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Cellulase Production Based on Hemicellulose Hydrolysate from Steam-Pretreated Willow

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ABSTRACT

The production cost of cellulolytic enzymes is a major contributor to the high cost of ethanol production from lignocellulosics using enzymatic hydrolysis. The aim of the present study was to investigate the cellulolytic enzyme production of *Trichoderma reesei* Rut C 30, which is known as a good cellulase secreting micro-organism, using willow as the carbon source. The willow, which is a fast-growing energy crop in Sweden, was impregnated with 1–4% SO_2 and steam-pretreated for 5 min at 206°C. The pretreated willow was washed and the wash water, which contains several soluble sugars from the hemicellulose, was supplemented with fibrous pretreated willow and used for enzyme production. In addition to sugars, the liquid contains degradation products such as acetic acid, furfural, and 5-hydroxy-methylfurfural, which are inhibitory for microorganisms. The results showed that 50% of the cellulose can be replaced with sugars from the wash water. The highest enzyme activity, 1.79 FPU/mL and yield, 133 FPU/g carbohydrate, was obtained at pH 6.0 using 20 g/L carbon source concentration. At lower pHs, a total lack of growth and enzyme production was observed, which probably could be explained by furfural inhibition.

Index Entries: Cellulase enzyme production; *Trichoderma reesei* Rut C 30; lignocellulosics; furfural inhibition.

INTRODUCTION

Enzymatic conversion of willow, considered a promising raw material for large scale production of ethanol in Sweden, has been investigated during the last decade *(1,2)*. A typical process configuration consists of pre-

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treatment, enzyme production, enzymatic hydrolysis, fermentation, and ethanol refining as the major process steps. The raw material is first steam exploded with high pressure saturated steam to make the cellulose more accessible to enzymatic attack. In the hydrolysis step the cellulose is converted to glucose with cellulolytic enzymes, which are produced from a part of the pretreated material using *T. reesei*. The hydrolysate is then separated from the solid residue and fermented with baker's yeast to ethanol. The solid residue, mostly lignin, can be used as a solid fuel for production of process steam. Finally the ethanol is refined using distillation.

The raw material constitutes a large fraction of the total production cost in the bioconversion of lignocellulosic materials to ethanol *(3)*. A high degree of utilization of the raw material is thus required. When hardwood, such as willow, is used, the hemicellulose part, which constitutes 23% of the dry matter, is underutilized. During the steam-pretreatment of willow, the hemicelluloses are degraded mostly to monosaccharides of which 55% is xylose *(4)*. In addition to soluble sugars, by-products are also formed, which are inhibitory to fermentation *(5)*. When ordinary baker's yeast is used for alcoholic fermentation, the xylose fraction of the raw material leaves the process unchanged, which reduces the total yield of ethanol based on the amount of saccharides available in the raw material. There are also other yeasts, (such as *Pichia stipitis, Candida shehatae)*, which are capable of converting the pentoses to ethanol, but they do not ferment well in undetoxified hydrolysates *(6)*. Another possible way to utilize the pentoses is to use them for production of cellulolytic enzymes. This results in an increased ethanol yield as a part of the cellulose, which would be used for enzyme production that can be replaced with the xylose-rich liquid. The xylose utilization of *Trichoderma reesei* Rut C 30 was investigated by Mohagheghi et al. *(7)* using the mixture of Solka Floc and xylose in the medium. It was clearly shown that *T. reesei* can utilize the xylose, but for sufficient enzyme production the medium must be supplemented with either fibrous cellulose, or other inducers such as sorbose *(8)*.

In a previous study, it was shown that steam-pretreated willow is a suitable carbon source for enzyme production using *T. reesei* Rut C 30 *(9)*. The aim of the present work was to examine the enzyme production of the same strain on various mixtures of fibrous and liquid fractions from steam-pretreated willow as carbon sources. The effects of the composition, concentration of carbon source, stirring speed, and pH were investigated.

