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β-Glucosidase Production by *Trichoderma reesei*

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Abstract

The hydrolysis of cellulose to the water-soluble products cellobiose and glucose is achieved via synergistic action of cellulolytic proteins. The three types of enzymes involved in this process are endoglucanases, cellobiohydrolases, and β-glucosidases. One of the best fungal cellulase producers is *Trichoderma reesei* RUT C30. However, the amount of β-glucosidases secreted by this fungus is insufficient for effective cellulose conversion. We investigated the production of cellulases and β-glucosidases in shake-flask cultures by applying three pH-controlling strategies: *(1)* the pH of the production medium was adjusted to 5.8 after the addition of seed culture with no additional pH adjustment performed, *(2)* the pH was adjusted to 6.0 daily, and *(3)* the pH was maintained at 6.0 by the addition of Tris-maleate buffer to the growth medium. Different carbon sources—Solka Floc 200, glucose, lactose, and sorbitol—were added to standard Mandels nutrients. The lowest β-glucosidase activities were obtained when no pH adjustment was done regardless of the carbon source employed. Somewhat higher levels of β-glucosidase were measured in the culture filtrates when daily pH adjustment was carried out. The effect of buffering the culture medium on β-glucosidase liberation was most prominent when a carbon source inducing the production of other cellulases was applied.

Index Entries: β-Glucosidase; cellulase; *Trichoderma reesei* RUT C30; Tris-maleate buffer; endoglucanase activities.

Introduction

Lignocellulose materials, which are the most abundant renewable resources on Earth, represent an enormous potential as raw materials for various industries. An estimated 10^{12} metric t of cellulose is produced by plants each year (*1*). Although cellulosic materials are available in vast

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amounts, biotechnological exploitation of these materials based on enzymatic hydrolysis to provide fermentable sugars is still at a low level of utilization. Native cellulose, the major structural polysaccharide in plants, is highly resistant to enzymatic degradation. Various microorganisms, including fungi and bacteria that can utilize cellulose, have developed a complex set of enzymes (i.e., cellulases) that can convert the water-insoluble cellulose into individual glucose monomers. Although these enzymes are capable of providing cellulolytic microorganisms with a sufficient amount of sugars to support their life cycle, the slow rate and low extent of hydrolysis are preventing industrial exploitation of these enzymes in the conversion of lignocellulosics into glucose. To some extent, the enzymatic digestibility of native lignocellulosics can be improved by various pretreatment techniques, yet there are several unsolved issues related to enzymatic saccharification of lignocellulosics, precluding large-scale application of cellulases. The fact that enzymatic reaction takes place in a solid-liquid phase system is the most prominent feature that sets the technical limit to exploitation of enzymatic cellulose hydrolysis. Furthermore, product inhibition of cellulases and unproductive adsorption of cellulases onto the surface of accompanying materials, such as lignin, require large enzyme dose to achieve satisfactory high conversion rates and yields *(2)*. Therefore, owing to the high market price of cellulases, the bioconversion of lignocelluloses to fermentable sugars is currently not economic.

Cellulolytic fungi, especially *Trichoderma* strains, are superior to cellulolytic bacteria. Currently, research and development of cellulase production is dominated by the soft-rot fungus *Trichoderma reesei* for several reasons *(3–5)*. These include high titers, yields, and production rates of extracellular enzymes, which make downstream processing cost-effective *(6)*. Since the beginning of the 1960s, when Mandels and Weber reported fungal cellulolytic activity *(7)*, many efforts have been made in genetic modification of *Trichoderma* strains and optimization of culture conditions *(8)* aimed at more efficient cellulase production. *T. reesei* RUT C30, which is a partially catabolite derepressed, hypercellulolytic mutant, is the successful outcome of the mutagenic program carried out by Montenecourt and Eveleight *(9)*. Although it is capable of producing cellulases with high titers and yields, the secreted enzyme cocktail is unbalanced regarding the β-glucosidase-to-cellulase ratio *(10)*. Typically, the relative amount of β-glucosidase to cellulases is in the range of 0.3–0.5, which is relatively low compared with the optimal 1:1 required for efficient cellulose conversion into glucose *(11,12)*.

