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Studies on Hemicellulase System in *Aspergillus niger*
— Bioconversion of Cellulosic Wastes for the Production of D-xylose —

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*Aspergillus niger*의 Hemicellulase계 효소에 관한연구
-생물전환공정에 의한 D-Xylose의 생산-

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Systematic bioconversion process for the production of xylose from agricultural wastes such as barley straw and corn cobs was studied. After the pretreatment in 1% NaOH solution for 24 hours at 30°C, enzymatic hydrolysis of barley straw for 48 hours at 30℃ resulted in the liberation of 15.8% of reducing sugar which is equivalent to 87% of total D-xylose content. Among various agricultural wastes, corn cob as well as barley straw was demonstrated to be potent sources for the production of D-xylose by the process of enzymatic conversion.

The primary objective of the article is to develop systematic bioconversion processes for the production of useful chemicals from agricultural wastes. The major constituents of agricultural wastes such as straws are cellulose, hemicellulose, and lignin with the contents of 36%, 25%, and 15% on dry weight basis. Much research has been conducted through out the world for the bioconversion process of cellulose into glucose and further to liquid fuel such as alcohol as well as feed stuff such as single cell protein[1,2].

The major constituent of hemicellulose is xylan, which is a pentosan composed of D-xylose. D-xylose can be utilized as a substrate of acetone-butanol fermentation or for the production of single cell protein[3,4,5]. D-Xylose is also considered to be useful source for the ethanol fermentation in addition to the cellulosic source[6]. It can also be converted into xylitol, an artificial sweetener by hydrogenation[7].

In the previous paper, a potent microorganism *Aspergillus niger* KG-79 was isolated for the purpose of efficient degradation of barley straw xylan and the enzymatic conversion of isolated D-xylan was reported[8,9]. In this study, a complete bioconversion process including pretreatment of the substrate for the production of D-xylose directly from barley straw was carried out. The applicability of the process to other agricultural wastes was also tested.

Materials and Methods

Preparation of crude xylanase

For the purpose of a large scale enzyme production, solid culture on wheat bran koji was adopted. The koji culture media was prepared with 1.6Kg wheat bran and 0.4Kg barley straw mixed with 1.5Kg water. After sterilization and inoculation the koji was evenly spread in a pan. Three days of culture at 26°C demonstrated the maximum yield of the enzyme productivity. The crude extract of D-xylanase was obtained by extraction of the Koji culture with 5 volumes of water at 30°C for 2 hours[8].

Assay method of D-xylanase

D-Xylanase activity was determined with 0.25%

solution of D-xylan as a substrate in 50mM acetate buffer (pH 4.5) at 40°C[8]. Reducing sugar was assayed the method of Somogyi-Nelson method[9].

Pretreatment of barley straw

Barley straw cut with a hammer-mill to 2.5 mesh was soaked in the NAOH solution at a liquid to solid ratio of 5 (w/w) and 30°C.

Enzymatic hydrolysis

Digestibility of the substrate was tested after neutralization and adjustment of pH to 4.5 with HCl solution. Forty units of the crude enzyme extract produced by koji culture of *Aspergillus niger* KG79 was added to one g of the substrate. Temperature of hydrolysis was 30°C. The solid/liquid ratio was maintained at 1/10 (w/w).

Analysis of sugar components

Component sugar was analyzed by the high performance liquid chromatography (Varian model 5021) with a micropak-NH_2 column. Using acetonitrile-H_2O as an eluent, the effluent was detected by a refrative index detector or a UV detector at 192nm.

Materials

Xylan from larch wood was purchased from Sigma Chemical Co., U.S.A. Barley and rice straws were collected at Suwon area in Korea.

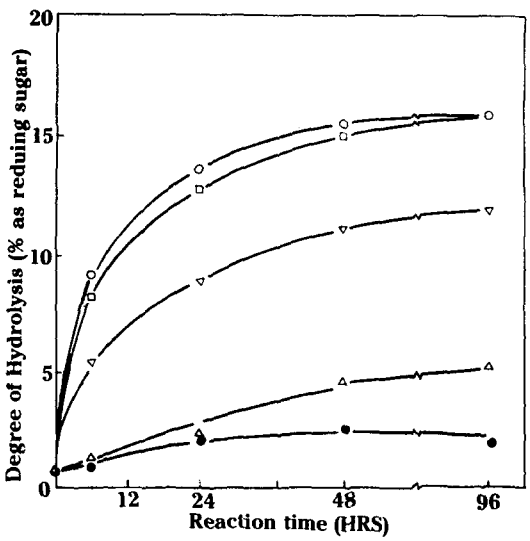

Fig. 1. Yield of the enzymatic hydrolysis of barley straw (2.5 mesh) pretreated with various concentration of NaOH solution.