MATERIALS AND METHODS

Pretreatment

In the present study, *Salix caprea*, a fast-growing willow species, was used for production of cellulolytic enzymes. Chopped and screened willow was first presteamed for 40 min with saturated steam at 1 bar. After

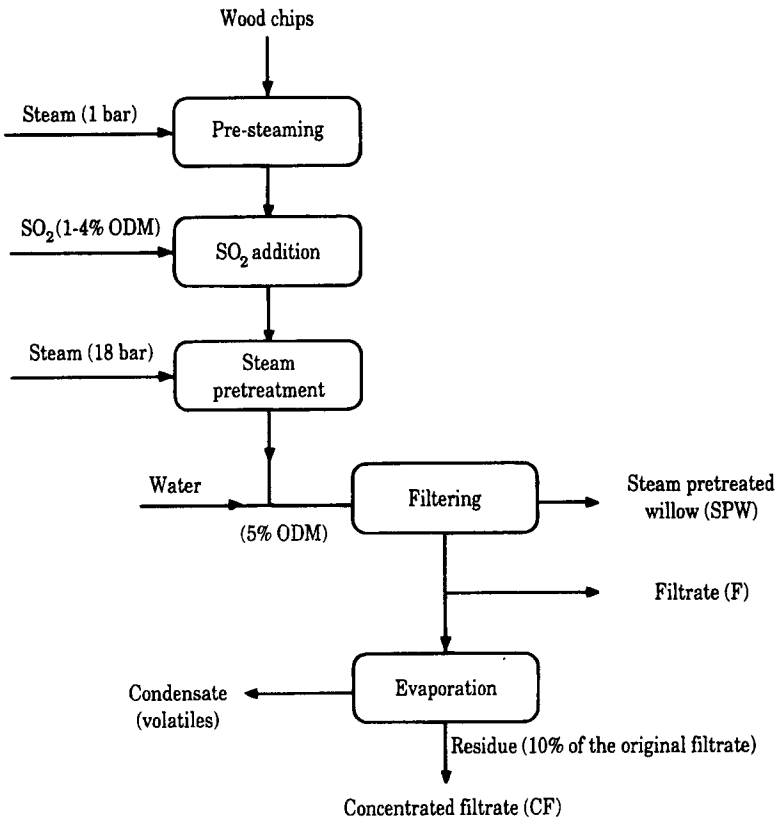

Fig. 1. Schematic flowsheet of carbon source preparation.

presteaming, the hot material was transferred into plastic bags and impregnated with 1–4% SO_2, based on oven-dried material (ODM), and stored overnight at 4°C. The steam treatment was performed at 206°C for 5 min *(1,2)*. The pretreated material was diluted with tap water to approx 5% ODM and filtered using a PF 0.1H2 (Larox OY, Finland) filter press unit *(10)*. The filtrate, i.e., the hydrolyzed hemicellulose part of the raw material, was divided into two fractions. One fraction was concentrated by removing 90% of the liquid by vacuum evaporation at 80°C and pH 2.8–3.0 using a Büchi RE 121 rotavapor. The concentrated filtrate (CF) had tenfold higher concentration of nonvolatiles, whereas most of the volatile compounds were removed. The fibrous material (SPW), the CF, and the unconcentrated filtrate (F) were all used as carbon sources for enzyme production. Figure 1 shows the schematic procedure for the preparation of the three fractions and the nomenclature that will be used. The composition of the pretreated willow was determined using Hägglund's method *(11)*, with the modification that the sugar content of the acid hydrolysate was also analyzed, and from that the cellulose content was calculated. The

SPW contained 48.2% cellulose based on ODM. The liquids were analyzed on HPLC for different sugars and inhibitory compounds.

