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Effect of Acetic Acid and Furfural on Cellulase Production of *Trichoderma reesei* RUT C30

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Abstract

Because of the high temperature applied in the steam pretreatment of lignocellulosic materials, different types of inhibiting degradation products of saccharides and lignin, such as acetic acid and furfural, are formed. The main objective of the present study was to examine the effect of acetic acid and furfural on the cellulase production of a filamentous fungus *Trichoderma reesei* RUT C30, which is known to be one of the best cellulase-producing strains. Mandels's mineral medium, supplemented with steam-pretreated willow as the carbon source at a concentration corresponding to 10 g/L of carbohydrate, was used. Four different concentration levels of acetic acid (0–3.0 g/L) and furfural (0–1.2 g/L) were applied alone as well as in certain combinations. Two enzyme activities, cellulase and β-glucosidase, were measured. The highest cellulase activity obtained after a 7-d incubation was 1.55 FPU/mL with 1.0 g/L of acetic acid and 0.8 g/L of furfural added to the medium. This was 17% higher than that obtained without acetic acid and furfural. Furthermore, the results showed that acetic acid alone did not influence the cellulase activity even at the highest concentration. However, β-glucosidase activity was increased with increasing acetic acid concentration. Furfural proved to be an inhibiting agent causing a significant decrease in both cellulase and β-glucosidase production.

Index Entries: *Trichoderma reesei* RUT C30; cellulase; steam-pretreated willow; inhibition; acetic acid; furfural.

Introduction

The production of fuel ethanol from lignocellulosic materials has been a focus of interest over the past 10 years owing to the increased demand for

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environmentally beneficial and cost-efficient liquid fuels *(1–3)*. One of the most promising processes is based on the enzymatic breakdown of the cellulosic fraction of raw material *(4,5)*. To obtain an enzymatically digestible substrate for cellulases, the starting material must be pretreated. A method frequently used to achieve this is high-pressure steam pretreatment, which achieves good yields on both hardwoods and softwoods. During this process, the lignin part becomes structurally modified, and the hemicellulose part is degraded to monosaccharides, resulting in an enzymatically more digestible lignin–cellulose complex *(6–8)*. However, a wide spectrum of degradation products are liberated, which, apart from decreasing the sugar yield, can have an inhibitory effect on microorganisms and cellulase enzymes *(9–11)*. These by-products can be divided into three distinguishable groups: aliphatic acids, such as acetic acid (HAc), formic acid, and levulinic acid; furan derivatives, such as furfural and 5-hydroxy-methylfurfural (HMF); and phenolic compounds. Furfural and HMF are formed when monosaccharides (pentoses and hexoses), liberated during the hemicellulose hydrolysis, are broken down thermochemically. Furfural and HMF can undergo further degradation to formic acid and levulinic acid, respectively. HAc is formed via deacetylation of hemicellulose, while phenolic components are liberated from lignin *(12,13)*.

In this study, the effect of HAc and furfural on the production of cellulase and β-glucosidase enzymes by *T. reesei* RUT C30 was investigated using steam-pretreated willow (SPW) as the carbon source. These two particular substances were chosen because hardwood hemicellulose is highly acetylated and contains large amounts of pentoses, especially xylose, which is a potential precursor of furfural.

Materials and Methods

Pretreatment of Willow

A fast-growing willow species, *Salix caprea* Q082, was used as the raw material for the production of cellulase enzyme. The willow was first chopped and fractionated. The fraction between chip size 1 and 3.5 mm was used in the pretreatment. The material had a dry matter content of 94.4 wt%. The raw material was analyzed for lignin, cellulose, and hemicellulose content by the Swedish University of Agricultural Sciences in Ultuna *(14)*. The composition of the willow fraction used can be found elsewhere *(15)*.

