited conditions, the presence of 10 g l^{-1} acetic acid in the synthetic fermentation medium containing 50 g l^{-1} $_D$-xylose caused a serious reduction in the ethanol production by *Pichia stipites* (33% reduction in the ethanol yield and 56% reduction in the volumetric ethanol productivity). The authors also observed that, when sugarcane bagasse hydrolyzate was treated with a weak basic anion exchange resin, 84% of the acetic acid in the hydrolyzate was removed and the subsequent ethanol fermentation was greatly improved. In this study, the birch wood hydrolyzate detoxified with activated char was further

treated with an anion exchange resin (Amberlite IRA 67) at 30°C for 1 h. A large portion of the acetic acid (68%) present in the hydrolyzate detoxified with the stem-activated charcoal could be removed by the anion exchange resin, as shown in Table 10. It has been reported that 3.7 g l^{-1} of acetic acid in sugar cane hemicellulose hydrolyzate was completely consumed by *C. tropicalis* [16]. Although the sensitivity to acetic acid is yeast species dependent, the acetic acid concentration of the birch wood hydrolyzate treated with the anion exchange resin decreased by its harmless level to *C. magnoliae* (3.7 g l^{-1}). The anion exchange resin is, however, too expensive to use for detoxifying the birch wood hemicellulose hydrolyzate. In conclusion, the dehydration product prepared from the birch wood hydrolysis residue can be used as inexpensive sorbent for removing furan derivatives and lignin degradation products in the birch wood hydrolyzate. To overcome the inhibition caused by acetic acid, the adaptive response of the yeast to acetic acid should be considered.

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Table 7. Effect of treatment with various sorbents on the concentrations of neutral sugars in the hydrolysate of Japanese white birch wood[1]

Sorbent	Dose ($g\ l^{-1}$)	Concentration ($g\ l^{-1}$)				
		D-Xylose	L-Arabinose	D-Glucose	D-Mannose	D-Galactose
Birch wood carbon[2]	0	42.9	1.8	3.1	1.7	4.8
	100	39.1	2.6	2.4	1.8	4.6
	125	38.7	2.7	2.4	1.8	4.5
	150	37.6	2.5	2.3	1.8	4.4
Steam-activated	10	41.5	1.7	2.8	1.6	4.8
charcoal[3]	15	41.3	2.7	2.7	1.9	4.8
	20	41.3	1.7	2.7	1.6	5.0
	30	42.3	1.6	2.8	1.6	3.8
$ZnCl_2$-activated	10	38.9	2.5	2.4	1.8	4.6
charcoal[4]	20	38.0	2.2	2.4	1.7	4.5
	30	37.5	1.7	2.4	1.6	4.9

[1] Sorption experiments were conducted by agitating 20 ml of the hydrolyzate with desired amounts of sorbents in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h.

[2] The ground wood of Japanese white birch (*Betula platyphylla* var. *japonica*) was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 ($g\ g^{-1}$) at 120°C for 60 min. The resulting hydrolysis residue was treated with concentrated sulfuric acid according to the method of Namasivayam and Kadirvelu [11].

[3] Steam-activated charcaol (Shirasagi M, Japan EnviroChemicals, Ltd., Osaka, Japan).

[4] $ZnCl_2$-activated charcoal (Wako Pure Chemicals Industries, Ltd., Osaka, Japan).

Table 8. Effect of treatment with various sorbents on the concentrations of inhibitors in the hydrolyzate[1]

Sorbent	Dose ($g\ l^{-1}$)	A_{280}[5]	Concentration ($g\ l^{-1}$)	
			Acetic acid	Furfural
Birch wood carbon[2]	0	0.19	12.6	1.2
	100	0.05	10.5	0.1
	125	0.03	10.4	0.1
	150	0.03	10.4	0.1
Steam-activated charcoal[3]	10	0.06	11.6	0.2
	15	0.03	11.5	0.2
	20	0.02	11.8	0.1
	30	0.01	11.7	-[6]
$ZnCl_2$-activated charcoal[4]	10	0.06	11.6	0.5
	20	0.05	11.5	0.3
	30	0.03	11.4	0.2

[1-4] See Table 7.

[5] Absorbance at 280 nm.

[6] Less than detection limit (< 0.01 $g\ l^{-1}$).

Table 9. Chemical composition and BET surface area of carbonaceous sorbents

Sorbent	C (%)	H (%)	N (%)	Ash (%)	Surface area (m^2 g^{-1})
Birch wood carbon[1]	44.3	3.7	0.9	0.1	5
Steam-activated charcoal[2]	93.3	0.5	0.3	2.5	959
$ZnCl_2$-activated charcoal[3]	85.6	2.2	0.4	0.6	988

[1-3] See Table 7.

Table 10. Chemical composition of Japanese white birch (*Betula platyphylla* var. *japonica*) wood hydrolyzates

Components	Concentration (g l^{-1})		
	Original[1]	Detoxified with activated charcoal[2]	Detoxified with activated charcoal followed by treatment with anion exchange resin[3]
Arabinose	1.8	2.7	1.4
Xylose	42.9	41.3	37.6
Galactose	4.8	4.8	2.9
Glucose	3.2	2.7	2.4
Mannose	1.7	1.9	1.3
Acetic acid	12.6	11.5	3.7
Furfural	1.2	0.2	0.1
Phenolics[4]	0.186	0.029	0.028

[1]The ground wood was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120ºC for 1 h.

