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Use of Hemicellulose Hydrolysate for β-Glucosidase Fermentation

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ABSTRACT

Hydrolysis of cellulose by *Trichoderma* cellulases often results in a mixture of glucose, cellobiose, and low-mol-wt cellodextrins. Cellobiose is nonfermentable for most yeasts, and therefore it has to be hydrolyzed to glucose by β-glucosidase prior to ethanol fermentation. In the present study, the β-glucosidase production of one *Penicillium* and three *Aspergillus* strains, which were previously selected out of 24 strains, was investigated on steam pretreated willow. Both steam-pretreated willow and hemicellulose hydrolysate, released during steam explosion of willow, were used as carbon sources. Reference cultivation runs were performed using prehydrolyzed Solka Floc and glucose. The four strains were compared with *Trichoderma reesei* regarding sugar consumption and β-glucosidase production. *Aspergillus niger* and *Aspergillus phoenicis* proved to be the best enzyme producers on hemicellulose hydrolysate. The maximum β-glucosidase activity, 4.60 IU/mL, was obtained when *A. phoenicis* was cultivated on the mixture of hemicellulose hydrolysate and steam-pretreated willow. The maximum yield of enzyme activity, 502 IU/g total carbohydrate, was obtained when *Aspergillus foetidus* was cultivated on the hemicellulose hydrolysate.

Index Entries: β-glucosidase production; hemicellulose hydrolysate of willow; *Trichoderma; Penicillium; Aspergillus.*

INTRODUCTION

Different techniques are available for the conversion of lignocellulosic materials to fuel ethanol. During the past decade, process alternatives based on enzymatic hydrolysis have been the focus of interest, showing

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good yields of fermentable sugars from both soft- and hardwoods (*1–3*). Most of these processes consist of the following five major process steps: pretreatment of raw material, including chopping, screening, and prehydrolysis of hemicellulose; cellulase enzyme production; enzymatic hydrolysis of the cellulose fraction; fermentation of the hydrolysate, using a suitable microorganism; and ethanol refining (*4*). One method for prehydrolysis of the hemicellulose fraction, which has been used for a large variety of woody materials, is high-pressure steam pretreatment (*5*). During the steam-pretreatment of wood, the hemicellulose part of the material is degraded mostly to monosaccharides (xylose, mannose, galactose, glucose, and arabinose). The fibrous material remaining after pretreatment consists of cellulose and structurally modified lignin. As a result of the steam explosion, the pretreated material is more accessible to enzymatic attack.

The cellulose fraction of the fibrous material is further hydrolyzed to cellobiose and glucose by means of cellulolytic enzymes. Mainly, three groups of hydrolytic enzymes are involved in the hydrolysis of cellulose to glucose: endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidase/cellobiase (*6,7*). The cellobiase enzyme has an important role in the hydrolysis of cellulose. It cleaves the β-glucosidic bond in the cellobiose, which is released by EG and CBH from the cellulose. The conversion of cellobiose, which is inhibitory to CBH and EG (*8*), increases the yield considerably (*9*).

The β-glucosidase activity in most *Trichoderma* cellulase preparations has been shown to be much lower than what is required for an efficient conversion of cellulose to glucose (*10*). Although the cellobiase production of *Trichoderma reesei* Rut C 30 can be enhanced by changing the pH and temperature profile of the fermentation (*11*), addition of external cellobiase from other microorganisms is required. The optimal ratio of β-glucosidase: cellulase activity required for sufficient hydrolysis of cellulose was reported to be in the range of 0.8–1.5 IU of β-glucosidase per filter paper unit of cellulase activity (*12*).