The wood chips, weighing 1000 g, were first presteamed for 60 min using 1 bar of saturated steam. The steamed willow chips had a dry matter content of 58.2 wt%. The hot material was transferred into a plastic bag and impregnated with approx 35 g of SO_2. After storing the chips overnight at room temperature, the SO_2 uptake was determined by weighing. The SO_2 uptake was 3.8 wt% based on the moisture content of the chips. The impregnated material was steam pretreated at 207°C for 5 min. The pretreated material was diluted with hot tap water to approx 5 wt% water-insoluble

Table 1
Typical Composition
of Hydrolysate from SPW

Compound	Concentration (g/L)
Cellobiose	0.17
Glucose	3.22
Xylose	6.42
Galactose	0.60
Arabinose	0.37
Mannose	0.99
HAc	2.77
Furfural	0.78
HMF	0.26
Total sugar	10.78

dry matter, stirred for 30 min, and then filtered on a PF 0.1 H2 (Larox OY, Finland) filter press unit. The filtrate, comprising the hydrolyzed hemicellulose fraction, was analyzed by high-performance liquid chromatography (HPLC) for sugars, HAc, furfural, and HMF. Table 1 presents the composition of a typical filtrate. The filter cake was washed thoroughly with hot tap water until the filtrate was free of sugars *(16,17)*. The cellulose and lignin content of the washed SPW was determined using a modified Hägglund *(18)* method, in which the acid hydrolysate obtained in the assay was analyzed by HPLC for cellobiose and glucose, which were used to calculate the cellulose content of the SPW. The SPW contained 48.8 wt% cellulose and 38.4 wt% lignin based on dry matter.

Preparation of Inoculum

The fungus *Trichoderma reesei* RUT C30 (ATCC no. 56765) was stored on agar slants containing 20 g/L of malt extract, 5 g/L of glucose, 1 g/L of proteose peptone, and 20 g/L of bacto agar. After 15 d at 30°C, the conidia were harvested by suspending in 5 mL of sterile water. The spore suspension (1.5 mL) was pipeted into a 1-L baffled Erlenmeyer flask containing 200 mL of sterilized Mandels's medium *(19)*, in which the concentrations of nutrients were 0.3 g/L of urea, 1.4 g/L of $(NH_4)_2SO_4$, 2.0 g/L of KH_2PO_4, 0.3 g/L of $CaCl_2$, 0.3 g/L of $MgSO_4$, 0.25 g/L of yeast extract, and 0.75 g/L of proteose peptone together with 7.5 g/L of Solka Floc 200 (FS&D, Urbana, IL) cellulose powder. The medium was supplemented with the following trace elements: 0.5 mL/L of $FeSO_4 \cdot 7H_2O$, 2 mL/L of $CoCl_2$, 0.16 mL/L of $MnSO_4$, and 0.14 mL/L of $ZnSO_4$ in 1 wt% aqueous solutions. Prior to sterilization, the pH of the medium was adjusted to 5.4 by the addition of either 10 wt% NaOH or 10 wt% H_2SO_4 solutions. After 4 d at 30°C and 400 rpm in an LSR/L-V orbital rotary shaker incubator (Adolf Kühner AG, Birsfelden, Switzerland), the inoculum was ready.

Table 2
Experimental Conditions (Concentrations of Furfural and HAc) and Summary of Obtained Average pH and Largest pH Deviations (LD)[a]

Experiment no.	HAc (g/L)	Furfural (g/L)	Average pH	LD
1	—	—	5.49	–2.70
2	—	0.4	5.60	–2.43
3	—	0.8	5.57	–2.57
4	—	1.2	5.45	–2.40
5	1.0	—	6.03	–0.47
6	1.0	0.4	5.95	–1.10
7	1.0	0.8	5.85	–1.53
8	1.0	1.2	5.80	–2.33
9	2.0	—	6.17	1.17
10	2.0	0.4	6.12	0.67
11	2.0	0.8	6.07	0.35
12	2.0	1.2	6.03	0.27
13	3.0	—	6.21	1.63
14	3.0	0.4	6.17	0.63
15	3.0	0.8	6.08	–0.20
16	3.0	1.2	5.97	–0.20

[a]LD, largest difference between the measured pH and 6.0.

Shake Flask Experiments

The mycelia obtained from the inoculum were used to initiate growth in 1-L baffled Erlenmeyer flasks containing 200 mL of modified Mandels's medium in which the yeast extract and proteose peptone were replaced with 0.38 g/L of dried yeast *(20)*. The enzyme production was performed using SPW as the sole carbon source at a concentration corresponding to 10 g/L of cellulose. After sterilization and addition of the necessary amount of HAc and freshly vacuum-distilled furfural, the pH was adjusted to 6.0. Table 2 gives the concentrations of the two chemicals. The inoculum constituted 10% of the medium. The cultivation was carried out in an LSR/L-V (Adolf Kühner AG) laboratory rotary shaker incubator at 30°C and 400 rpm for 7 d. The pH of the cultivation was checked once a day and adjusted to 6.0 by the addition of either sterile 10 wt% NaOH or 10 wt% H_2SO_4 solutions. Samples were withdrawn every day, before pH adjustment, and centrifuged at 3400*g* for 10 min using an A.L.C. laboratory desktop centrifuge (A.L.C. International S.r.I., Cologno Monzese, Italy). Half of the sample by volume was saved for the measurement of enzyme activity, whereas the other half was analyzed by HPLC. The experiments were terminated after 7 d.