The amounts of cellulase enzyme components found in the culture filtrate of *T. reesei* RUT C30 are strongly dependent on culture conditions *(13,14)*. The quality and concentration of carbon source are very important. In addition, the composition of the culture medium, especially the amount of organic Nitrogen sources (yeast extract and peptone), as well

as the physical parameters of the fermentation (temperature, aeration, and pH) are important. For instance, several reports have shown the importance of pH and the applied pH-controlling strategy on cellulase production of *T. reesei* strain RUT C30. Tangnu et al. *(15)* showed that high cellulase activity could be observed when the pH was controlled at about 5.0. Juhasz et al. *(16)* found that a slightly higher pH, about 6.0, was better for cellulase production in terms of productivity; however, deactivation was observed during the final stage of fermentation. Regulation of pH in shake-flask cultures is mostly limited to manual pH adjustment. In our previously published work *(16)*, several organic acid buffer systems were applied to overcome the task of manual pH control. The unexpected consequence of the experiments was a culture filtrate in which the β-glucosidase activity sometimes exceeded the overall activity of the cellulases.

In the present study, the influence of maleic acid on cellulase secretion with special emphasis on β-glucosidase formation was investigated. Cellulase fermentations were performed on Solka Floc 200 purified cellulose powder, glucose, lactose, and sorbitol. Control experiments containing these carbon sources were carried out in a modified complete Mandels medium, and in another set of cultivations, the same medium was supplemented with Tris-maleate buffer. Extra-cellular, intracellular, and cell-bound enzyme activities were measured.

Materials and Methods

Preparation of Inoculum and Enzyme Production in Shake Flasks

T. reesei RUT C30 (ATCC# 56765) stock cultures were maintained 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 3 wk at 28°C, spores were suspended in 5 mL of sterile water and 1 mL of this suspension was transferred aseptically to a 750-mL Erlenmeyer flask containing 200 mL of the sterile medium prepared as modified Mandels medium in which the concentrations of nutrients were 0.40 g/L of urea, 1.87 g/L of $(NH_4)_2SO_4$, 2.67 g/L of KH_2PO_4, 0.53 g/L of $CaCl_2 \cdot 2H_2O$, 0.40 g/L of $MgSO_4$, 0.33 g/L of yeast extract, and 1.00 g/L of proteose peptone together with 10.00 g/L of Solka Floc cellulose powder. The medium was also supplemented with the following trace elements: 7 mg/L of $FeSO_4 \cdot 7H_2O$, 20 mg/L of $CoCl_2$, 2 mg/L of $MnSO_4$, 2 mg/L of $ZnSO_4$ *(17)*. Solka Floc 200 cellulose powder in a concentration of 10.00 g/L was added as carbon source. After 4 d at 28°C and 350 rpm the inoculum was ready.

A total volume of 20 mL of mycelium suspension, obtained from the seed cultures, was used to initiate growth in 750-mL Erlenmeyer flasks containing 200 mL of sterile Mandels medium *(17)*. The carbon source added to the production medium was either 10.00 g/L of Solka Floc 200 or 11.00 g/L of glucose or 11.00 g/L of lactose or 11.00 g/L of sorbitol.

After inoculation, the Erlenmeyer flasks were incubated on an orbital shaker at 28°C and 350 rpm for 7 d. Samples were withdrawn daily at the same hour of the day, and when it was necessary, the pH in the flasks was manually adjusted using sterile, 10 wt% solutions of NaOH or H_2SO_4. In shake-flask cultures, basically three pH controlling strategies were applied: (*1*) starting from pH values of 5.8 no manual adjustment was carried out at all, (*2*) the pH was adjusted to its initial value of 5.8, and (*3*) the pH was kept at between 5.6 and 6.0 by supplementing the medium with 0.1 *M* Tris-maleate buffer (11.60 g/L of maleic acid, 12.10 g/L of Tris, 1.64 g/L of NaOH). Each experimental condition was performed in triplicate.

