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Production of 2,3-Butanediol from Pretreated Corn Cob by *Klebsiella oxytoca* in the Presence of Fungal Cellulase

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ABSTRACT

A simple and effective method of treatment of lignocellulosic material was used for the preparation of corn cob for the production of 2,3-butanediol by *Klebsiella oxytoca* ATCC 8724 in a simultaneous saccharification and fermentation process. During the treatment, lignin, and alkaline extractives were solubilized and separated from cellulose and hemicellulose fractions by dilute ammonia (10%) steeping. Hemicellulose was then hydrolyzed by dilute hydrochloric acid (1%, w/v) hydrolysis at 100°C at atmospheric pressure and separated from cellulose fraction. The remaining solid, with 90% of cellulose, was then used as the substrate. A butanediol concentration of 25 g/L and an ethanol concentration of 7 g/L were produced by *K. oxytoca* from 80 g/L of corn cob cellulose with a cellulase dosage of 8.5 IFPU/g corn cob cellulose after 72 h of SSF. With only dilute acid hydrolysis, a butanediol production rate of 0.21 g/L/h was obtained that is much lower than the case in which corn cob was treated with ammonia steeping prior to acid hydrolysis. The butanediol production rate for the latter was 0.36 g/L/h.

Index Entries: Ammonia steeping; 2,3-butanediol; corn cob; *Klebsiella oxytoca*; simultaneous saccharification and fermentation (SSF).

INTRODUCTION

2,3-Butanediol is a colorless and odorless liquid having a high boiling point of 180–184°C and a low freezing point of –60°C. This petroleum-based product has diverse industrial use, particularly as a polymeric

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substance in addition to its use for manufacturing butadiene or antifreeze. Currently, butanediol is enjoying 4–7% annual growth, buoyed by the increased demand for polybutylene terephthalate resins, gamma-butyrolactone, and spandex and its precusors *(1)*. Butanediol is also a potentially valuable fuel additive. The heating value of butanediol (27,198 J/g) is similar to other liquid fuels such as ethanol (29,055 J/g) and methanol (22,081 J/g). It can also be used as octane booster for gasoline or as high-grade aviation fuel after converting it to methyl ethyl ketone (MEK). Furthermore, butanediol can also be converted to diacetyl form for flavoring in food products *(2)*.

Industrially, butanediol is produced from petroleum-based feedstocks. It can also be produced from simple sugars such as glucose and xylose by bacterial fermentation. *Klebsiella oxytoca* (*K. pneumonia*), one of a few bacterial species that utilizes all the major sugars (hexoses and pentoses), produces butanediol in high yield and high concentration (up to 10%, w/v) under optimum conditions, e. g., temperature, dissolved oxygen, and so forth *(3,4)*. The production of butanediol from lignocellulosic materials has been considered as an alternative approach in the conversion of biomass substrates to liquid fuels and chemical feedstocks *(5,6)*.

Cellulosic biomass is a complex mixture of carbohydrate polymers from plant cell wall known as cellulose and hemicellulose, plus lignin and a smaller amount of minor compounds known as extractives. A key problem in utilization of cellulosic materials for fuel and chemical production is the poor yields of glucose from cellulose by acids or enzymes. Many pretreatment techniques have been used to improve cellulose hydrolysis. These techniques can be characterized as either chemical or physical in nature. The power required for physical treatments, including grinding and milling, is too costly. Chemical treatments with strong acids or bases are also expensive and compounded by the necessity of chemical recovery. Other treatment methods include the ammonia explosion *(7)*, steam explosion *(8,9)*, and ammonia-recycled percolation *(10)* processes. The ammonia explosion process allows the explosion of cellulosic materials at a relatively low temperature to avoid sugar decomposition. However, it is difficult to recover all the feed ammonia for reuse. The steam explosion process requires considerable thermal energy. And in the ammonia-recycled percolation method, the pretreatment is conducted at relatively high temperature (150°C) and pressure (325 psi), causing considerable degradation of hemicellulosic carbohydrates. However, the ammonia recovery rate can be as high as 99% *(10)*.