Measurement of Enzyme Activity

The enzyme activity of the samples was characterized by using two different methods: filter paper activity (FPA) using Mandels's *(21)* procedure and β-glucosidase activity using Berghem's *(22)* method. The enzyme

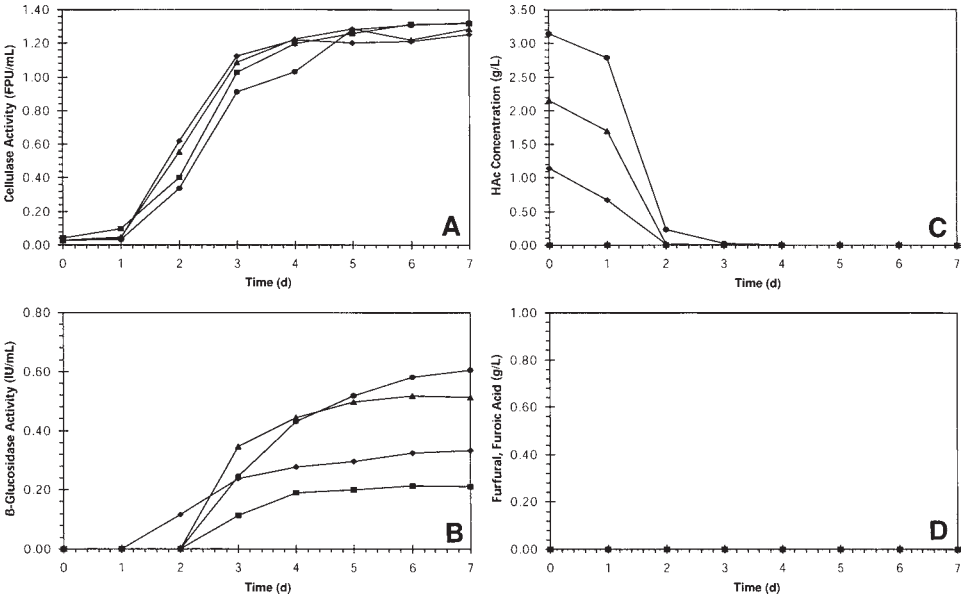

Fig. 1. Cellulase production at 0 (■), 1.0 (◆), 2.0 (▲), and 3.0 g/L (●) HAc concentrations. The furfural concentration was 0 g/L in all experiments. **(A)** Cellulase activity; **(B)** β-glucosidase activity; **(C)** HAc concentration; **(D)** furfural and furoic acid concentrations.

production of *T. reesei* was defined as the enzyme activities measured in the supernatant of the fermentation broth. The enzyme productivity was based on these values and calculated after a residence time of 3 d.

HPLC Analysis

Prior to HPLC analysis, all samples were filtered through an MFS-13 cellulose-acetate membrane filter of 0.2-µm pore diameter (Advantec MFS, Plesanton). Cellobiose, glucose, xylose, furfural, HAc, and furoic acid were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65°C using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu). The mobile phase was a 5 m*M* aqueous solution of H_2SO_4 at a flow rate of 0.5 mL/min.

Results and Discussion

The cellulase and β-glucosidase activities obtained after 7 d of cultivation for the 16 different experimental runs shown in Table 2 are summarized in Figs. 1–3. Each run was done in triplicate, and the mean values of all measured parameters (i.e., enzyme activities; concentrations of HAc, furfural, and furoic acid) as well as the relative standard deviations were calculated. The relative standard deviation for all the parameters determined was under 5%.