When hardwood, in which the xylan content varies between 15–23% based on dry matter (*13*), is used for ethanol production, the xylose fraction leaves the process unchanged. This reduces the overall ethanol yield based on carbohydrates, since it cannot be fermented by ordinary baker's yeast. There are other pentose fermenting microorganisms, which are capable of converting C5 carbohydrates to ethanol, but these are sensitive to inhibitory compounds formed during the steam pretreatment (*14,15*). Another option for utilizing the xylose-rich liquid is to use it for cellulase enzyme production. In a previous study, in which *T. reesei* Rut C 30 was cultivated on the hemicellulose hydrolysate, supplemented with fibrous pretreated willow at a total carbohydrate concentration of 20 g/L, a cellulolytic enzyme activity of 1.79 filter paper activity (FPU)/mL, and only 0.43 IU/mL β-glucosidase activity, was obtained (*16*). An alternative is to utilize the hydrolyzed hemicellulose fraction for cellobiase production, which can

be used for supplementing the *Trichoderma* fermentation broth with β-glucosidase. When *Aspergillus niger* VKMF-2092 was cultivated on a 50–50% mixture of glucose and wheat bran at 10 g/L total carbohydrate concentration, an activity of 3.25 IU/mL was obtained after 148 h residence time *(17)*. *Aspergillus wentii* cultivated on various carbon sources at 30 g/L carbohydrate concentration yielded β-glucosidase activities in the range of 0.35–1.10 IU/mL *(18)*.

In the present study, four different β-glucosidase producers, *A. niger*, *Aspergillus phoenicis*, *Aspergillus foetidus*, and *Penicillium ochro-chloron* were investigated. The yield of β-glucosidase, using these fungal strains grown on glucose, prehydrolyzed Solka Floc 200 (FS&D, Urbana, OH), and hemicellulose hydrolysate from willow, were determined and compared with the yield obtained with *T. reesei*.

MATERIALS AND METHODS

Pretreatment of Willow

Salix caprea, a fast-growing willow species, was used for enzyme production. The raw material contained 36.8% cellulose, 23.0% hemicellulose, 20.7% lignin, and 19.5% other compounds. The willow was chipped and sieved, and the fraction between 1 and 3.5 mm was used. First, the willow chips were presteamed for 40 min with 1 bar saturated steam. The hot presteamed material was immediately transferred into plastic bags, impregnated with 1% SO_2, based on oven-dried material (ODM), and stored overnight at room temperature. The steam pretreatment of the impregnated material was performed at 207°C for 5 min with saturated steam *(13,19)*. The pretreated material was diluted to approx 5% ODM with hot tap water, stirred for 30 min, and filtered using a PF 0.1H2 (Larox OY, Finland) filter press unit *(4)*. The filtrate comprised of hydrolyzed hemicellulose, was divided into two fractions. One fraction was used without further treatment. The other fraction was concentrated by vacuum evaporation, removing 75% of the water at 80°C and pH 2.8–3.0, using a Büchi RE 121 rotavapor (Büchi Labortechnik AG, Switzerland), thus removing the volatile compounds that have been shown to be inhibitory to *T. reesei* *(16)*. The composition of the steam-pretreated willow (SPW), regarding cellulose and lignin content, was determined by Hägglund's method *(20)*, with the modification that the sugar content of the acid hydrolysate was analyzed as well for total sugars, which was used to calculate the cellulose content. The steam-pretreated willow contained 50% cellulose and 37% lignin, based on ODM. Both the original filtrate (F) and the concentrated filtrate (CF) were analyzed for sugar content, using phenol sulfuric acid method *(21)*. The total sugar concentration was 64 g/L and 267 g/L for F and CF, respectively. The fibrous material (SPW), the F, and the CF were all used as carbon (C) sources for β-glucosidase production. The amounts of SPW, F, and CF added to the medium were based on the cellulose and sugar contents given above.

Prehydrolysis of Cellulose

The prehydrolysis of cellulose powder (Solka Floc) was carried out in an NBS G24 (New Brunswick, NJ) rotary shaker using 500 mL E-flasks. A total volume of liquid of 300 mL, containing 20 g/L Solka Floc, 8 mL/L Celluclast 1.5 L enzyme preparation (Novo A/S, Denmark), and tap water, was incubated at 50°C, pH 4.8, and 150 rpm for 20 h. The obtained slurry, i.e., the prehydrolyzed Solka Floc (PSF), was diluted twice with tap water, and used as a C source for β-glucosidase production.

Fungal Strains

The following five fungal strains were used for cellobiase production: *A. niger* BKM F-1305, *A. phoenicis* QM329, *A. foetidus* Biogal 39 (strain collection Dr. G. Szakacs, Technical University of Budapest, Dept. of Agricultural Chemical Technology, Budapest, Hungary), *P. ochro-chloron* WFPL 175A, and *T. reesei* Rut C 30, respectively. The strains were obtained from the Department of Agricultural Chemical Technology, Technical University of Budapest.