0.25%(△), 0.5% (▽), 1.0% (□), 2.0% NaOH solution (○), and water (●).

Results

Effect of pretreatment

The pretreatment of barley straw was carried out with sodium hydroxide solution in various concentration at room temperature. The yield of enzymatic hydrolysis of the pretreated barley straw depended upon the alkaline strength of NaOH solution. Alkaline solution above 1.0% did not significantly improve the yield of xylose production (Fig. 1).

The particle size of straw affected the enzymatic conversion yield to some extent at lower concentration of NaOH solution. The effect of the size of straw was, however, insignificant as the substrate was pretreated with alkaline solution higher than 1.0% (Fig. 2).

The treating time of barley straw with 1.0% NaOH solution also affected the yield of hydrolysis (Fig. 3). It required at least about 24 hours at room temperature.

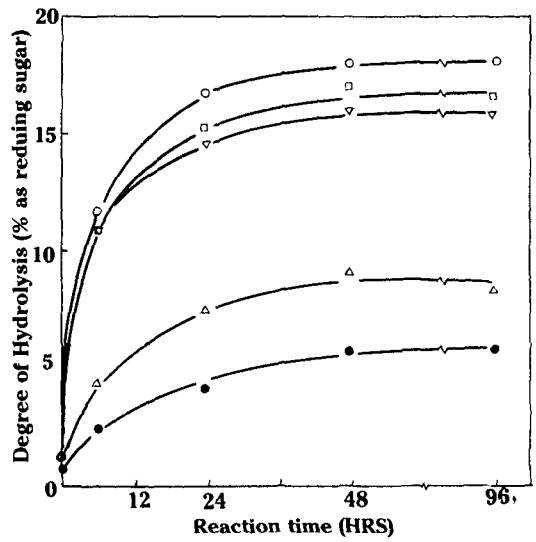

Fig. 2. Yield of the enzymatic hydrolysis of barley straw (40 mesh) pretreated with various concentration of NaOH solution.

0.25%(△), 0.5% (▽), 1.0% (□), 2.0% NaOH solution (○), and water (●).

Effect of enzyme concentration

Hydrolysis was carried out over 48 hours at 30°C at a liquid/solid ratio of 10(w/w) (Fig. 1). The amount of enzyme required for reasonable conversion yield within 48 hours was determined to be 4 U/ml reaction mixture or 40 U/g substrate (Fig. 4).

Conversion yield

The conversion yield of 86.8% or more was obtained

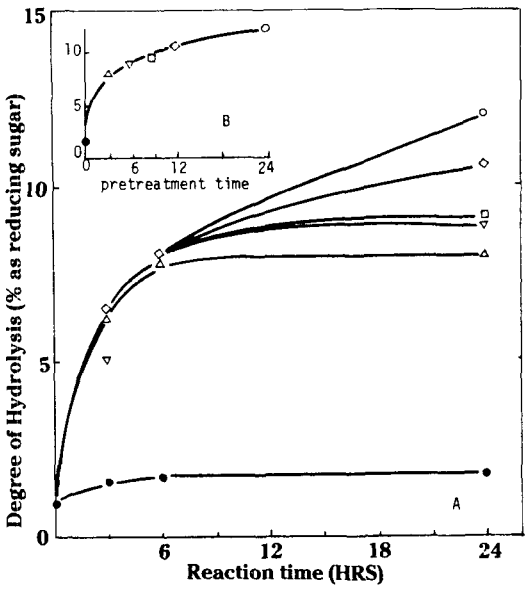

Fig. 3. Effect of pretreatment time with 1% NaOH solution on the conversion yield.

A. Time course of the enzymatic conversion with different time of pretreatment.
B. A plot of the conversion yield vs. pretreatment time after 24hrs reaction time.
3 hours (△); 6 hours (▽) hours (□); 12 hours (◇); 24 hours (○); no pretreatment (●).

under the above condition as the pentosan content in barley straw was considered to be 18.2% by TAPPI standard method. The final sugar concentration of the extract was 15.8g/Kg as a reducing sugar.