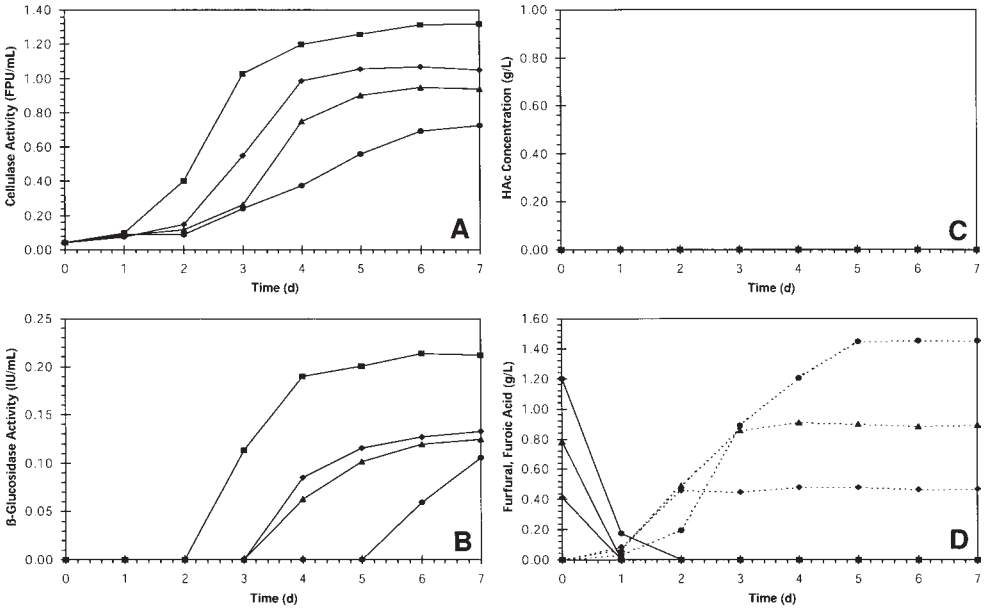

Fig. 2. Cellulase production at 0 (■), 0.4 (◆), 0.8 (▲), and 1.2 g/L (●) furfural concentrations. The HAc concentration was 0 g/L in all experiments. **(A)** Cellulase activity; **(B)** β-glucosidase activity; **(C)** HAc concentration; **(D)** furfural (solid lines) and furoic acid (broken lines) concentrations.

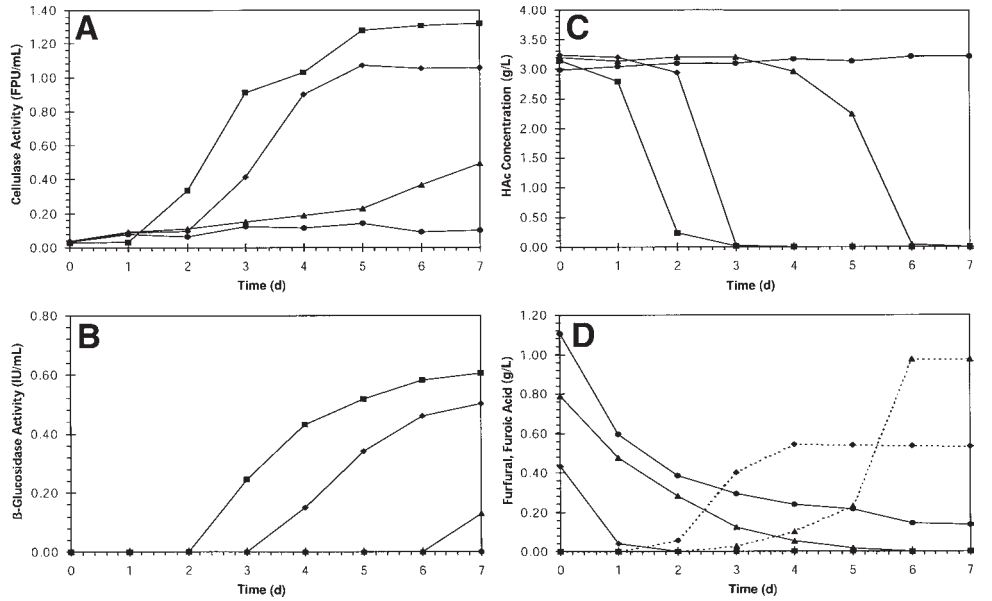

Fig. 3. Cellulase production at 0 (■), 0.4 (◆), 0.8 (▲), and 1.2 g/L (●) furfural concentrations. The HAc concentration was 3.0 g/L in all experiments. **(A)** Cellulase activity; **(B)** β-glucosidase activity; **(C)** HAc concentration; **(D)** furfural (solid lines) and furoic acid (broken lines) concentrations.