[2]The original hydrolyzate was treated with a steam-activated charcoal (15 g l^{-1}) in a reciprocal shaker (160 strokes min^{-1}) at 30 ºC for 1 h.

[3]The original hydrolyzate was treated with a steam-activated charcoal (15 g l^{-1}) followed by treatment with an anion exchange resin (80 g l^{-1}).

[4]The overall content of phenols was evaluated by the absorbance at 280 nm (A_{280}) at pH 12.

CHAPTER IV. ADAPTATION OF PARENT STRAIN

Introduction

The economic feasibility of using lignocellulose hydrolyzates in industrial fermentation depends on the efficient fermentation of sugars in the hydrolyzates and the tolerance of yeasts to fermentation inhibitors. Treatment with carbonaceous sorbents is an efficient method for the reducing the concentrations of furan derivatives and lignin degradation products. However these sorbents could not decrease the acetic acid concentration by its harmless level. Although ion exchange resins are useful for eliminating acetic acid from the hydrolyzates, they are too expensive to employ as a sorbent for detoxifying the hydrolyzates. As an alternative method to overcome inhibition caused by the toxic compounds in the hydrolyzates, the use of yeast strains adapted to the hydrolyzates by repeated recycling improved fermentabilities of D-xylose [1], sugarcane bagasse hydrolyzate [2], and hardwoods hydrolyzates [3-6]. Parajo *et al*. [7] reported a 3.6-fold increase in xylitol volumetric productivity and a two-fold increase in xylitol yield after five fermentations of neutralized eucalyptus wood hydrolyzates with recycled cells of *Debaryomyces hansenii*. In this study, adaptation of the parent strain of *C. magnoliae* was performed by sequentially transferring and growing cells in media containing increasing

concentrations of the culm hydrolyzate of bamboo (*P. pubescens*). The effect of adaptation on the fermentation performance is evaluated.

Material and Methods

Preparation of fermentation substrate

The ground culms (P32 R82 mesh) of bamboo (*P. pubescens*) were composed of 18.0% pentosan (including 16.8 % xylan), 38.4% glucan, 27.8% lignin (including 1.4% acid soluble lignin) and 1.3% ash. The ground culms were directly hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120°C for 1 h. The hydrolyzate was treated with a steam-activated charcoal (Shirasagi M) in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h.

Microorganism and inoculum

Cells of *C. magnoliae* TISTR5663 (deposited in the National Institute of Bioscience and Human-Technology, Tsukuba, as FERM P-16522) were grown on an agar slant containing malt extract (3 g l^{-1}), yeast extract (3 g l^{-1}), peptone (5 g l^{-1}), D-glucose (10 g l^{-1}) and agar (20 g l^{-1}) at 4°C for 3 d. A spoon of a slant culture was transferred to 5 ml of the pre-culture medium

containing xylose (20 g l^{-1}), casamino acids (1 g l^{-1}), Difco yeast nitrogen base without amino acids and ammonium sulfate (1.7 g l^{-1}) and urea (2.27 g l^{-1}), and cultivated at 30°C for 24 h.

Adaptation

Adaptation of the yeast was performed according to the method of Amartey and Jeffries [8]. A loop full of a slant culture was transferred to 5 ml of the pre-culture medium containing increasing concentrations of the hydrolyzate (25%, 50%, 75% and 100%) supplemented with $_{D}$-xylose (30 g l^{-1}, 20 g l^{-1}, 10 g l^{-1} and 0 g l^{-1}), casamino acids (1.0 g l^{-1}), yeast nitrogen base without amino acids and ammonium sulfate (1.7 g l^{-1}) and urea (2.2 g l^{-1}). Cultivation was performed at 30°C for 24 h.

Experimental set-up

Batch fermentation runs were performed in a BMZ-P type culture installation (ABLE Corp., Tokyo, Japan) containing baffles and two sets of disk turbines with six and four flat-blades and with a working volume of 1.5 l of medium. This installation was equipped with pH, temperature, dissolved oxygen, and aeration rate controllers. Ding and Xia [9] reported that the two-phase

aeration is more effective than one-phase aeration for xylitol production. In this study, two phase aeration process was employed. The first step was carried out under aerobic conditions to improve glucose consumption through cell propagation in the medium [10, 11]. The second step under limited oxygen conditions is intended to increase the xylitol accumulation.

At the fixed temperature (30°C), to consume $_{D}$-glucose in the medium, the aerobic phase was applied in the first 22 h, and then the aeration rate was reduced. In the second aeration phase, the agitation and aeration were set at 325 min^{-1} and at 0.67 vvm (volume of air per volume of medium per minute), respectively.

The volumetric oxygen transfer coefficient (K_La) was determined by the dynamic method [12]. The dissolved oxygen concentration (DOC) of the medium was decreased to zero by nitrogen sparging and the K_La was calculated from the rate of DOC increase during subsequent aeration. The oxygen transfer rate (OTR) was calculated from the relationship:

$$OTR = K_La\ (C^* - C)$$

where C^* and C are saturated DOC and DOC, respectively.