Analyses

Aseptically taken samples were centrifuged at 3400*g* for 10 min. The collected supernatants were analyzed for total protein content and various enzyme activities. All samples were analyzed in triplicate and the mean values were calculated. The relative standard deviation of measurements was always below 5%. The protein content of the samples was determined using a Coomassie assay *(18)*. The total cellulase activity of the samples was determined as filter paper activity (FPA) expressed in filter paper units (FPU) using Mandels's procedure *(19)*. Because the amount of β-glucosidase in the supernatants varied significantly, cellulase activities in some samples were determined by supplementing the supernatants with β-glucosidase enzyme (Novozymes 188; Novozyme, Bagsvaerd, Denmark) to a β-glucosidase activity of 1.4 IU/mL (highest measured in the samples) *(20)*. β-Glucosidase activity was assayed using 4-nitrophenyl-β-D-glucopyranoside substrate according to Berghem's method *(21)*. Endoglucanase activity was measured against hydroxyethylcellulose substrate using a method based on the procedure of the International Union of Pure and Applied Chemists *(22)*. Supernatants collected from cultivations carried out in Tris-maleate buffered media were assayed for maleic acid using high-performance liquid chromatography. Prior to analysis, samples were filtered through a 0.45-μ*m*-pore-size regenerated cellulose filter. The analysis was performed using an Aminex HPX-87H (Bio-Rad, Hercules, CA) organic acid column at 65°C. The mobile phase was 5 m*M* sulfuric acid in ultrapure water at a flow rate of 0.5 mL/min. The analytical column was protected with a Cation-H (Bio-Rad) precolumn. For the detection of maleic acid, a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan) was used.

Intracellular and cell-bound enzyme activities in samples taken on 7 d were determined using the following procedure: A 15-mL aliquot of each culture broth was centrifuged at 3400*g* for 10 min. The enzyme activities assayed in the supernatant were considered extracellular enzyme activities. The insoluble fraction, consisting of residual carbon source residues (Solka Floc 200) and fungal mycelium, was washed by subsequent repeated resuspension in acetate buffer (0.05 *M*, pH 4.8) and centrifugation.

Table 1
Experimental Setup, Average pH Values, and Protein Concentrations

Experiment	Carbon source	pH control	Average pH	Protein concentration (mg/mL)[a]	
				d 4	d 7
A	Solka Floc	Daily	5.7	0.83	0.88
B	Solka Floc	—	3.9	0.53	0.87
C	Solka Floc	Buffer (0.10 *M*)	5.9	1.01	1.46
D	Glucose	Daily	5.7	0.24	0.39
E	Glucose	—	3.7	0.13	0.31
F	Glucose	Buffer (0.10 *M*)	6.0	0.41	0.65
G	Lactose	Daily	5.7	0.31	0.46
H	Lactose	—	4.9	0.26	0.39
I	Lactose	Buffer (0.10 *M*)	6.0	0.72	0.91
J	Solka Floc	Buffer (0.05 *M*)	5.6	n.m.	n.m.
K	Solka Floc	Buffer (0.10 *M*)	5.9	n.m.	n.m.
L	Solka Floc	Buffer (0.15 *M*)	5.8	n.m.	n.m.

[a]n.m., not measured.

After two washes, the insoluble fraction was resuspended to a total volume of 15 mL in acetate buffer (0.05 *M*, pH 4.8). A homogeneous sample was removed, out and the dry matter content was determined gravimetrically. The fungal mycelium in a 2-mL aliquot of suspension was subjected to cell disintegration: 2 min of grinding with 2 g of glass beads (710–1180 μ*m*). The mixture was centrifuged at 3400*g* for 10 min. Enzyme activities measured in the supernatant were considered intracellular enzyme activities. The solid residue was washed in a manner similar to the one just described. After two cycles of subsequent resuspension and centrifugation, the solid residue was suspended in 2 mL of acetate buffer (0.05 *M*, pH 4.8). Cell-bound enzyme activities were determined from this suspension comprising the cell debris.

Results

Extracellular Enzyme and Protein Production

Cellulase production by *T. reesei* RUT C30 was investigated utilizing three different carbon sources—Solka Floc 200, lactose, and glucose—of which two are known to induce cellulase induction (Solka Floc 200 cellulose powder, lactose). Three pH control strategies were applied for each carbon source, as described in Materials and Methods. Table 1 provides the experimental setup.