The FPAs were in the range of 0.10–1.55 FPU/mL, and the β-glucosidase activity varied between 0 and 0.61 IU/mL. The complete inhibition of microbial growth was observed only in one case (experiment no. 16), which had the highest concentration of HAc and furfural. Maximum cellulase activity, 1.55 FPU/mL, was reached for the condition in experiment no. 7. However, the highest β-glucosidase activity of 0.61 IU/mL was obtained in experiment no. 13.

HAc appeared to be noninhibiting at pH 6.0 up to the highest concentration investigated, i.e., 3.0 g/L (experiment nos. 1, 5, 9, and 13). The cellulase production did not seem to be affected at all, and an activity of about 1.3 FPU/mL was reached after 7 d of cultivation. However, the β-glucosidase activity obtained increased with increasing concentration of HAc, from 0.21 to 0.61 IU/mL. The cellulase productivity was about 14.5 FPU/(L × h). Graphs of enzyme activity vs time are shown in Fig. 1. In all cases HAc was taken up completely by *T. reesei* RUT C30 within 3 d (Fig. 1C). Weak aliphatic acids, such as HAc, are widely used to prevent microbial growth at low pH. Despite their various chemical structures, the mechanism inhibiting cell growth is based on the same principle. Only the associated acid form is liposoluble and can therefore penetrate through the plasma membrane. Inside the cell, where the pH is neutral, the immediate dissociation of the aliphatic acids occurs, causing a decrease in the intracellular pH, which can lead to cell death. Because these compounds are weak acids, the concentration of the associated acid formed, and thereby their inhibiting effect, depend on the extracellular pH. At lower pH, a higher inhibiting effect can be expected *(23)*. In the present study, pH 6.0 was chosen to keep the concentration of the undissociated HAc low, thereby minimizing its inhibiting effect.

When only furfural was added to the culture medium, both cellulase and β-glucosidase activities decreased with increasing furfural concentration (Fig. 2). The cellulase activity decreased from 1.32 to 0.73 FPU/mL when the concentration of furfural was increased from 0 to 1.2 g/L. The corresponding values for β-glucosidase activity were 0.21 and 0.11 IU/mL, which represents a 50% decrease in activity. The metabolism and effects of furfural in eukaryotic cells have been investigated for yeast cells. However, not much can be found in the literature regarding other eukaryotes such as filamentous fungi. In yeast cells, the conversion of furfural depends on the rate of oxygenation. In yeast, furfural is oxidized to furoic acid under aerobic conditions, and it is reduced to furfuryl alcohol in anaerobic fermentation *(24)*. In the present study, furfural was converted to furoic acid stoichiometrically (*see* Figs. 2D and 3D).

At an HAc concentration corresponding to 1.0 g/L, a slight increase in both final cellulase and β-glucosidase activities was observed when the furfural concentration was increased from 0 to 0.4 g/L, but the productivity values did not differ greatly (data not shown). When the initial furfural concentration was 0.8 g/L (experiment no. 7), the final FPA increased further, reaching a value of 1.55 FPU/mL. However, both the β-glucosidase

and cellulase productivity were lowered significantly. At a furfural concentration of 1.2 g/L, considerably lower values of the activities and cellulase productivity were observed.

When the initial concentration of HAc was 2.0 g/L and the amount of furfural was increased stepwise (experiment nos. 9–12), the cellulase activity after 7 d of cultivation was not affected at all and an average of 1.31 FPU/mL was reached (data not shown). However, the cellulase productivity decreased substantially from 15.1 to 3.4 FPU/(L × h). The production of β-glucosidase decreased by 21% from 0.51 (experiment no. 9) to 0.40 IU/mL (experiment no. 12).

At the highest concentration of HAc (3.0 g/L), a reduction in the production of both cellulase and β-glucosidase was observed with increasing concentration of furfural in the culture medium. At a furfural concentration of 1.2 g/L, no enzyme was produced (Fig. 3). Neither the formation of furoic acid nor the consumption of HAc was observed. However, the concentration of furfural was reduced to 0.14 g/L from the initial 1.2 g/L (Fig. 3D).