Analytical methods

Neutral sugars, xylitol, ethanol, furfural, 5-hydroxymethylfurfural, and acetic acid were determined by HPLC Furfural, 5-hydroxymethylfurfural (5-HMF) and acetic acid were determined by HPLC as described previously. The overall content of lignin degradation products was estimated by ultraviolet spectroscopy as described previously.

Results and Discussion

The ground culms of *P. pubescens* were hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120°C for 1 h to afford a sugar solution containing 36.9 g l^{-1} xylose, 2.3 g l^{-1} arabinose and 7.5 g l^{-1} glucose (Table 11). Besides neutral sugars, the original hydrolyzate also contained significant amounts of the fermentation inhibitors such as acetic acid (10.2 g l^{-1}), furfural (0.98 g l^{-1}), 5-hydroxymethylfurfural (0.25 g l^{-1}) and low molecular weight phenols, as shown in Table 11. These compounds inhibit microbial metabolism. Delgenes *et al*. [13] found that 0.5 g l^{-1} of furfural reduced the cell growth of the yeast, *Pichia stipitis* by 25%. Further, the A_{280} value of the hydrolyzate (0.209) diluted 1:1000 with water at acidic pH was too high to perform microbial conversion of the sugar solution. Tada *et al*. [14] reported that successful xylitol production from corn cob hydrolyzates diluted 1:1000 with water by *C.*

magnoliae required the A_{280} value to be less than 0.02. In this study, the hydrolyzate was treated with a commercially available steam-activated charcoal at 30ºC for 1 h.

When the hydrolyzate was treated with 20 g l^{-1} of activated charcoal, the concentrations of furfural and 5-hydroxymethylfurfural decreased from 0.98 g l^{-1} to 0.09g l^{-1} and from 0.25 g l^{-1} to 0.04 g l^{-1}, respectively. The A_{280} value of the hydrolyzate also decreased from 0.186 to 0.029. The results indicate that the pretreatment with activated charcoal can eliminate large parts of furan derivatives and low molecular weight phenols from the hydrolyzate. In contrast, the concentrations of each neutral sugar and acetic acid (originating from xylan) stayed almost constant after treatment with the activated charcoal. Although acetic acid acts as an inhibitor of microbial metabolism, the sensitivity to acetic acid is yeast species dependent. Pessoa *et al*. [15] reported that 3.7 g l^{-1} of acetic acid in sugar cane hemicellulose hydrolyzate was completely consumed by *C. tropicalis*. *C. guilliermondii* was also able to assimilate significant amounts of acetic acid in hemicellulose hydrolyzates of eucalyptus wood and sugar cane bagasse in amounts of 40% and 50%, respectively [16, 17]. Ferrari *et al*. [18] reported that acetic acid in the *Eucalyptus* wood hydrolyzate (10.1-10.4 g l^{-1}) was partially consumed by *P. stipitis*. Yeast cultures adapted to hardwood hemicellulose hydrolyzates by repeated recycling were used to overcome the inhibition of microbial metabolism caused by the toxic compounds present in the hydrolyzates [2-7]. In this study, adaptation of the parent strain was performed by sequentially

transferring and growing cells in media containing increasing concentrations of the hydrolyzate. Xylitol fermentation valuables of the detoxified bamboo hydrolyzate using adapted cells and parent cells are listed in Table 12**.** Compared to the parent culture, xylitol fermentation with the adapted culture shows a great improvement in the yield, maximum concentration, and volumetric productivity. Further acetic acid in the detoxified hydrolyzate was completely assimilated by the adapted cells, whereas 3.7 g l^{-1} of acetic acid still remained in the parent culture.

One of the most common inhibitor in ethanol fermentation is acetic acid. In general, hemicellulose hydrolyzates prepared from hardwoods and bamboo (*P. pubescens*) culm contain significant amounts of acetic acid (usually more than 10%). However, regard to xylitol production, no negative influence of acetic acid has been recognized. The concentration of acetic acid decreased continuously until its complete depletion, when the adapted cells of *Debaryomyces hansenii* [19], *C. guilliermondii* [20], and *C. magnoliae* [21] were grown on hemicellulose hydrolyzates. Culture with adapted or recycling cells of yeasts eliminates the inhibition caused by acetic acid and allows significant improvements in the xylitol fermentation of the hemicellulose hydrolyzates. In conclusion, the strain improvement through cell adaptation is an important consideration in efforts to develop industrial fermentation procedures for the lignocellulosic hydrolyzates.

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Table 11. Concentration of components in the culm hydrolyzates of bamboo (*Phyllostachy pubescens*)

Component	Concentration (g l^{-1})	
	Original[1]	Detoxified[2]
$_{D}$-Xylose	36.9	36.7
$_{D}$-Glucose	7.5	7.5
$_{L}$-Arabinose	2.3	2.3
Acetic acid	10.2	9.7
Furfural	0.98	0.09
5-Hydroxyfurfural	0.25	0.04
A_{280}[3]	0.209	
A_{280}[4]	0.186	0.029

[1] The ground culm of *P*. *pubescens* was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120°C for 1 h.

[2] The original hydrolyzate was treated with 20 g l^{-1} of a steam-activated charcoal (Shirasagi M) in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h.