As can be seen in Fig. 1A, B, there were major differences in the amounts of extracellular cellulases (FPA, endoglucanase activity) measured

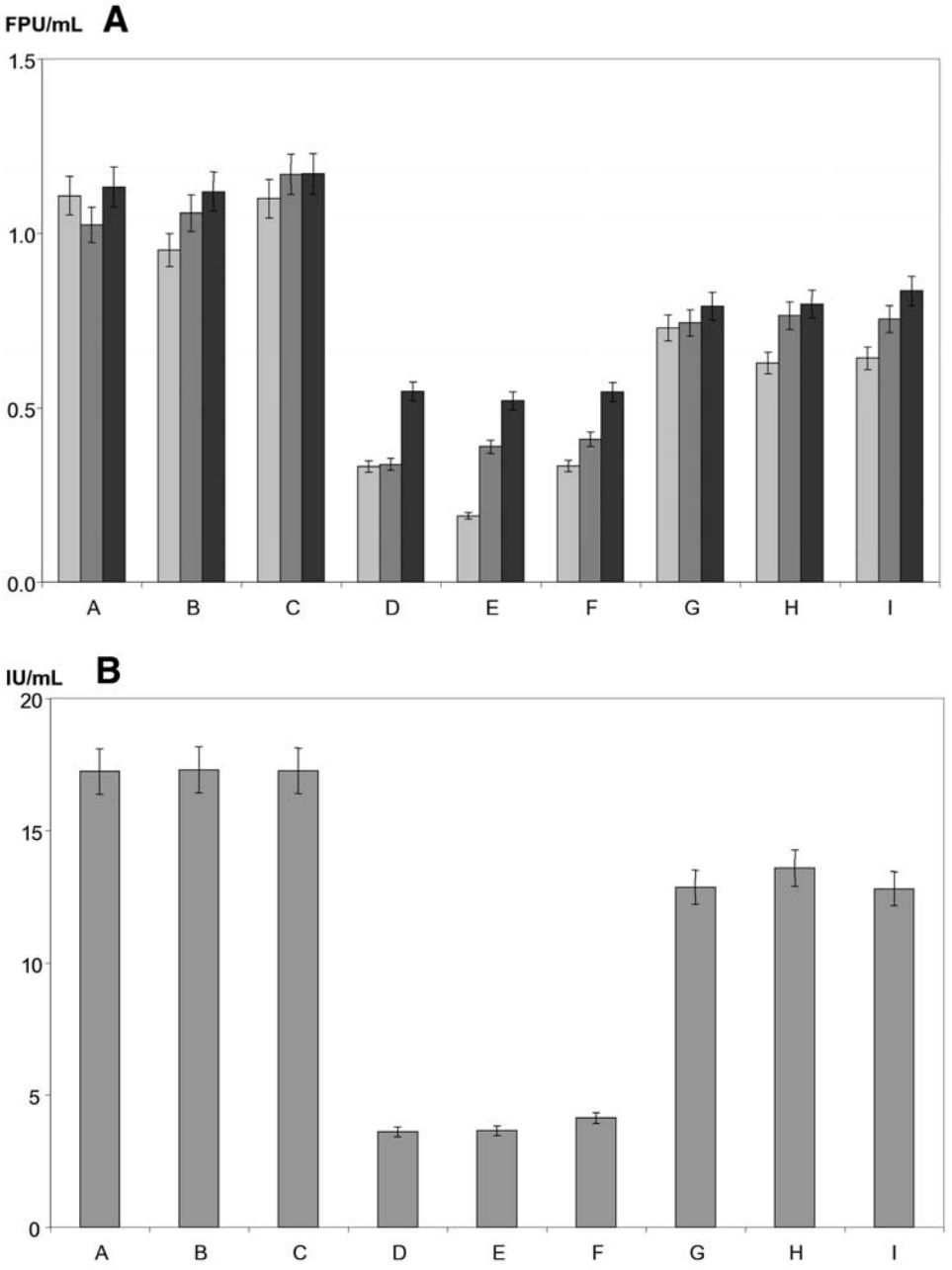

Fig. 1. (A) FPAs (light grays bars, d 4; medium gray bars, d 7; dark gray bars, d 7 with added β-glucosidase); **(B)** endoglucanase activities (d 7).

on d 4 and d 7, depending on the carbon source used. The highest FPA values, about 1.15 FPU/mL, were reached on Solka Floc 200. As already known, lactose is a carbon source provoking cellulase synthesis. However, the obtained FPAs were 40% less than measured on Solka Floc 200.