Figure 4 summarizes how the cellulase (Fig. 4A) and β-glucosidase (Fig. 4B) activities obtained after 7 d of cultivation changed with the furfural concentration at different levels of HAc in the medium. Assuming linear pH profiles between the pH values measured daily, an average pH could be calculated for each condition. Despite the daily pH adjustment, these average values showed a smaller or larger deviation from the desired value of pH 6.0, depending on the concentration of HAc and furfural added (Fig. 4C, Table 2). In the absence of HAc, the average pH was considerably below 6.0. The largest deviation from pH 6.0 (LD) was between –2.70 and –2.40 pH units. The acidification was moderated when 1.0 g/L of HAc was added to the medium. The average pH values were considerably higher, between 5.80 and 6.03, compared with those obtained at zero HAc concentration. However, a pH below 6.0 was observed in all cases in the early phase of the fermentation. In the case with 2.0 g/L of HAc in the medium, the average pH varied from 6.03 to 6.17, and the LDs were between 0.27 and 1.17. When the medium contained 3.0 g/L of HAc, the average pH varied between 5.97 and 6.21.

Several factors affect the pH of a fermentation. When the Mandels's medium is used without any addition of HAc and furfural, a significant drop in pH occurs at the beginning of the fermentation only to the acidification caused by the depletion of the inorganic nitrogen source *(25)*. However, after 4 to 5 d the pH starts to shift toward the alkaline region. The sum of these two opposite effects determines the average pH of a cultivation. Furthermore, two additional actions were superimposed on the effects just mentioned when HAc and furfural were added to the medium. Depending on the concentration of HAc, various amounts of NaOH were needed to adjust the pH to 6.0, before the medium was inoculated. When HAc was consumed, the equivalent amount of NaOH was "liberated," causing a significant increase in pH at the start of the fermentation. The furfural present in the medium was first taken up by the cells and converted in an

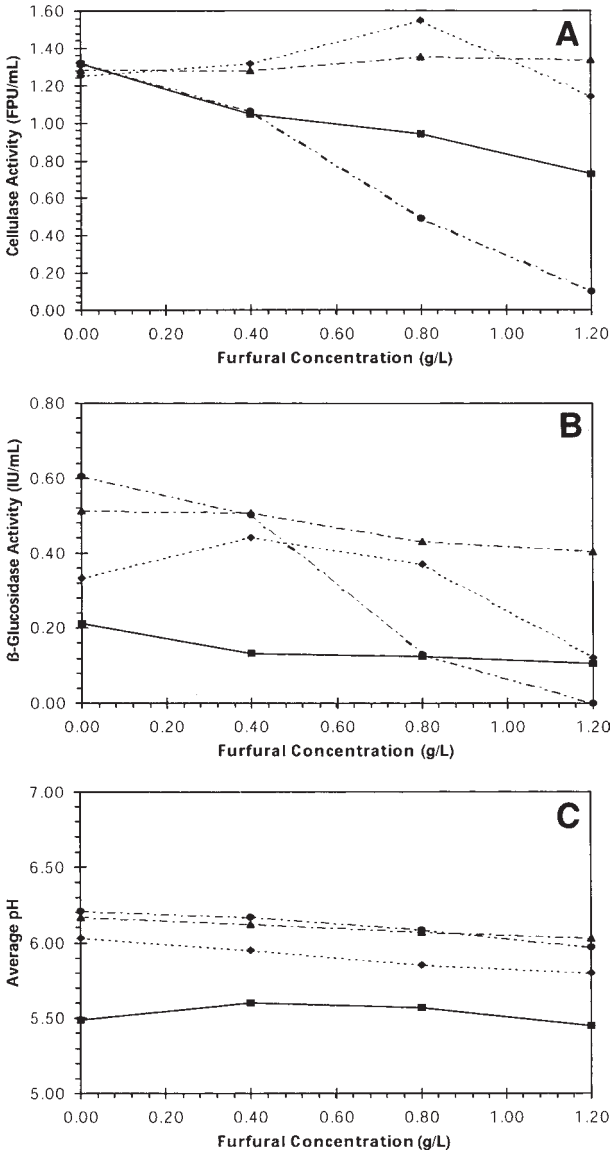

Fig. 4. Summary of enzyme production under various conditions as a function of furfural concentration. **(A)** Cellulase activity at 0 (—■—), 1.0 (– –◆– –), 2.0 (·–▲–·), and 3.0 g/L (· · –●– · ·) HAc; **(B)** β-glucosidase activity, legend as in (A); **(C)** average pH, legend as in (A).

oxidation process into furoic acid, which was released at a later phase of the cultivation (*see* Figs. 2D and 3D). Furoic acid, being a weak acid, increased the proton concentration in the fermentation broth, thereby shifting the pH toward the acidic region.