[3] Absorbance of diluted hydolyzate (pH) at 280 nm.

[4] Absorbance of diluted hydolyzate (pH 12) at 280 nm.

Table 12. Effect of adaptation on xylitol production from the detoxified culm hydrolyzates of bamboo

	Adapted	Unadapted
Initial xylose concentration (g l^{-1})	37.4	36.5
Final xylose concentration (g l^{-1})	0.2	2.5
Maximum xylitol concentration (g l^{-1})	23.8	16.1
Xylitol yield (g-xylitol g-$xylose^{-1}$)	0.67	0.46
Volumetric productivity (g l^{-1} h^{-1})	0.48	0.41
Initial acetic acid concentration (g l^{-1})	10.2	10.8
Final acetic acid concentration (g l^{-1})	0	3.7
Final ethanol concentration (g l^{-1})	0	5.1

Oxygen transfer rate (OTR) was set at 9.58 mmol-O_2 L^{-1} h^{-1} during the microaerobic phase

CHAPTER V. FERMENTATION OF DETOXIFIED HYDROLYZATES

Introduction

Traditionally, it was believed that yeasts could only ferment hexoses to ethanol or to polyols. In later years, *Pachysolen tannnophilus* [1] and many other yeast species [2-11] were shown to convert D-xylose to ethanol or to xylitol. Xylitol is a main byproduct of these yeasts during their D-xylose metabolism.

There are two possible metabolic routs for using D-xylose by microorganisms. In certain bacreria, the first step of the metabolism involves enzyme induction followed by D-xylose isomerization to D-xylulose. Thereafter D-xylulose is phosphorylated to form D-xylulose-5-phosphate. On the other hand, in D-xylose-fermenting yeasts, D-xylose is first reduced to xylitol by either NADPH- or NADH-linked xylose reductase. Next, xylitol is oxidized to D-xylulose by NAD^+- or $NADP^+$-linked xylitol dehydrogenase. In most yeast cell-free extracts, D-xylose reductase has a higher or even absolute preference for NADPH [12]. Xylitol dehydrogenase of most xylose-fermenting yeasts uses predominantly NAD^+ and very rarely NADP+ as a cofactor [12]. Then the D-xylulose formed is phosphorylated to afford D-xylulose-5-phosphate. It may subsequently enter into the pentose phosphate pathway. Glyceraldehyde-3-phosphate and fructose-6-phosphate are formed from D-xylulose-5-phosphate

through non-oxidative phase of the pentose phosphate pathway. Both of them are converted to pyruvate in the Embden-Meyerhof-Parnus pathway. The resulting pyruvate either converts to ethanol (ethanol fermentation) or enters into the tricarboxylic acid cycle to consume as an energy source (respiration). The pyruvate conversion depends on the oxygen supply condition used. The process of xylitol formation is unable to be stopped after the D-xylose reduction step. Regeneration of the cofactors through different metabolic pathway is necessary for both the xylitol production and cell growth of the yeasts. Therefore, for the successful xylitol production, the amount of xylose being converted to xylitol and the amount of xylitol being available for further metabolism, must be well balanced [12].

The microbial xylitol production depends on the fermentation conditions employed [13]. Together with the substrate concentration, oxygen supply condition is the most important of all environmental parameters that affect the microbial xylitol production [14, 15]. High degree of aeration promotes cell proliferation, whereas xylitol accumulation is repressed. Under anaerobic conditions, yeasts fail to grow on D-xylose to any appreciable extent. It was found that fermentation and growth occur simultaneously only under oxygen limitation [16]. In this study, the effects of aeration and initial D-xylose concentration on the xylitol production were investigated using the bamboo (*P. pubescens*) culm hydrolyzates.

Material and Methods

Preparation of fermentation substrate

The ground culms (P32 R82 mesh) of bamboo (*P. pubescens*) were directly hydrolyzed with 3% sulfuric acid with two liquor-to-solid ratio of 4 and 10 (g g^{-1}) at 120°C for 1 h. The hydrolyzates were detoxified with a steam-activated charcoal (Shirasagi M) in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h. The detoxified hydrolyzates were neutralized with calcium carbonate followed by filtration.

Microorganism and inoculum

A spoon of a slant culture (adapted or parent cells of *C. magnoliae* TISTR5663) was transferred to 5 ml of the pre-culture medium containing xylose (20 g l^{-1}), casamino acids (1 g l^{-1}), Difco yeast nitrogen base without amino acids and ammonium sulfate (1.7 g l^{-1}) and urea (2.27 g l^{-1}), and cultivated at 30°C for 24 h.

Experimental set-up

Batch fermentation runs were performed in a BMZ-P type culture installation (ABLE Corp., Tokyo, Japan) and with a working volume of 1.5 l of medium. In this study, two phase aeration

process was employed. At the fixed temperature (30°C), to consume D-glucose in the medium, the aerobic phase was applied in the first 12 h (initial D-xylose concentration: 19 g l^{-1}) or 17-20 h (initial D-xylose concentration: 35-37 g l^{-1}), and then the aeration rate was reduced. In the second aeration phase, for the fermentation media containing 19 g l^{-1} of D-xylose, the agitation and aeration were varied from 300 to 500 min^{-1} and from 0.33 to 1.00 vvm, respectively. For the fermentation media containing 35-37 g l^{-1} of D-xylose, the agitation was varied from 325 to 450 min^{-1} and aeration was set at 0.67 vvm. The OTR was calculated as described previously.