Inoculum Preparation

Prior to use, all fungi were stored on agar slants containing (in g/L) 20 malt extract, 5 glucose, 1 proteose peptone, and 20 bacto agar. After 2–3 wk at 30°C, the conidia were used to initiate growth in 750-mL E-flasks containing 150 mL medium. The original Mandels medium (22), in which the concentration of nutrients were (in g/L) 0.3 urea, 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 $CaCl_2$, 0.3 $MgSO_4$, 0.25 yeast extract, and 0.75 proteose peptone, together with 10 g/L Solka Floc cellulose powder, was used for *Trichoderma* inoculum preparation. Trace elements were also added (mg/mL): 5 $FeSO_4 \cdot 7H_2O$, 20 $CoCl_2$, 1.6 $MnSO_4$, and 1.4 $ZnSO_4$. The pH before sterilization was adjusted to 5.5–6.0. The inoculum was ready after 4 d at 30°C and 300 rpm. For Aspergilli; and *Penicillium*, the medium contained only 5% malt extract in tap water. Prior to sterilization, the pH was set to 5.5–6.0. After 2 d at 30°C and 300 rpm, the inoculum was ready.

Shake-Flask Cultivation

The mycelia obtained from the inoculum were used to initiate growth in 750-mL E-flasks containing 150 mL Mandels medium, including the various C sources, i.e., glucose, PSF, F, CF, and F, supplemented with fibrous SPW. Each strain was cultivated on each C source. The total concentration of the carbohydrates available in the medium was set to 10 g/L, except for F, in which it was 5 g/L. At this sugar concentration, the filtrate proved to be not inhibiting (16). The inoculum constituted 10% of the medium. The enzyme production was performed in a rotary shaker at 300 rpm and 30°C. Samples were withdrawn once a day, and, at the same time, the cultivation was adjusted to pH 6.0 with addition of either 10% NaOH

or 10% H_2SO_4 solutions, when required. The samples were centrifuged using a Janetzki T24 (Leipzig, Germany) centrifuge at C 6100*g* for 10 min. The supernatant was analyzed for enzyme activity and sugar content.

ANALYSIS

The enzyme activity of the samples from *Aspergillus* and *Penicillium* cultivation was determined as β-glucosidase activity, using Berghem's method *(23)*; the samples from the cultivation with *Trichoderma* were analyzed for both β-glucosidase activity, using Berghem's method, and FPU, using Mandels procedure *(24)*. The reducing-sugar content of the samples was determined with the DNS method *(25)*.

RESULTS AND DISCUSSION

The four strains of *Aspergillus* and *Penicillium* were selected after a screening of 24 strains of *Aspergillus, Penicillium, Trichoderma, Chaetomium, Geotrichum,* and *Paecilomyces*, using PSF as a C source. The four selected strains yielded the highest β-glucosidase activity after inoculation and 10 d of cultivation (results not shown). The aim of the present study was to utilize the pentose rich hydrolysate containing sugars only in form of monosaccharides for enzyme production.

Does β-Glucosidase Production Need to be Induced?

The biosynthesis, not only of the cellulases but of β-glucosidase in microorganisms, is, in most cases, subjected to catabolite repression *(26,27)*. The production of β-glucosidase is initiated only after the glucose or other easily metabolizable monosaccharides in the medium have been utilized. To investigate whether the β-glucosidase synthesis needed to be induced, all strains were cultivated in shake flasks, both on PSF and on glucose-containing media, at a total C source concentration of 10 g/L. For each experimental condition three fermentation runs were performed in parallel, and the mean values of the enzyme activity and the sugar concentration were calculated. The standard deviation (SD) was 0.05 IU/mL for the enzyme activity.

When *A. niger* was cultivated on PSF, a β-glucosidase activity of 2.46 IU/mL was obtained, compared with 2.80 IU/mL reached, when cultivated on glucose medium (Fig. 1A). After 2 d of cultivation, most of the sugars present in both media were consumed. The higher yield on glucose indicates that the β-glucosidase secretion of *A. niger* is not repressed by glucose.