One method of producing butanediol from biomass is the simultaneous saccharification and fermentation (SSF). This process is similar in principle and in practice to that used in producing ethanol from cellulosic biomass. In this process, a cellulose hydrolyzing enzyme (cellulase) is combined with a butanediol producing organism to carry out simultaneous hydrolysis of cellulose and hemicellulose to glucose, xylose, and a mixture

of minor sugars such as arabinose, and the conversion of sugars to butanediol. As a result, hydrolysis rates and yields of product are improved when compared to processes involving separate hydrolysis and fermentation steps *(11,12)*. As in the case of ethanol production by the SSF process, the cost of cellulase enzyme accounts for a large portion of the overall cost of conversion of biomass to butanediol. Therefore, a reduction in the cost of the enzyme usage would make this process more economically attractive. One way of reducing of enzyme usage is to remove lignin prior to SSF since it is known that lignin will adsorb cellulase and deactivate cellulase activity *(13–16)*. As a physical barrier, lignin also causes a higher cellulase dosage in order to achieve a reasonable cellulose hydrolysis rate. Another way is to recover and reuse cellulase. A prior removal of lignin will also allow a more complete cellulase recovery *(15,16)*. Likewise, the prior removal of hemicellulose will also enhance the reactivity of cellulose fraction *(17)*.

An effective utilization of xylose, arabinose, and other minor sugars in addition to glucose is important in the process economics. *K. oxytoca* ATCC 8724 has been studied the Laboratory of Renewable Resources Engineering *(3,4)*. This bacterial strain is capable to produce butanediol from both hexoses and pentoses with good yield. In this study, we combined *K. oxytoca* with a relatively easy and effective pretreatment technique to demonstrate the viability of producing butanediol from cellulosic biomass using ground corn cob as a model authentic lignocellulosic material.

MATERIALS AND METHODS

Materials

Ground corn cob (8% moisture) with an average particle diameter of 3.2 mm was purchased from Andersons Inc., Maumee, OH and was used for the experiments unless otherwise indicated. The corn cob (dry basis) has the following composition: 44.88% cellulose, 32.68% xylan, 7.41% lignin, and 2.51% acetate *(18)*. The liquid cellulase preparation with a specific activity of 170 IFPU/mL was provided by Iogen (Ottawa, Canada). Aqueous ammonia (30%) and hydrochloric acid (37%) were purchased from Mallinckrodt Chemical (Paris, KY). 2,3-Butanediol and other carbohydrates were purchased from Sigma Chemical (St. Louis, MO).

Organism and Medium

Klebsiella oxytoca ATCC 8724 was purchased from American Type Culture Collection, Rockville, MD and was maintained on YMA (Difco) slants. The medium (YMP) for cell growth contained the following: yeast extract (Difco), 3 g; malt extract (Difco), 3 g; peptone (Difco), 5 g; glucose, 10 g; and distilled water, 1 L. Sterilization was accomplished by auto-

claving at 15 lb/in^2 for 15 min. Bacterial cells were grown aerobically in 250-mL Erlenmeyer flasks containing 100 mL growth medium at 25°C on a rotary shaker at 150 rpm for 24 h.

Methods

Ammonia Steeping to Remove Lignin

Corn cob (20 g) was mixed with 100 mL aqueous ammonia (10% ammonia) in a 250-mL Erlenmeyer flask and was incubated in a shaker for 24 h at ambient temperature. The mixture was then filtered to separate corn cob from ammonia solution. The corn cob was then washed twice by deionized water to remove residual ammonia from the surface of the particles. The delignined corn cob preparation was then obtained by vacuum evaporation to remove residual ammonia. The lignin fraction was precipitated out of solution upon ammonia removal and collected by filtration.

Dilute Acid Hydrolysis to Remove Hemicellulose

Delignined corn cob that comprises mainly cellulose and hemicellulose fractions was then treated with a 1% hydrochloric acid solution at 100–108°C for 1 h. The acidic hemicellulose hydrolyzate obtained can be neutralized as pentose-rich stream. The remaining solid (cellulose fraction) was then washed with deionized water to remove residual acid.

Enzymatic Hydrolysis of Cellulose Fraction

To the cellulose fraction (9.23 g) obtained from 20 g of corn cob, 50 mL water and 1.0 mL cellulase enzyme (equivalent to 8.5 IFPU/g corn cob cellulose) were added in a 250-mL flask. The saccharification was carried out at 50°C for 48 h.