Analytical methods

Neutral sugars, xylitol, acetic acid, furfural, 5-hydroxymethylfurfural, and ethanol were determined by HPLC as described previously. The overall content of lignin degradation products was estimated by ultraviolet spectroscopy as described previously.

Results and Discussion

The culm of bamboo (*P. pubescens*) was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 10 (g g-1) to give a sugar solution containing 19 g l^{-1} $_D$-xylose. The hydrolyzate was detoxified with a steam-activated charcoal (20 g l^{-1}) to give a sugar solution containing 19.4 g l^{-1} $_D$-xylose. The A_{280} value of the dilute hydrolyzate decreased from 0.145 to 0.006 after detoxification with the activated char. The results indicate that the sorbent selectively removes furan derivatives and lignin degradation products from the hydrolyzate by their harmless level.

Table 13 shows the fermentation parameters obtained in experiments conducted with the fermentation medium containing 19.4 g l^{-1} $_D$-xylose, 3.6 g l^{-1} $_D$-glucose, 1.2 g l^{-1} $_L$-arabinose, and nutrients supplemented. No attempt was made for the determination of acetic acid. $_D$-Glucose in the fermentation media (3.5-3.9 g l^{-1}) was completely consumed during the first 12 h. *C. magnoliae* grew in the detoxified hydrolyzate and accumulated xylitol at different rates under the microaerobic conditions employed. As shown in Figure 3, the highest level of biomass (17.4 g l^{-1}) was attained with the highest OTR (27.6 mmol-O_2 l^{-1} h^{-1}), whereas the xylitol secretion was strongly repressed. At the OTR of 15.0 mmol-O_2 l^{-1} h^{-1}, the best fermentation performance (xylitol concentration: 10.5 g l^{-1}; xylitol yield: 0.59 g-xylitol g-$xylose^{-1}$; xylitol volumetric productivity: 0.42 g l^{-1} h^{-1}) could be attained. Vandeska *et al*. [17] reported that an OTR of 14

mmol-O_2 l^{-1} h^{-1} was optimal for xylitol production from a synthetic medium using *C. boidinii* (xylitol yield: 0.48 g-xylitol g-xylose^{-1}; xylitol volumetric productivity: 0.24 g l^{-1} h^{-1}).

Table 14 shows the fermentation parameters obtained in experiments conducted with the fermentation medium containing 37.6 g l^{-1} $_D$-xylose, 7.5 g l^{-1} $_D$-glucose, 2.3 g $_L$-arabinose, 9.7 g l^{-1} acetic acid, and nutrient supplements. The medium also contained 0.09 g l^{-1} furfural, 0.04 g l^{-1} 5-hydroxymethylfurfural, and lignin degradation products. However no inhibition caused by these inhibitors was observed in the fermentation experiments. To avoid catabolite repression, $_D$-glucose in the medium was almost completely consumed by the yeast under the aerobic conditions employed (first 17-20 h). Further most of the acetic acid in the medium was also assimilated by the yeast during the aerobic phase. Therefore inhibition caused by acetic acid present in the medium can be overcome by a suitable combination of aerobic and oxygen limited aeration process as well as the adaptation of the parent strain to the hydrolyzate used. However $_D$-xylose was slightly consumed before the complete assimilation of $_D$-glucose.

Figure 4 shows time course of xylitol yield, xylose, acetic acid and dry cell concentration in batch xylitol fermentation of detoxified bamboo culm hydrolyzates by *Candida magnoliae* under various microaerobic conditions. At the second aeration phase, the OTR was varied from 8.0 to 22.3 mmol-O_2 l^{-1} h^{-1}. At the OTR of 9.6 mmol-O_2 l^{-1} h^{-1}, the highest xylitol concentration (23.8 g l^{-1}) was obtained, whereas the xylitol volumetric productivity was the lowest under the

aeration conditions tested. At the OTR ranging 11.2 to 14.5 mmol-O_2 l^{-1} h^{-1}, $_D$-xylose in the medium also successfully converted to xylitol. At the OTR of 22.3 mmol-O_2 l^{-1} h^{-1}, both the xylitol yield and concentration markedly decreased compared with other aeration condition, whereas the highest concentration of cell was attained. Under aerobic condition, xylose-fermenting yeasts metabolize mainly $_D$-xylose for energy production. $_D$-Xylose absorbed into yeast cells is first reduced to xylitol by $_D$-xylose reductase. At the most aerobic condition (22.3 mmol-O_2 l^{-1} h^{-1}), it is clear that a portion of the synthesized xylitol is secreted from the cell and the remainder is oxidized by xylitol oxidase. Xylulose generated through the latter process is used as a carbon source for energy production under the aerobic condition. The aeration condition is, therefore, undesirable for the microbial xylitol production.