The sugar composition of the hydrolyzate was analyzed to be 87% xylose, 9% arabinose, 3% glucose, and 1% ribose respectively (Fig. 5).

Application of different substrate sources

Various different sources of agricultural wastes other than barley straw were also tested for the productivity of D-xylose under the same conversion process studied above. It was noted that corn cobs was one of the potent source for the xylose production whereas rice bran demonstrated the lowest yield (Table 1). The production yield of xylose from corn cobs was 19.6%.

Process Description

On the basis of available data from the experiments described above, a complete process was designed for the enzymatic hydrolysis of 1M/T barley straw per day.

A flow diagram of the overall process for the production of xylose is shown in Fig. 6. The primary plant feed

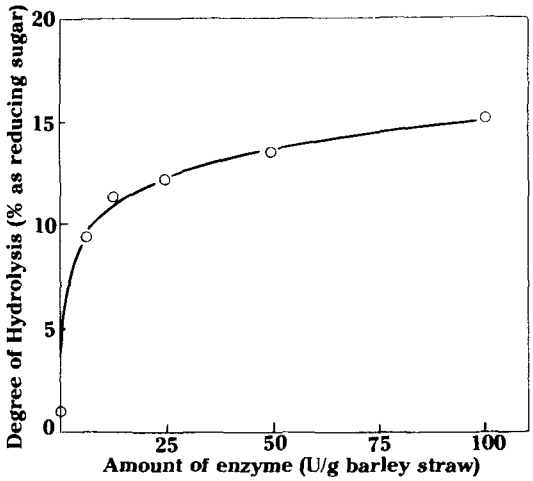

Fig. 4. Effect of enzyme concentration on the conversion yield.

The enzymatic hydrolysis was carried out for 48 hours with various amount of the enzyme after the pretreatment with 1.0% NaOH for 24 hours.

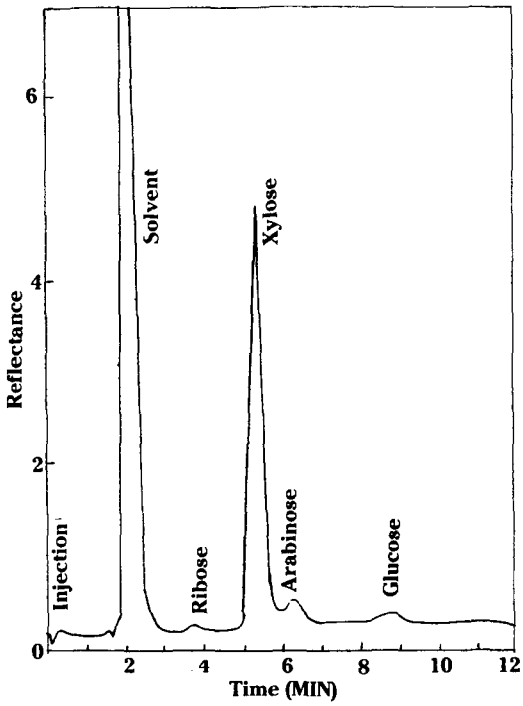

Fig. 5. High performance liquid chromatogram of barley straw hydrolyzate.

Micropak NH_2 column was used with acetonitrile: H_2O = 80:20 as a solvent system. The flow rate was 2ml/min. The detector was RI, 8X.

considered is 1M/T per day of barley straw. The size of feed is cut to approximately 2.5 mesh by means of ham-

mer milling. The size of feed is not critical so long as the substrate soaked in aqueous suspension which can be mixed and filtered.

The pretreatment and hydrolysis can be carried out in a single tank at a mild condition of pH 4.5 and 30 °C. According to this process one can obtain the final conversion yield as high as 86.8% on the basis of the content of pentosan.