Simultaneous Saccharification and Fermentation (SSF) of Cellulose Fraction

Pretreated corn cob (8 g, dry equivalent) was added to a 250-mL Erlenmeyer flask with 50 mL YMP medium. It was autoclaved for 15 min at 121°C. The total volume of water in the flask was 80 mL, after taking into account the water content of the pretreated corn cob. The initial pH was adjusted to 5.5 by phosphate buffer. Following this, 20 mL of actively growing bacterial cells that were previously grown in the YMP medium and cellulase (8.5 IFPU/g corn cob cellulose) were added to initiate SSF of the cellulose at 35°C in a shaker at 250 rpm. Samples (0.2 mL) were taken at 12 h intervals over 96 h of incubation.

Analysis

Glucose, xylose, 2,3-butanediol, ethanol, acetate, glycerol and xylitol were analyzed by HPLC (column: 300 × 7.8 mm HPX-87H, Bio-Rad, Richmond, CA). Lignin was determined as Klason lignin by the weight method (*19*). Cellulose and hemicellulose (as xylan) were determined

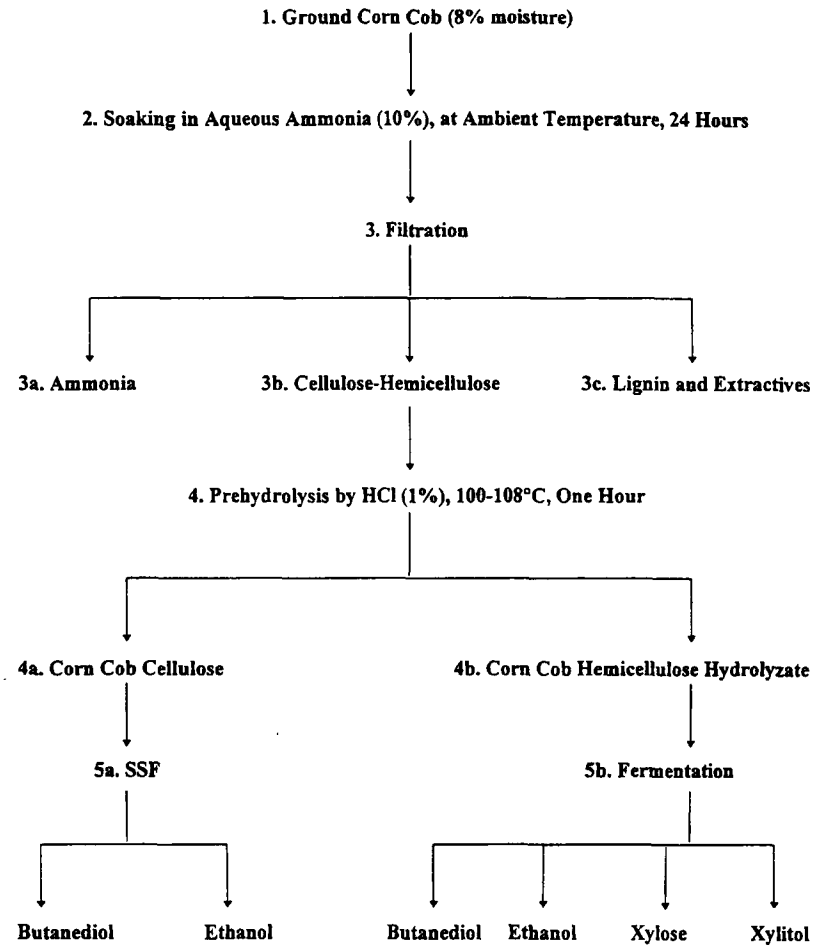

Fig. 1. A schematic representation of the procedures used for the preparation of substrate from ground corn cob.

according to the methods described *(19)*. The residual ammonium ion in the solution was determined with a 05800-05 Solution Analyzer equipped with an ammonium electrode (Cole-Parmer Instrument, Niles, IL).