From the fermentation experiments using the bamboo culm hydrolyzates, it is clear that the microbial xylitol production is significantly affected by oxygen supply condition monitored by OTR as well as initial $_D$-xylose concentration of the medium. $_D$-Glucose concentration of the hydrolyzate is also an important factor for the microbial xylitol fermentation, because $_D$-glucose is used for the proliferation of yeast during the aerobic phase of fermentation. In conclusion, among the environmental factors that affects the microbial xylitol production by $_D$-xylose-fermenting yeasts, the dissolved oxygen concentration is most important and must be carefully controlled.

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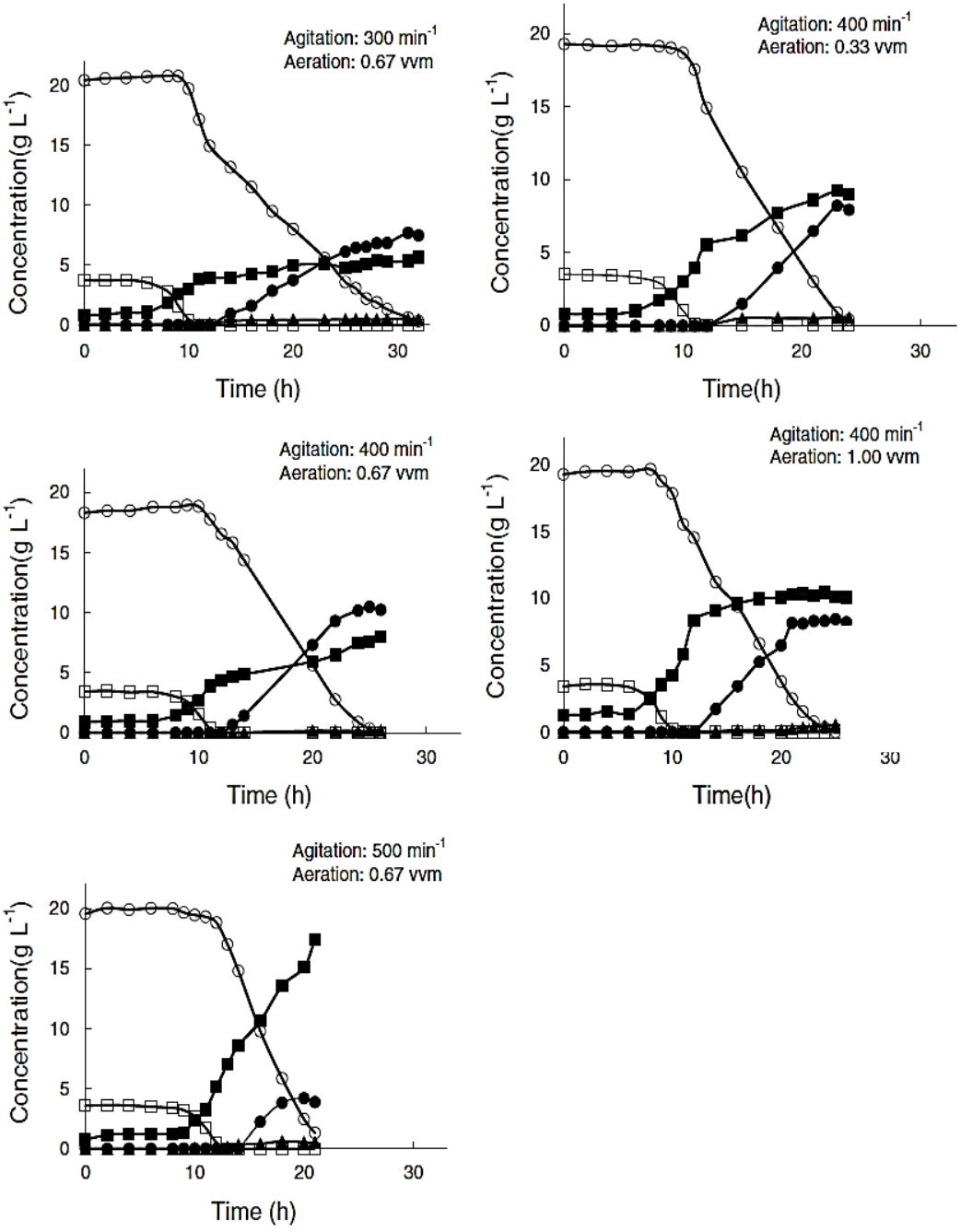

Figure 3. Time course of D-xylose, D-glucose, xylitol, ethanol, and biomass concentrations in batch xylitol fermentations by Candida magnoliae from detoxified bamboo (*Phyllostachys pubescens*) culm hydrolyzates (Initial D-xylose concentration: 19.4 g l^{-1})