Higher average pHs can thus be expected at higher HAc concentrations than at lower concentrations. This was counteracted when the fur-

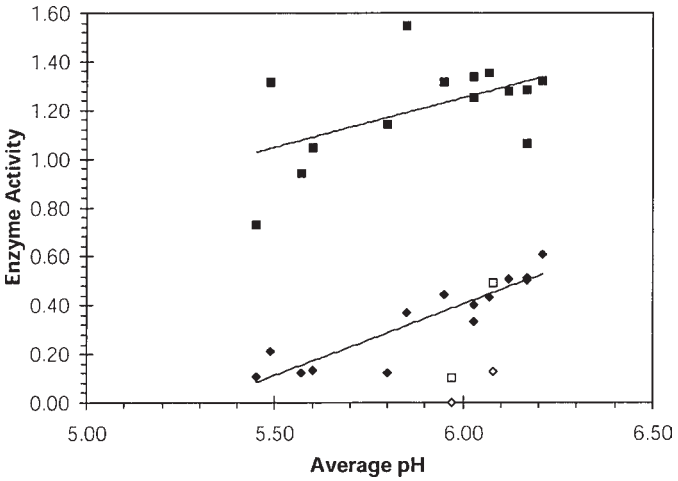

Fig. 5. Cellulase activity (■) and β-glucosidase (◆) activity as a function of average pH. Open symbols were neglected when linear regression was applied.

fural concentration was increased, owing to the formation of furoic acid (*see* Fig. 4C). The enzyme activities obtained after a 7-d cultivation showed an increasing tendency with increasing average pH (Fig. 5). The plotted line in Fig. 5 was obtained by linear regression of all points with the exclusion of experiment nos. 15 and 16, which had negligible enzyme production. This shows that the extracellular pH plays an important role. The enhancement of enzyme production of *T. reesei* owing to elevated pH in the culture medium has been observed by many researchers *(25–28)*. Furthermore, at higher pH, less inhibition by HAc is expected. However, the present results may suggest that there was no inhibition caused by HAc (*see* Fig. 1) and that the differences in enzyme activities are owing to differences in cultivation pH and furfural concentration.

Conclusion

The effect of an increased amount of furfural on the enzyme production of *T. reesei* RUT C30 was dependent on the HAc concentration in the culture medium (*see* Fig. 4A,B). Decreased levels of both cellulase and β-glucosidase activities were observed when only furfural was added to the culture medium, which cannot be explained by differences in the pH of cultivation, but by furfural inhibition of enzyme production (Fig. 4C). However, when HAc was added to the medium to give a concentration of 1.0 g/L, an enhancement of cellulase production was observed, resulting in a cellulase activity of 1.55 FPU/mL, which was 17% higher than the activity obtained in the reference fermentation. When the furfural concentration in the culture medium containing 2.0 g/L of HAc was increased, no difference was observed in the final cellulase activities. However, the cellulase productivity decreased considerably. When the medium contained 3.0 g/L of

HAc, increased furfural concentration resulted in decreased cellulase activity, even to a greater extent than when only furfural was added. Furthermore, furfural seemed to increase the lag phase of the cultivation, and the enzyme production did not start until furfural was completely oxidized.

HAc had no effect on the cellulase production of *T. reesei* RUT C 30 when no furfural was present, but it increased β-glucosidase production. Furthermore, in the presence of furfural, HAc had a positive effect when applied at low concentrations, canceling the inhibition caused by furfural.

A clear connection could be established between the average pH of the cultivation and the enzyme production of *T. reesei* RUT C30 (Fig. 5). The cause-and-effect relations are not quite clear, because of the numerous variables affecting the cellulase production. However, it is evident that the average pH plays an important role, which in the present study was affected by the chemicals and experimental conditions applied, i.e., the pH adjustment strategy.

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