RESULTS AND DISCUSSION

The procedures for the substrate preparation for butanediol production by *K. oxytoca* are summarized in Fig. 1. In this pretreatment process, lignin and other alkaline extractives were solubilized and separated from corn cob after steeping in the dilute ammonia (10%) solution at ambient temperature. This was followed by hydrolysis using a dilute acid at 100–108°C to hydrolyze the hemicellulose. The changes in composition of corn cob after ammonia steeping are shown in Table 1. The original lignin content of corn cob was reduced by approx 90% from 0.074 to 0.0085 g/g after steeping. In addition, other alkaline extractives such as acetate and

Table 1
Composition of Corn Cob

Materials	Cellulose (%)	Xylan (%)	Lignin (%)	Acetate (%)	Other (%)
Original	44.88	32.68	7.41	2.51	12.53
After Ammonia Steeping	56.2	38.5	0.85	0.0	4.45
After Acid Hydrolysis	90.4	5.29	0.91	0.0	3.4

alkaline extractable materials were removed during steeping (Table 1). The content of hemicellulose, measured as xylan, remained more or less unchanged. Based on the composition of the treated corn cob dry weight, xylan content was increased from 32.68 to 38.5% after lignin removal. Likewise, cellulose content was increased from 44.88 to 56.2%. The retention of hemicellulose after ammonia steeping is in contrast to other treatment processes that resulted in the partial loss of the hemicellulose fraction. This is probably because of the mild condition (ambient temperature and atmospheric pressure) was employed during steeping.

The steeping process was followed by dilute acid hydrolysis. In this step, treated corn cob was incubated in the dilute acid solution at 100–108°C for 1 h to hydrolyze the hemicellulose. Hemicellulose solubilized by this treatment was about 87% since hemicellulose content dropped from 0.38 to 0.05 g/g dry corn cob (Table 1). A clean, light-amber colored hemicellulose hydrolyzate with carbohydrate concentration of 81.8 g/L was obtain with xylose comprising over 90% of the carbohydrates. This hemicellulose hydrolyzate had no acetate and alkali extractives present (Fig. 2). This is important for the utilization of hemicellulosic carbohydrates since acetate, even at a very low concentration of around 5 g/L, is known to inhibit ethanol *(20,21)* and butanediol fermentations *(22,23)*. The inhibition can be even more acute in the present of the fermentation product, ethanol, or butanediol (unpublished observation). This hemicellulose hydrolyzate has been used as a substrate for ethanol production by a xylose-fermenting yeast strain *(23)*. The same hydrolyzate has also been used as the substrate for the production of high-value sweetener, xylitol *(24)*, or as a raw material for xylose production.

The overall possible effect of sequential ammonia steeping and dilute acid treatment is to increase the exposure of cellulose to cellulase. The results from an increase in the surface area of cellulose available for the enzymatic hydrolysis because of the swelling of cellulose *(10,16)*, and also

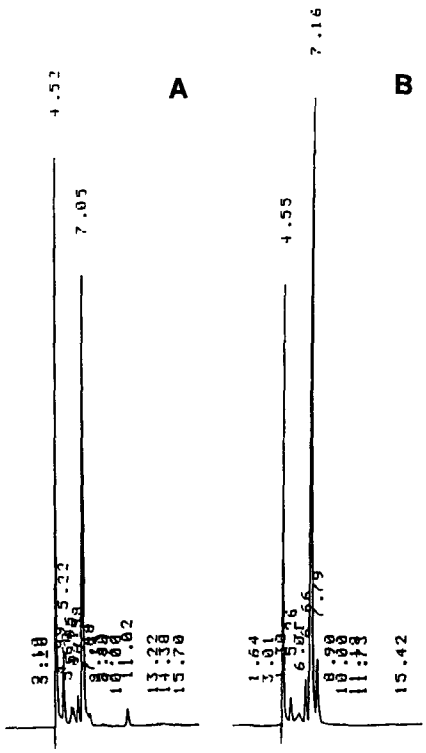

Fig. 2. LC profiles of dilute acid hydrolyzates before ammonia **(A)** and after ammonia steeping **(B)**. Retention time (min): Acid (4.52); Xylose (7.05); Arabinose (7.79); Acetic Acid (11.02).

increase in the porosity, thereby enhanced the accessibility of cellulose by cellulase (25). Upon drying of the treated cellulose fraction, the beneficial effect of alkaline swelling of cellulose was reduced. This effect is shown in Fig. 3, dry cellulose no longer is as susceptible to cellulase as when it had not been dried. The material after lignin and hemicellulose removal has a cellulose content of 90.4% as based on dry weight (Table 2). This is an increase of cellulose content of over 100% from the original 0.45 g/g corn cob.