For *A. phoenicis*, the maximum β-glucosidase activities obtained after 6 d were 2.80 IU/mL and 3.60 IU/mL, when cultivated on glucose and on PSF, respectively (Fig. 1B). In both cases, the soluble sugar content of the medium was reduced to 0 g/L after 2 d, and, at the same time, the enzyme production was started. The somewhat lower enzyme activity

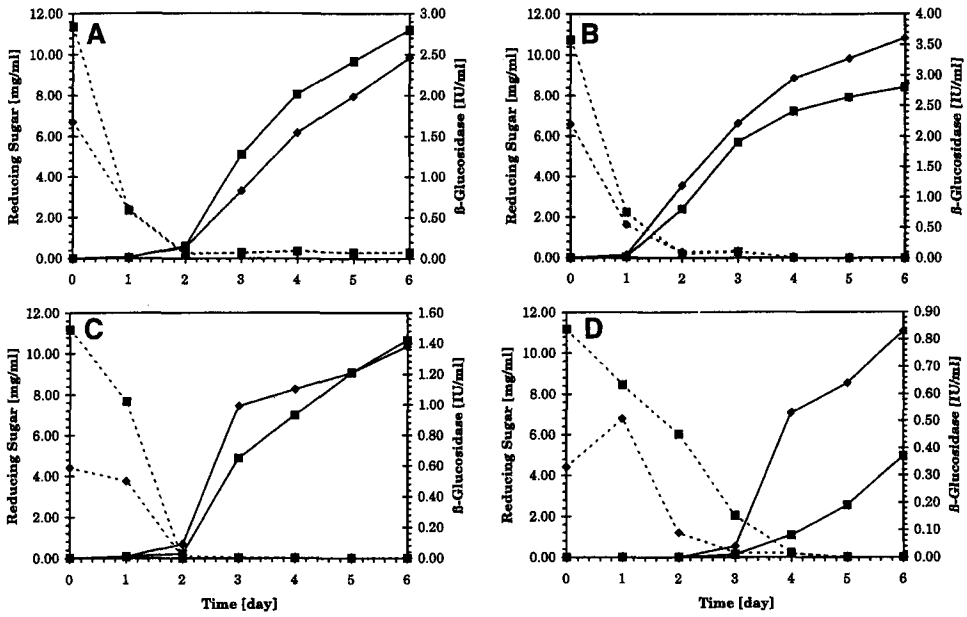

Fig. 1. β-Glucosidase activity and reducing sugar concentration vs time for cultivation of **(A)** *A. niger*, **(B)** *A. phoenicis*, **(C)** *A. foetidus*, and **(D)** *P. ochro-chloron*, on glucose medium (■), and on PSF medium (◆). Solid lines = enzyme activity, broken lines = sugar concentration.

reached after 6 d on glucose cannot be explained with catabolite repression. A significant amount of enzyme was produced on glucose medium, with an initial production rate equal to that obtained with PSF. After 4 d, the production rate started to decline (Fig. 1B), presumably because of depletion of nutrients. The cell mass produced on glucose was higher than that produced on PSF, resulting in a higher consumption of nutrients.

There was no significant difference between the two C sources when *A. foetidus* was cultivated, but only 1.40 IU/mL activity was reached on both media (Fig. 1C).

For the *P. ochro-chloron*, the possibility of catabolite repression cannot be excluded (Fig. 1D). There was a significant difference in enzyme activity after 5 d of cultivation for the two C sources. The enzyme activity reached was 237% higher on PSF than on glucose. The final activity obtained on glucose was 0.37 IU/mL, which is 55% less than that reached on PSF. Furthermore, the sugar consumption rate was lower on glucose than on PSF, which indicates catabolite repression.