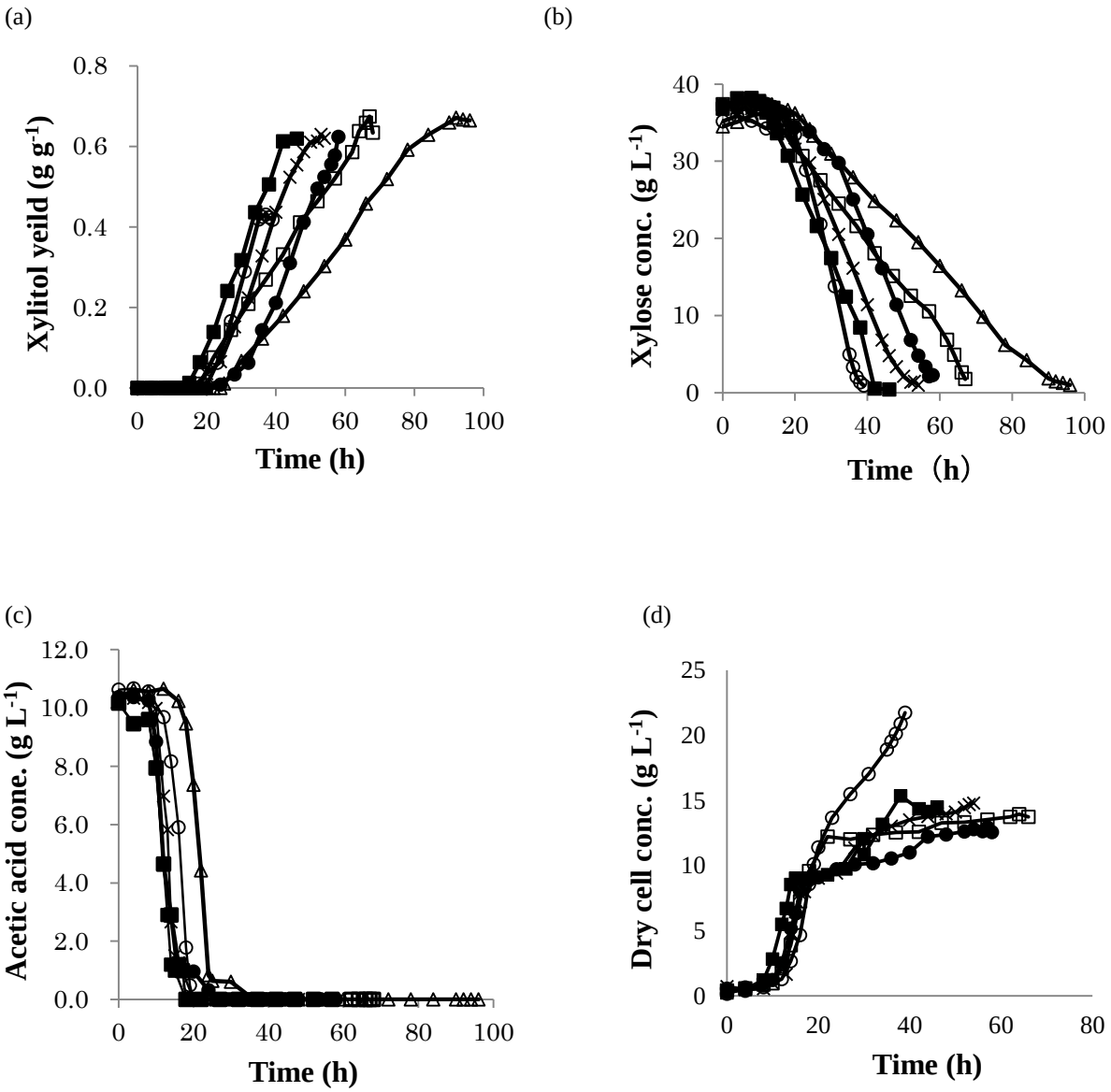

Figure 4. Time course of xylitol yield (a), xylose (b), acetic acid (c) and dry cell (d) concentration in batch xylitol fermentation of detoxified bamboo culm hydrolyzates by *Candida magnoliae* under various microaerobic conditions.

Table 12. Effects of oxygen transfer rate (OTR) on the xylitol production from the culm hydrolyzate of bamboo (*P. pubescens*) by *Candida magnoliae*[1]

	OTR (mmol-O_2 l^{-1} h^{-1})				
	8.4	12.3	15.0	17.2	27.6
Xylitol concentration (g l^{-1})	7.7	8.2	10.5	8.4	4.2
Xylitol yield (g-xylitol g-xylose^{-1})	0.37	0.46	0.59	0.49	0.23
Xylitol productivity (g l^{-1} h^{-1})	0.25	0.36	0.42	0.34	0.21
Cell concentration (g l^{-1})	5.7	9.0	7.9	10.1	17.4

[1] The ground culm of *P. pubescens* was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 10 (g g^{-1}) at 120°C for 1 h. The resulting hydrolyzate was detoxified with 20 g l^{-1} of a steam-activated charcoal (Shirasagi M) in a reciprocal shaker (160 strokes min^{-1}) at 30°C for 1 h.