Since most of the lignin, acetate, alkali extractives, and hemicellulose have been removed by prior process steps, one would expect the remaining corn cob cellulose to be more reactive to cellulase hydrolysis. This is supported by the cellulase hydrolysis results. Figure 3 shows the effect of different stage of treatment on cellulase hydrolysis of corn cob when cellulase dosage of 8.5 IFPU per g corn cob cellulose was used. The results show that the combination of ammonia steeping and dilute acid hydrolysis gave the highest glucose yield of 91.8% based on dry cellulose within 48 h. The ammonia treated or dilute acid treated sample showed similar, but lower reactivity toward the cellulase. The sample without any treatment was least susceptible to cellulase hydrolysis.

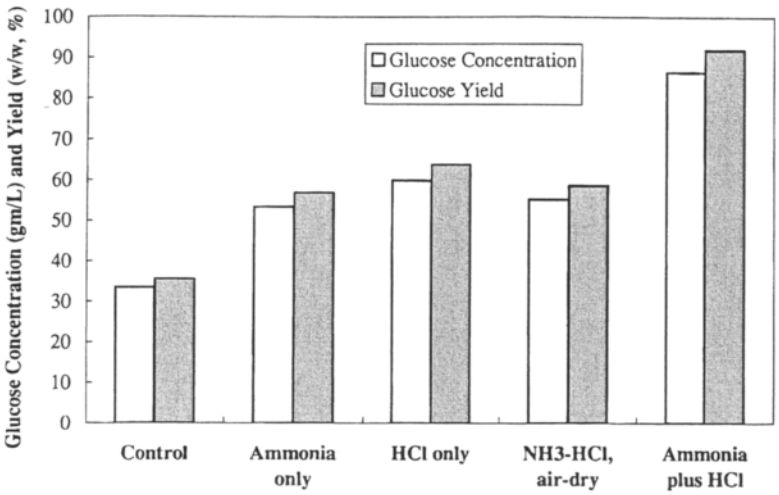

Fig. 3. Cellulase hydrolysis of corn cob after different treatment.

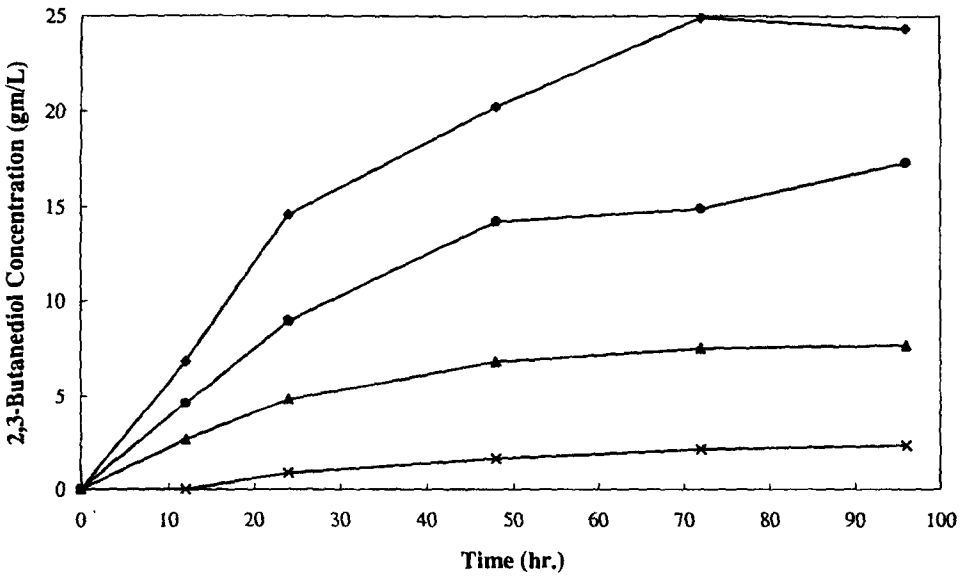

Fig. 4. Effect of different treatment on butanediol production from corn cob using SSF. -◆- Ammonia plus HCl, -●- HCl only, -▲- Ammonia only, -×- Control.