Inoculum Preparation

The fungus *Trichoderma reseei* Rut C 30 was stored on agar slants containing (in g/L): 20 malt extract, 5 glucose, 1 proteose peptone, and 20 bacto agar, prior to usage. After 30 d at 30°C, the conidia were suspended in 5 mL of sterile water and 1.6 mL of the suspension was pipetted into a 1 L baffled E-flask containing 200 mL sterilized Mandels medium *(12)*, in which the concentrations of the nutrients in g/L were 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 7.5 Solka Floc. Trace elements were also added (mg/L): 5 $FeSO_4 \cdot 7H_2O$, 20 $CoCl_2$, 1.6 $MnSO_4$, and 1.4 $ZnSO_4$, respectively. The pH before sterilization was adjusted to 5.4. After 4 d at 30°C and 300 rpm the inoculum was ready.

Enzyme Production in Shake Flasks

The mycelia from the inoculum was used to initiate growth in 1 L baffled flasks containing 200 mL of a modified Mandels medium where the yeast extract and the proteose peptone were replaced with 0.38 g/L dried yeast *(9)*. The inoculum constituted 10% of the total volume. The enzyme production was performed at two different carbon source concentrations corresponding to 10 and 20 g/L of total available carbohydrate. For the higher loading the amount of nutrients and inoculum were doubled. The enzyme production was performed in a rotary shake-incubator at 30°C and 300 rpm. The pH was adjusted if it was below 4.4 with addition of sterile 10 wt% NaOH solution. Samples were taken once a day and centrifuged at 2500g for 10 min using a Winfug (AB Winkelcentrifug, Sweden) centrifuge. The supernatant was then analyzed for enzyme activity and the concentration of sugars and some volatile inhibitors were also determined.

Analysis

The enzyme activity of the samples was determined both as filter paper activity (FPU) using Mandels procedure *(13)* and β-glucosidase activity using Berghem's method *(14)*.

The centrifuged samples from the enzyme production were filtered through 0.2 μm membrane filters (MFS-13, Micro Filtration System) and analyzed on an HPLC unit (Shimadzu, Japan) equipped with a refractive index detector. Cellobiose, glucose, xylose, acetic acid, 5-hydroxy-methylfurfural, and furfural were separated on an Aminex HPX-87H column at 65°C using 5 m*M* H_2SO_4 as eluent, at a flow rate of 0.5 mL/min.

RESULTS AND DISCUSSION

Enzyme Production using Concentrated Hemicellulose Hydrolysate

Two main series of experiments were performed in shake flasks to investigate the cellulase production of *T. reesei* using the concentrated filtrate, CF, supplemented with fibrous pretreated willow, SPW. In the first series, the effect of the initial pH was studied. The initial pHs were set to 4.8, 5.3, and 5.8 before sterilization. The concentration of the sugars in the concentrated filtrate was set to 5 g/L by dilution with tap water, and the medium was supplemented with pretreated willow at an amount corresponding to a cellulose concentration of 5 g/L. The concentrated and rediluted filtrate to 5 g/L total sugar contained in g/L: 0.07 cellobiose, 1.48 glucose, 3.35 xylose, 0.63 acetic acid, and 0.13 5-hydroxi-methylfurfural. The concentration of furfural was below the detection limit.

In the second set of experiments, the effect of the concentration of carbon source and nonvolatile inhibitors were examined at two carbon source levels, 10 and 20 g/L of total carbohydrates. In both series the cellulose constituted 50% of the total carbon source. Control fermentations were run with SPW concentrations corresponding to 10 and 20 g/L of cellulose. For each conditions three fermentations were run in parallel and the mean values were calculated.

The enzyme activity and the total sugar concentration vs time are shown in Fig. 2 for the first series of fermentations. The mean value of the standard deviation was 0.04 FPU/mL, for the enzyme activity measurement and 0.30 g/L, for total sugar analysis in all experiments. The pH has a significant effect on enzyme production and in all cases the cellulase production was repressed while sugars were present in the medium. This can be explained by the presence of easily metabolisable sugars, which increase the intracellular level of glucose-6-phosphate or its analogues and repress the cellulase production *(15)*. Thus the cellulolytic enzyme production rate is a function of the sugar consumption (*see* Fig. 2A, B). At the lowest pH, 4.8, a very long lag phase can be seen, which is reduced by increasing pH. Also, the final enzyme yields differed although the initial carbon source concentration was the same in all experiments. The highest filter paper activity after 7 d cultivation, 0.89 FPU/mL, was obtained when the starting pH was 5.8, and the lowest 0.64 FPU/mL, for pH 4.8. It seemed that the pH had a double effect on cellulolytic enzyme production when the medium contained soluble sugars. Both the growth rate of the microorganism and the amount of secreted cellulases increased with increasing pH. The β-glucosidase production showed the same trend, with a final activity of 0.46 IU/mL obtained at pH 5.8 and 0.16 IU/mL at the lowest pH level.