Enzyme Production on Concentrated Filtrate

The enzyme production on concentrated filtrate was investigated at a carbohydrate concentration of 10 g/L. The highest β-glucosidase activity after 6 d cultivation, 3.75 IU/mL, was obtained with *A. phoenicis*, and the

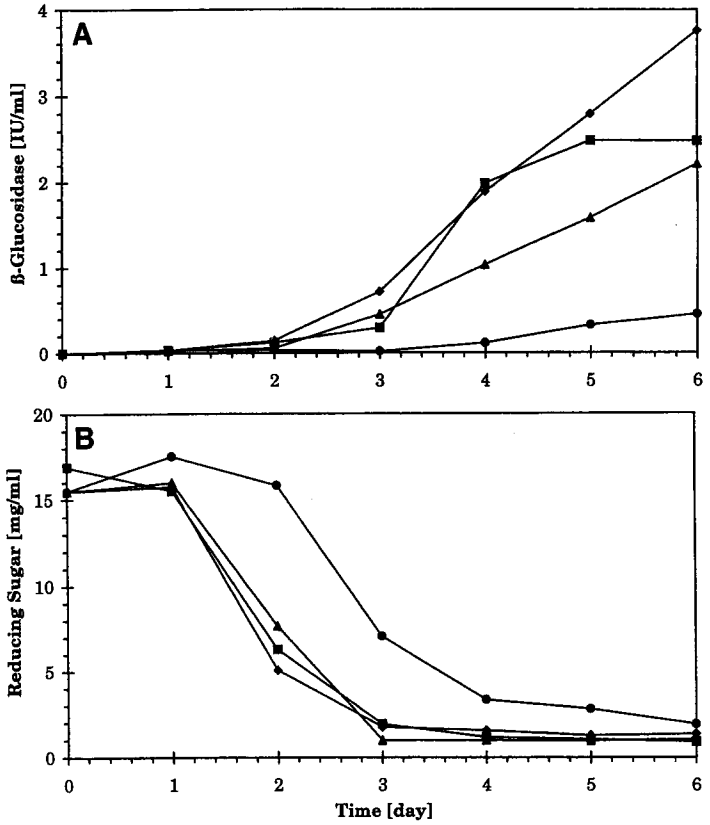

Fig. 2. β-Glucosidase activity and reducing sugar concentration vs time for various fungal strains cultivated on CF at a sugar content of 10 g/L. **(A)** β-Glucosidase activity. **(B)** Sugar concentration. *A. niger* (■), *A. phoenicis* (◆), *A. foetidus* (▲), *P. ochro-chloron* (●).

lowest, 0.45 IU/mL, for *P. ochro-chloron* (Fig. 2). The enzyme yield of *A. niger* obtained on CF was somewhat lower than that reached on pure glucose medium. All the other strains produced significantly more enzyme on CF than on glucose (Table 1), even though the sugar consumption rates were lower on the CF medium. Although the yield of enzyme activity for *P. ochro-chloron* reached on CF was increased with 22%, compared to that obtained on glucose, it was much lower than for all the other strains (Fig. 2).

Enzyme Production Using Original Filtrate

Two main series of experiments were performed to study the β-glucosidase production, using F as a C source. In the first series, the total concentration of sugars in the medium was set to 5 g/L by dilution with tap water. In the second series, the same diluted F was used, but it was

Table 1
β-Glucosidase Activities and Yields Based of Total Carbohydrates After 6 d of Cultivation Using Glucose, PSF, and F- and CF-Containing Media

	Glucose		PSF		F		CF	
Strain	Activity (IU/mL)	Yield (IU/g)	Activity (IU/mL)	Yield (IU/g)	Activity (IU/mL)	Yield (IU/g)	Activity (IU/mL)	Yield (IU/g)
A. niger	2.80	280	2.46	246	1.90	380	2.48	248
A. phoenicis	2.80	280	3.60	360	2.11	422	3.75	375
A. foetidus	1.42	142	1.38	138	2.51	502	2.21	221
P. ochro-chloron	0.37	37	0.83	83	0.24	48	0.45	45

Table 2
β-Glucosidase Activities Obtained After 6 d of Cultivation Using the Mixture of Diluted Filtrate and Steam Pretreated Willow

Strain	Activity (IU/mL)	Yield (IU/g soluble CH)	Yield (IU/g total CH)
A. niger	2.90	580	290
A. phoenicis	4.60	920	460
A. foetidus	2.02	404	202
P. ochro-chloron	0.69	138	69

supplemented with fibrous SPW at a cellulose concentration corresponding to 5 g/L. For each strain, three parallel runs were performed. The average SD in enzyme activity was 0.05 IU/mL. The results are presented as average values in Table 1 and Table 2.