The SSF process is a viable option for conversion of biomass to ethanol *(16)* and butanediol *(6)*. The SSF process improves the hydrolysis rates and product yields in comparison to processes involving separate hydrolysis and fermentation. To demonstrate the effectiveness of the current pretreatment process, SSF experiments were performed using bacterial culture *K. oxytoca* and corn cob samples after different steps of treatment. As shown in Fig. 4, the combination of ammonia steeping and

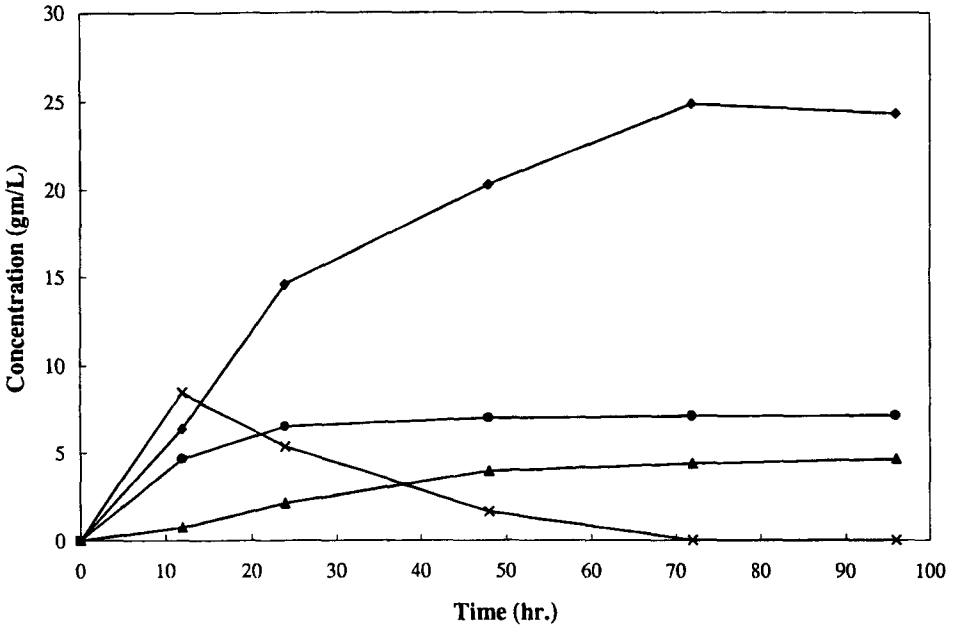

Fig. 5. Time course of SSF of cellulose fraction of corn cob. -◆- 2,3-Butanediol, -●- Ethanol, -▲- Cellubiose, -×- Glucose.

dilute acid hydrolysis gives the best results. The rate of butanediol production is at least threefold higher than those with ammonia, but without acid treated sample. Compared to the untreated sample, a tenfold increase in butanediol accumulation was obtained with ammonia-acid treated sample.

The time course of product formation during SSF with ammonia and acid treated sample is shown in Fig. 5. During 10 h of incubation, the glucose concentration increased to about 9 g/L and then declined as would be expected as a result of the presence of bacterial cells. The amount of cellobiose also increased slowly and reached the level of about 4 g/L at 48 h. Ethanol concentration increased to 7 g/L (accounting for about 17% substrate) by 24 h and stayed more or less unchanged throughout the remainder of the fermentation. The butanediol concentration increased almost linearly and reached a level of 25 g/L after 72 h. This is a yield of 62.5% based on total dry cellulose (80 g/L). The relatively low yield is due in part to coproduction of other products such as ethanol.

The stepwise removal of lignin and hemicellulose fractions from lignocellulose appears to be effective in the utilization of the cellulose fraction for butanediol production. The pretreatment can have the following beneficial effects: the solubilization of lignin and the separation of lignin from cellulose fraction, the chemical swelling of cellulose *(10,16)*, disruption of the crystalline structure of cellulose, and increase in the accessible surface area for cellulase *(25)*. Consequently, the cellulase dosage required for cellulose hydrolysis is 50% or less than those reported in the literature *(26)*.

Another advantage to be gained by the current procedure is the obtaining of a lignin- and acetate-free hemicellulose hydrolyzate. This fraction can be converted to products in the separate streams. This is significant in view of the fact that pentose utilization by micro-organisms is slower and its utilization by micro-organisms is often subjected to glucose inhibition even in the presence of low concentrations of glucose.

Unlike lignin recovered from acid hydrolysis or conventional pulping process, the lignin fraction removed by ammonia steeping remains chemically unchanged. This is because low temperatures and low pressures are used during ammonia steeping. The lignin residues after ammonia removal can be useful for synthesis of chemicals and polymers. Based on our estimation, at least 98% of ammonia is recoverable for reuse. The large scale ammonia recovery technology currently in practice in fertilizer and ammonia industries can be used for this purpose.

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