The second series of fermentations were performed with the initial pH 5.8 based on the results of the previous experiments. Table 1 shows the final

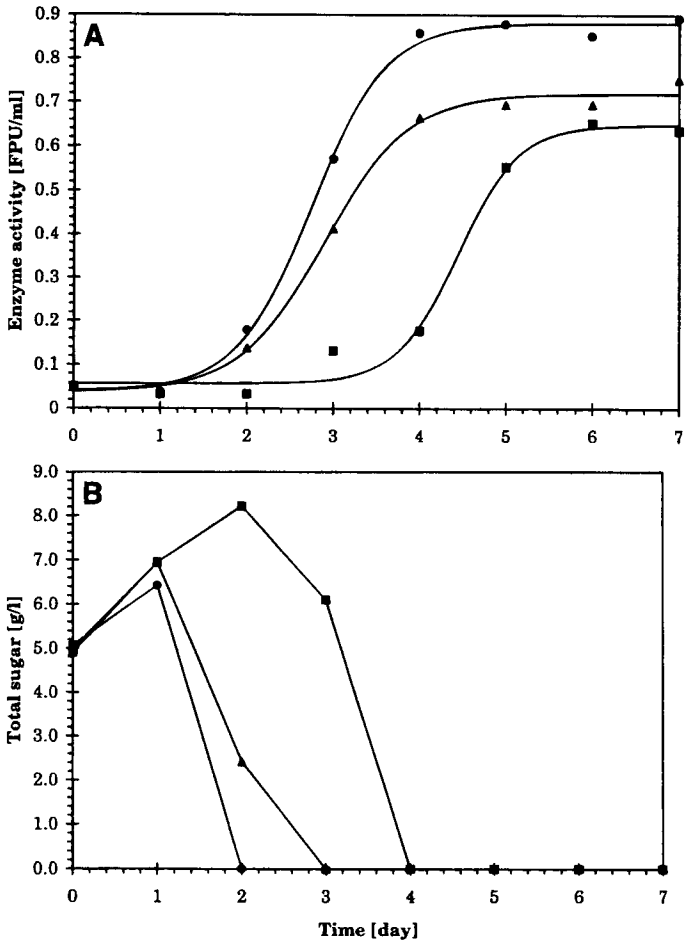

Fig. 2. Enzyme activity vs time for various staring pHs. **(A)** Cellulase activity; **(B)** total sugar concentration. ■ = pH 4.8, ▲ = pH 5.3, ● = pH 5.8.

Table 1
Enzyme Activities and Yields after 8 d Cultivation for Concentrated Filtrate (CF) with Initial pH 5.8

No.	FPU [IU/ml]	$Stdev_{FPU}$ [IU/ml]	Yield [FPU/g carbohydrate]	ß-gluc. [IU/ml]	$Stdev_{ß\text{-}gluc.}$ [IU/ml]
A	1.04	0.03	104	0.31	0.01
B	1.33	0.08	133	0.47	0.06
C	1.58	0.06	79	0.44	0.03
D	1.79	0.06	86	0.43	0.02