The β-glucosidase activities and yields after 6 d of cultivation with the F-containing medium are shown in Table 1. For comparison, data obtained on glucose and PSF are also shown. The highest β-glucosidase activity, 2.51 IU/mL, was obtained with *A. foetidus*. Also, for this C source, *P. ochro-chloron* showed the lowest production of β-glucosidase, resulting in a final activity of 0.24 IU/mL. Although the sugar consumption rate on d 1 was lower for all strains, compared to those obtained on glucose and PSF, 95% of the available carbohydrates were consumed within 2 d. The enzyme yields based on added carbohydrates were used for comparison of the β-glucosidase production on different C sources (Table 1). All strains gave the highest yields when cultivated with the F-containing medium. The highest yield of 502 IU/g carbohydrate was obtained with *A. foetidus*, which was 3.5× higher than that reached on pure glucose, or on PSF. The second best yield, 422 IU/mL, was obtained with *A. phoenicis* on F, which is only slightly higher than that obtained on PSF.

In the second series of experiments using the mixture of F and SPW, *A. niger*, *A phoenicis*, and *P. ochro-chloron* gave higher β-glucosidase activities than when cultivated on the filtrate (Table 2). For *A. phoenicis*, an activity of 4.60 IU/mL was obtained, which is more than twice that obtained on F. The yield based on total available carbohydrate (Table 2) is higher than that obtained on F, which indicates that most of the cellulose was utilized for β-glucosidase production. When *A. niger* was cultivated on the mixed carbon source, a β-glucosidase activity of 2.90 IU/mL was obtained, which is 1.5-fold higher than when cultivated on F, indicating that only a part of the cellulose was utilized for β-glucosidase production. The β-glucosidase production of *A. foetidus* was lower, 2.02 IU/mL, on the mixed C source than on F.

Table 3
Cellulase and β-Glucosidase Activities Obtained with *T. reesei* Cultivated on Different C Sources

Carbon source[a]	FPU (FPU/mL)	β-Glucosidase (IU/mL)
Glucose	0.30	0.11
PSF	1.06	0.40
CF	0.61	0.11
F	0.27	0.05
F + SPW	0.83	0.15

[a] For abbreviations, see text.

β-Glucosidase Production of *T. reesei*

For comparison, *T. reesei* Rut C 30 was cultivated on all five C sources. The obtained β-glucosidase and FPU activities after 6 d of cultivation on the different media are summarized in Table 3. The highest β-glucosidase and cellulase activities, 0.40 IU/mL and 1.06 FPU/mL, respectively, were obtained on PSF. This gives an enzyme activity ratio of 0.38 β-glucosidase activity to FPU activity. Although relatively high cellulase activity, 0.83 FPU/mL, was reached on the mixture of F and SPW, the β-glucosidase concentration was low, 0.15 IU/mL, resulting in a β-glucosidase:FPU ratio of 0.18. This ratio is considerably lower than that required for efficient hydrolysis of cellulose. On the media containing only soluble sugars (glucose, F, and CF), very low β-glucosidase activities were obtained.

CONCLUSIONS

The hemicellulose hydrolysate of willow obtained after steam pretreatment of the raw material proved to be a very good substrate for β-glucosidase production with three different Aspergilli strains. The highest β-glucosidase activity of 4.60 IU/mL was obtained when *A. phoenicis* was cultivated on the mixture of filtrate and steam-pretreated willow at a total carbohydrate concentration of 10 g/L. This activity is higher than shown by other reported data on *Aspergillus* strains cultivated on different C sources *(17,18)*. Cultivation of *A. foetidus* on filtrate alone, at a carbohydrate concentration of 5 g/L, resulted in a β-glucosidase activity of 2.51 IU/mL, with the highest yield of enzyme activity, 502 IU/g carbohydrate. All Aspergilli strains performed well on the concentrated filtrate, with a maximum β-glucosidase activity of 3.75 IU/mL reached in the cultivation of *A. phoenicis*.

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