Table 1. Yield of Enzymatic Conversion of Cellulosic wastes

Substrate	Yield (% w/w)
Rice bran	4.46
Sawdust	6.84
Rice straw	8.01
Corn cob	19.60
Barley straw	15.80

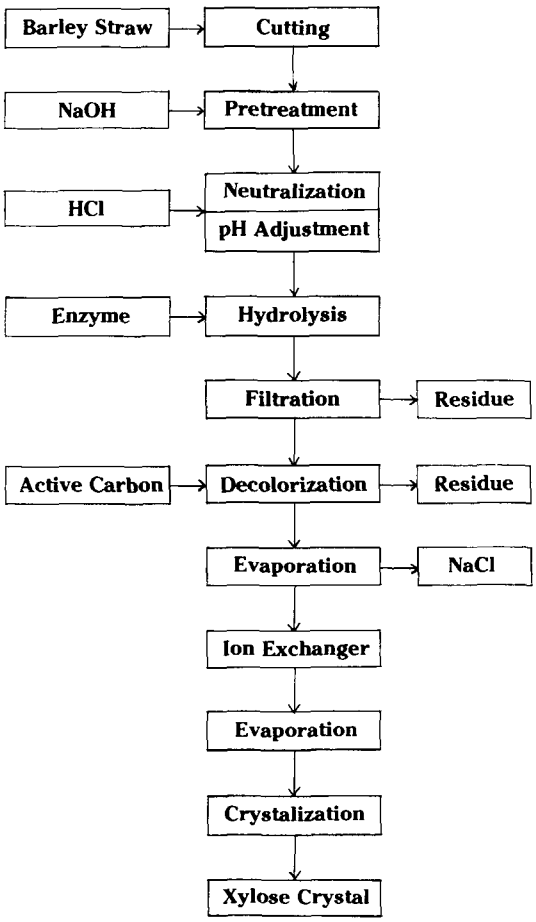

Fig. 6. A flow diagram of the hydrolysis and purification process.

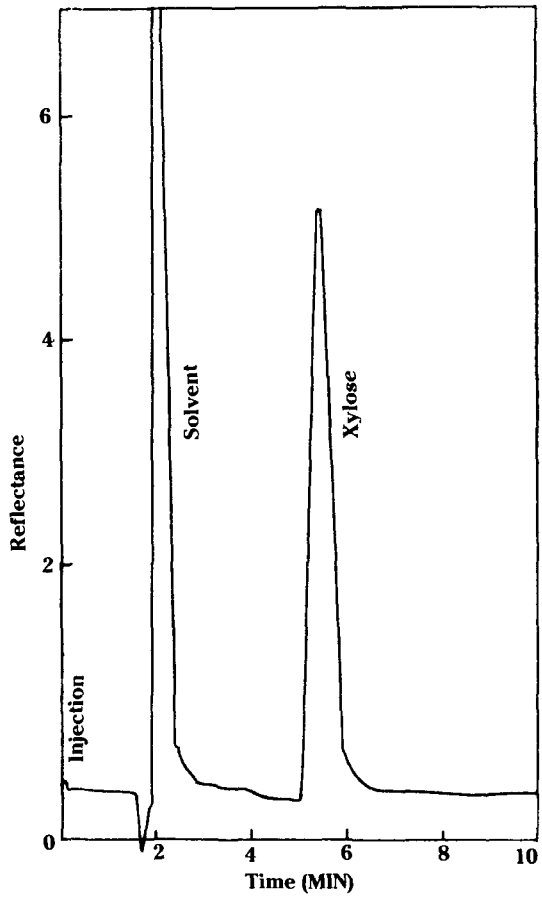

Fig. 7. High performance liquid chromatogram of the purified D-xylose.
The HPLC conditions were same as Fig. 5.

The xylose solution thus obtained was desalted and crystallized. The purity of the crystaline D-xylose was checked by HPLC and found to be homogeneous (Fig. 7).

Discussion

Apergillus niger has been known to be a potent microorganism which produces D-xylanases. Such xylanase system has been studied by many investigators[11-19]. For the production of D-xylanase wheat bran koji culture method was used. Wheat bran is a good source of xylan, which plays an important role for the induction of D-xylanase.

The rate and the extent of enzymatic hydrolysis depends upon the particular substrate and the type of pretreatment applied. The need for the pretreatment appears inevitable due to the content of lignin and cellulose in raw material[19-21]. As a result of unusual

structure of lignin it is highly resistant to the biological degradation. Lignin is a complex ploymer comprised of p-hydroxycinnamyl alcohols. The monomers are bound in such a way as to provide a complex three-dimensional structure which is interconnected with cellulose and hemicellulose. Such a structural characteristic reduces the accessibility of these compounds to the degradating enzymes.