A-10 g/L carbon source 100% SPW
B-10 g/L carbon source 50% SPW and 50% CF
C-20 g/L carbon source 100% SPW
D-20 g/L carbon source 50% SPW and 50% CF

enzyme activities and yields calculated on the total added carbon source (cellulose plus sugars). The FPU values were in the range 1.04 FPU/mL to 1.79 FPU/mL. The fermentations using a mixture of SPW and CF resulted in higher FPU activities compared with the fermentations where only SPW was used. One possible explanation is that the liquid (CF) contained some oligomeric saccharides, which were not considered, as only three carbohydrates were analyzed on HPLC. These media would then contain more carbon source than the media with SPW. The yields are lower for 20 g/L carbon source, than at 10 g/L concentration. The higest yield, 133 FPU/g added carbon source, was obtained with the medium containing the mixture of 5 g/L sugars from CF and 5 g/L cellulose from SPW. The lower yield at higher carbon source concentrations can be the effect of an increased mass transfer resistance when the concentration of the solids were doubled because of poor stirring in the shake flasks. The β-glucosidase activity varied less than the filter paper activity. It was in the range 0.43–0.47 IU/mL except for SPW with a cellulose content corresponding to 10 g/L, where a somewhat lower activity, 0.31 IU/mL, was obtained.

Enzyme Production Using Original Pretreated Filtrate

A central-composite face (CCF) experimental design was used to investigate the effect of three factors on enzyme production using the original F. The three factors were the pH, stirring speed, and the amount of soluble sugars in percent of the total carbon source (10 g/L), whereby the amount of inhibitors were also varied. Table 2 shows the levels of the three factors. The initial composition of the media are summarized in Table 3. The amount of added filtrate in the medium was calculated on the total amount of carbohydrates measured after enzymatic hydrolysis for 48 h with mixture of cellulolytic enzymes. This analysis showed that the total amount of the carbohydrates in the filtrate was 1.3 times higher than that of the amount of the sugars measured by HPLC. For each experimental setup three fermentations were run in parallel.

Unfortunately only six conditions, out of the 15, were successful, i.e., yielded a measurable amount of enzymes, thus the evaluation of the data using the CCF design was not possible, but general conclusions could be drawn by comparison of the individual fermentations. The final enzyme activities are shown in Table 4.

At the highest concentration of soluble sugars (8 g/L) no growth or enzyme production was observed even at the highest stirring speed and pH levels. The furfural concentration in the medium, which initially was around 1 g/L, decreased slightly during the cultivation, but was never reduced to 0.0 g/L. The concentration of sugars was increased during the fermentation, probably because of the action of the cellulases loaded with the inoculum. The level of acetic acid was unchanged during the whole cultivation.

Table 2
CCF Experimental Design Setup

Exp. No.	pH	Stirring speed [rpm]	Soluble carbon [% of total carbon source]
1	5.0	200	20
2	6.0	200	20
3	5.0	400	20
4	6.0	400	20
5	5.0	200	80
6	6.0	200	80
7	5.0	400	80
8	6.0	400	80
9	5.0	300	50
10	6.0	300	50
11	5.5	200	50
12	5.5	400	50
13	5.5	300	20
14	5.5	300	80
15	5.5	300	50

Table 3
Composition of Media Used in CCF Experimental Design*

Soluble carbon source [%]	CELL [g/l]	GLU [g/l]	XYL [g/l]	TS [g/l]	HAc [g/l]	HMF [g/l]	FUR [g/l]
20	0.05	0.32	1.18	1.55	0.72	0.06	0.27
50	0.12	0.91	3.11	4.14	1.78	0.16	0.73
80	0.15	1.35	4.65	6.15	2.63	0.23	1.08

*oligosaccharides are not shown

CELL = cellobiose, GLU = glucose, XYL = xylose, TS = total HPLC sugar, HAc = acetic acid, HMF = 5-hydroximethyl furfural, FUR = furfural

When the medium contained 2 g/L sugars, all conditions gave evaluable results. The enzyme activities were in the range 0.57–0.77 FPU/mL. As was stated before, there was no sense in evaluating the obtained data using the CCF design, but experiments 1–4 were calculated using a 2^2-type factorial design with the pH and stirring speed as variables, and the maximum