Table 13. Effects of oxygen transfer rate (OTR) on xylitol production from the detoxified bamboo hydrolyzates by *C. magnoliae*[1]

	OTR (mmol-O_2 l^{-1} h^{-1})					
	8.0	9.6	11.2	12.7	14.5	22.3
Initial xylose concentration (g l^{-1})	34.5	37.4	36.8	37.4	36.8	35.2
Final xylose concentration (g l^{-1})	1.0	0	2.3	0.95	0.43	0.93
Maximum xylitol concentration (g l^{-1})	22.9	23.9	22.7	21.7	21.7	14.6
Xylitol yield (g-xylitol g-xylose^{-1})	0.67	0.67	0.62	0.63	0.62	0.43
Volumetric productivity (g l^{-1} h^{-1})	0.33	0.48	0.55	0.59	0.68	0.81
Initial acetic acid concentration (g l^{-1})	10.3	10.2	10.4	10.4	10.2	10.6
Final acetic acid concentration (g l^{-1})	0	0	0	0	0	0
Dry cell concentration (g l^{-1})	-[2]	14.0	12.9	14.8	14.5	21.8

[1] The ground culm of *P. pubescens* was hydrolyzed with 3% sulfuric acid with a liquor-to-solid ratio of 4 (g g^{-1}) at 120°C for 1 h. The resulting hydrolyzate was detoxified with 20 g l^{-1} of a steam-activated charcaol (Shirasagi M) in a reciprocal shaker(160 strokes min^{-1}) at 30°C for 1 h. Adapted cells were used in the fermentation experiments.

GENERAL CONCLUSION

This thesis consists of microbial xylitol production from various hemicellulose hydrolyzates by the yeast *Candida magnolia*. In chapter 2, the author mentioned that fermentable sugar solutions with relatively high xylose concentration could be prepared from the culms of bamboo and bamboo grasses by hydrolysis with dilute sulfuric acid. Although glucose in the xylitol fermentation medium generally causes catabolite inhibition, when the aerobic condition is employed during the first stage of the fermentation process, glucose is assimilated preferably by the yeast. The yeast proliferates using the energy obtained from the respiration. The concentration of D-xylose in the hydrolyzates markedly increased with decreasing the liquid-to-solid ratio. However, when lower liquid-to-solid ratio (less than 5 g g^{-1}) was employed, the A_{280} was too high to perform the microbial conversion of D-xylose in the hydrolyzate. Tada *et al*. [1] reported that successful xylitol production from corn cob hydrolyzates required an A_{280} value below 0.02. Therefore, detoxification of the hydrolyzates before fermentation is necessary for the successful bioconversion of sugars in the hydrolyzates to xylitol or to ethanol.

When Japanese white birch wood was hydrolyzed with 3% sulfuric acid at 120ºC for 1 h, a sugar solution containing 42.9 g l^{-1} D-xylose could be obtained, as shown in chapter 3. However the hydrolyzate also contained significant amounts of fermentation inhibitors, such as acetic

acid, furfural and low molecular weight phenols originated mainly from lignin. Therefore, the hydrolyzate should be also detoxified for its successful fermentation. The author examined various carbonaceous sorbent for detoxification of the hydrolysate. Sorption capacities of the steam-activated charcoal were superior to other carbonaceous sorbents such as $ZnCl_2$-activated charcoal and commercially available charcoals. However activated charcoals are generally too expensive to employ as a sorbent for detoxifying lignocellulose hydrolyzates. On the other hand, mild hydrolysis of lignocelluloses produces waste fiber in large quantities. As an alternative, a carbonaceous sorbent could be prepared from the waste fiber by dehydration with concentrated sulfuric acid. When the hydrolyzate treated with the sorbent, the amounts of furfural and lignin degradation products decreased with increasing the amount of the birch wood hydrolysis residue added, whereas the concentrations of neutral sugars and acetic acid, which releases from acetyl groups of the birch wood xylan, could not be removed by treatment with the carbonaceous sorbents used. A large portion of the acetic acid (68%) present in the hydrolyzate detoxified with carbonaceous sorbents could be removed by the anion exchange resin.

From the view point of process cost, ion exchange resin is also too expensive to use for detoxifying the lignocellulosic hemicellulose hydrolyzate. To overcome the inhibition caused by acetic acid, the adaptive response of the yeast to acetic acid was considered in the chapter 4. Adaptation of the parent strain was performed by sequentially transferring and growing cells in

media containing increasing concentrations of the hydrolyzate. Compared to the parent culture, xylitol fermentation with the adapted culture shows a great improvement in the yield, maximum concentration, and volumetric productivity. Further acetic acid in the detoxified hydrolyzate was completely assimilated by the adapted cells, whereas 3.7 g l^{-1} of acetic acid still remained in the parent culture. The strain improvement through cell adaptation is an important consideration in efforts to develop industrial fermentation procedures for the lignocellulosic hydrolyzates.

A fermentable substrate with a relativity high xylose concentration (36.9 g l^{-1}) was prepared from the culm of bamboo by hydrolysis with 3% sulfuric acid under the relatively mild hydrolysis condition, as shown the chapter5. Except for acetic acid, inhibitors such as furfural and lignin degradation products were successfully removed by treatment with a steam-activated charcoal. Although acetic acid could not eliminate by the activated charcoal, it completely metabolized by adapted cells of *C. magnoliae* under aerobic conditions used. The detoxified hydrolyzate can be successfully converted to xylitol by the yeast. The best fermentative performance of the adapted cells of *C. magnoliae* in the hydrolyzate was obtained at the OTR of 9.6 mmol-O_2 l^{-1} h^{-1} (maximum xylitol concentration: 23.9 g l^{-1}; xylitol yield: 0.67 g-xylitol g-$xylose^{-1}$). Among the environmental factors that affects the microbial xylitol production by $_D$-xylose-fermenting yeasts, the dissolved oxygen concentration is most important and must be carefully controlled.

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