Thus, native ligno-cellulosic material requires pretreatment by physical and/or chemical means for the enhanced hydrolysis of the enzymes. Some of the mechanical treatments such as ball milling, steaming, steam explosion seem to cause exposing polysaccharide surface by destroying the structure of lignocellulosic materials without removing lignin[22-24]. The enhanced rate of hydrolysis by the pretreatment with an oxidizing agent (SO_2, chlorite etc.) or alkali (NaOH, NH_3 etc.) appears to be mainly due to disrupting the lignin-carbohydrate interaction, or *in situ* structural loosening and solubilization of hemicellulose and lignin[25-28]. Recently, selective solvent extraction methods of lignin has also been reported[29,30].

The pretreatment adds substantially to the cost of the biological conversion of hemicellulose. Since barley straw contained lower level of lignin (12.5% by TAPPI standard method T13m-54), mild treatment with 1% NaOH at the liquid/solid ratio of 5(w/w) was enough for attaining reasonable production yield of xylose by the subsequent hyrolysis.

It is of interest to note that the degree of the enzymatic conversion of barley straw xylan *in situ* was 87% whereas it was only 54% for the isolated barley straw xylan[8]. The results suggest that the raw barley straw is better source for the production of purified D-xylose by the enzymatic conversion process then the isolated ones. It appears that the isolated xylan from plant tissue matrix caused aggregation after some degree of enzymatic hydrolysis and thus became less accessible to the enzyme resulting in the limited degree of conversion[8]. On the contrary to the isolated xylan, xylan situated in the ligno-cellulosic matrix is likely to be readily accessible to the enzyme after the mild pretreatment. This result can be attributed to the effect of ligno-cellulose which seems to protect D-xylan from the agregation after enzymatic modification.

The conditions required for the acid hydrolysis of hemicelluloses are milder and the yield is considerally higher than those required for the hydrolysis of cellulose. The hydrolytic yield for cellulose is 50-55% while that for hemicellulose 70-90%. This process, nevertheless, occurs with simultaneous side reactions that produce a considerable amount of by-product. The xylose content of the acid hydrolyzate is lowered by undesired sugar contaminant and conversion to furfural which is an undesirable toxic substance for fermentation. There are considerable number of reports for the process of acid and enzyme hydrolysis studied previously. These are summarized in Table 2 and 3. It is likely that the enzymatic process is more efficient and specific for the production of D-xylose than the acid hydrolysis.

요 약

보리짚이나 옥수수 속과 같은 농산폐자원 으로부터 xylose를 생산하기 위한 생물전환공정에 관한 연구를 수행했다.

Table 2. A Summary of Acid hydrolytic Process of Hemicellulose

Starting material	Acid concentration	Temp. (°C)	Time	Liquid/solid ratio (w/w)	Yield (%)	Purity (%)	Ref.
Rice hull	0.1N HCl	120	2 hrs	20	—	68.4	28
Corn cobs	0.9-1.0% H_2SO_4	114-124	3 hrs	10	95	—	31
Corn cobs	0.2-10.0% H_2SO_4	90-140	4-14 hr	—	52	98.6	32
Corn cobs	water	140	90 min				
	0.2% SO_2	160	20 min	—	80	—	33
Corn stover	4.4% H_2SO_4	110	50 min	17	94	77.8	34
Maize cob	0.5% H_2SO_4	150-170	40 min	—	72	70-80	35
Rye grass	2% H_2SO_4	150	10 min	10	74	60.0	36
Wheat straw	1% H_2SO_4	100	5.5 hrs	13	70	67.4	37

Table 3. A Summary enzymatic Hydrolysis Process of Pentosan

Starting material	Pretreatment condition	Hydrosis condition	Yield (%)	Purity (%)	Ref.
Corn cob	5% NH_3 30°C 5days	55°C, 24hr	72	79.9	38
	2% NaOH 30°C 6hrs	55°C, 24hr			11
White birth	3% NaOH 30°C 5days	55°C, 24hr	86	92.7	38
Rice straw	5% NH_3 30°C 5days	55°C, 24hr	59	70.8	38
Barley straw	1% NaOH 30°C 1day	30°C, 48hr	86.8	87	this process

보리짚을 1% 가성소다용액으로 24시간 30℃에서 전처리한후 30℃에서 48시간 효소 가수분해를 시킨결과 15.8%의 환원당을 유리시켰다. 이 환원당의 량은 보리짚에 들어있는 전 D-xylose 함량에 87%에 해당하는 것이다. 여러가지 농산폐자원의 효소전환공정을 시험한 결과, 보리짚과 옥수수속이 D-xylose 생산에 가장 적합한 원료가 될수 있음을 관찰하였다.

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