Table 4
Final FPU Activities for the Successful CCF Experiments

Exp. No.	FPU [IU/ml]	$Stdev_{FPU}$ [IU/ml]
1	0.57	0.05
2	0.77	0.04
3	0.63	0.02
4	0.76	0.03
10	0.59	0.04
13	0.64	0.04

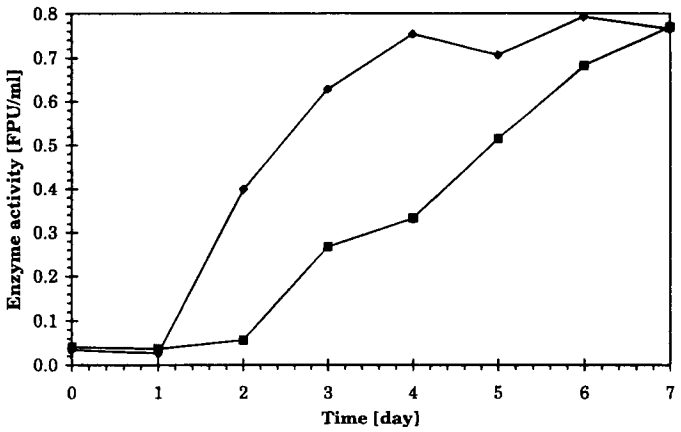

Fig. 3. CCF experimental design. Comparison of conditions No2. and No4. (*see* Table 2). ■ = FPU (No2., 200 rpm), ♦ = FPU (No4., 400 rpm).

FPU activity values obtained after 7-d cultivation as the dependent variable. The result of the regression analysis of the data comprising to fitting a polynomial equation and significance test of obtained parameters showed that the stirring has no significant effect on the final yield. The final equation obtained was $Y = 0.68 + 0.08x_1$, where Y is the response (FPU activity) and x_1 = [pH-5.5]/0.5. Although the stirring has no effect on the final yield as shown in Fig. 3, it has a significant effect on the enzyme production rate.

The effect of increased pH can be seen in Fig. 4, where the enzyme production, sugar concentration, and furfural concentration vs time are shown for conditions 1 and 2 (*see* Table 2). The final cellulolytic activity, after 7 d cultivation, increased from 0.57 FPU/mL at pH 5.0 to 0.77 FPU/mL at pH 6.0. In both cases the utilization of sugars was not started until the furfural was consumed, which can be considered to be a detoxification period. The soluble sugars were utilized to support the growth of the microorganism.

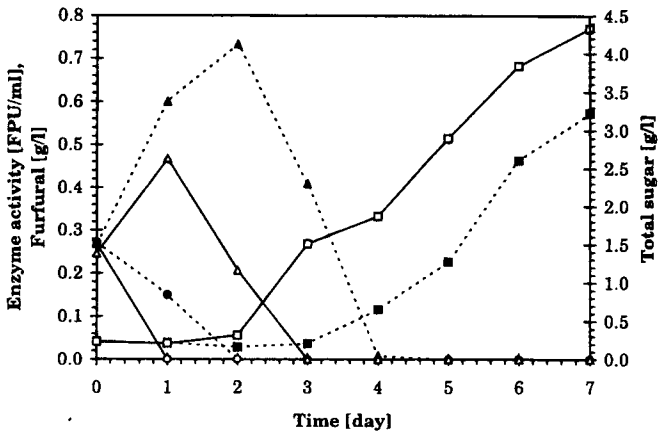

Fig. 4. CCF experimental design. Comparison of conditions No1. and No2. (*see* Table 2). ■ = FPU (No1.), ▲ = Total sugar (No1.), ● = Furfural (No1.), □ = FPU (No2.), Δ = Total sugar (No2.), ○ = Furfural (No1.).

During this period no cellulase production was observed. This was followed by the cellulose utilization phase, with the effective cellulase production. At pH 6.0 the furfural was consumed already within 1 d, whereas it took about 2 d at pH 5.0. This gave a higher enzyme production rate although it seems that the production was not completed after 7 d in either of the two conditions. At pH 6.0 there is no difference between the final enzyme activities at various stirring speeds, but the fermentation performed at 400 rpm was finished after 4 d cultivation, whereas at 200 rpm, the FPU activity increased during the whole fermentation (Fig. 3).

There was only one fermentation with 50% F, which resulted in a measurable enzyme production (Exp. 10). This was performed at pH 6.0 and 300 rpm. The furfural concentration, which initially was around 0.70 g/L, decreased to 0.0 g/L during the first 2 d (*see* Fig. 5). The level of the acetic acid was constant for 2 d, but was then consumed completely within 4 d. The total sugar concentration increased during the first 2 d and then decreased to 0.0 g/L. After 2 d the cellulase production started and the final activity after 7 d cultivation was 0.59 FPU/mL. This clearly shows that the acetic acid is utilized together with the other soluble carbohydrates in the medium and that it has no effect on growth at least up to a concentration of 1.8 g/L and if the pH is high enough. The somewhat lower FPU activity, compared with the results at 20% soluble carbohydrates is probably the result of the higher concentration of furfural in the medium.

CONCLUSIONS

The experiments on concentrated filtrate show that 50% of the cellulose can be replaced with soluble sugars derived from the hemicellulose fraction of the willow. A yield of 133 FPU/g added carbohydrates was obtained using the mixture of 5 g/L SPW and 5 g/L sugars from CF,

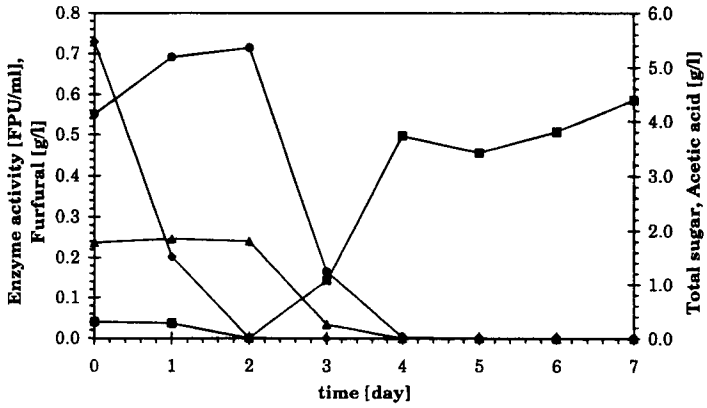

Fig. 5. CCF experimental design. Enzyme activity, acetic acid, total sugar and furfural concentration versus time for condition No10. (*see* Table 2). ■ = FPU, ◆ = Furfural, ▲ = Acetic acid, ● = Total sugar.

whereas the cultivation on 100% SPW gave 104 FPU/g. The yields at 20 g/L carbohydrates concentration were lower, 79 FPU/g for SPW and 86 FPU/g for the SPW and CF mixture. The yields obtained on media containing 50% soluble sugars of the total carbohydrates were higher than the yields on pure SPW. The yields at higher solids concentrations were lower than at lower solids concentrations, thus, it may be concluded that the improved mass transfer in the case of lower solids concentrations yielded an increased cellulase production.

It is evident that the evaporation of water solutions is expensive because of the high energy demand. From an economical point of view it is preferable to use the original filtrate. The effect of the pH, stirring speed, and amount of soluble sugars on enzyme production was investigated with a CCF experimental design. Only six conditions gave evaluable enzyme activities, thus the evaluation of the data with CCF model was not possible. There was no enzyme production when 8 g/L sugars were added to the medium, which corresponds to a furfural concentration of 1 g/L. Only one condition with 5 g/L sugars provided cellulases with an activity of 0.59 FPU/mL after 7 d cultivation. The experiments at a concentration of 2 g/L sugars indicate that furfural has an effect on growth and that is implified by a low pH. At pH 6.0 the furfural was rapidly consumed, yielding the highest cellulolytic activity, 0.77 FPU/mL. The result of the statistical analysis of data obtained at 2 g/L sugar concentration shows that only the pH has effect on cellulase yield at constant solids concentrations and the effect of stirring can be neglected.

ACKNOWLEDGMENT

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