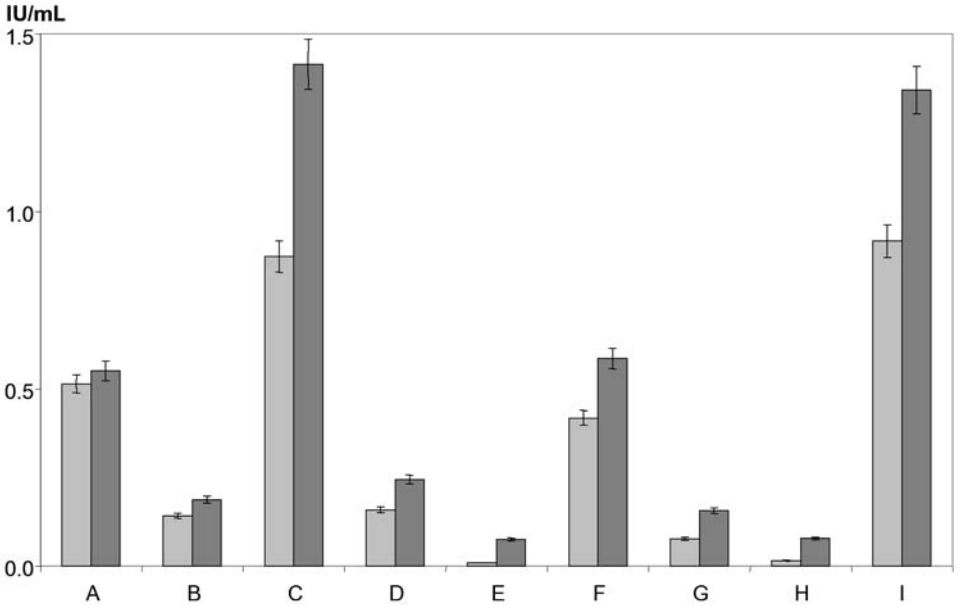

Fig. 2. Extracellular β-glucosidase activities (light gray bars, d 4; dark gray bars, d 7).

Significantly lower, about 0.40 FPU/mL, cellulase activities were obtained on glucose containing medium. FPAs measured with additional β-glucosidase were higher than determined without β-glucosidase supplementation, and there were minor differences within the experiments in which the same carbon source was used. There were minor differences regarding endoglucanase activities using different pH strategies on the same carbon source. The highest endoglucanase activities were obtained on Solka Floc 200, whereas on lactose and glucose 30 and 75% lower activities were reached, respectively.

The results summarized in Fig. 2 show that the highest extracellular β-glucosidase activities measured on d 7 were obtained when the production medium was supplemented with Tris-maleate buffer, irrespective of the carbon source. The highest activities (1.4 IU/mL) were reached on Solka Floc 200 and lactose, whereas 60% lower β-glucosidase activity was obtained on buffered glucose medium. In buffered cultures, the activities were 140% higher on Solka Floc 200 and glucose, and a 5.5-fold increase was observed on lactose compared to nonbuffered, pH-adjusted cultures. The β-glucosidase activities in pH-adjusted cultures were two and three times higher than in cultures without pH adjustment.

In buffered cultures not only β-glucosidase activities but also extracellular protein concentrations were considerably higher compared with nonbuffered cultivations (see Table 1). In experiments with Tris-maleate buffer, protein concentrations measured on d 7 were almost twofold higher than obtained in nonbuffered cultures. Generally, lower protein concentrations were obtained in non-pH-adjusted cultures compared with pH-adjusted cultivations.

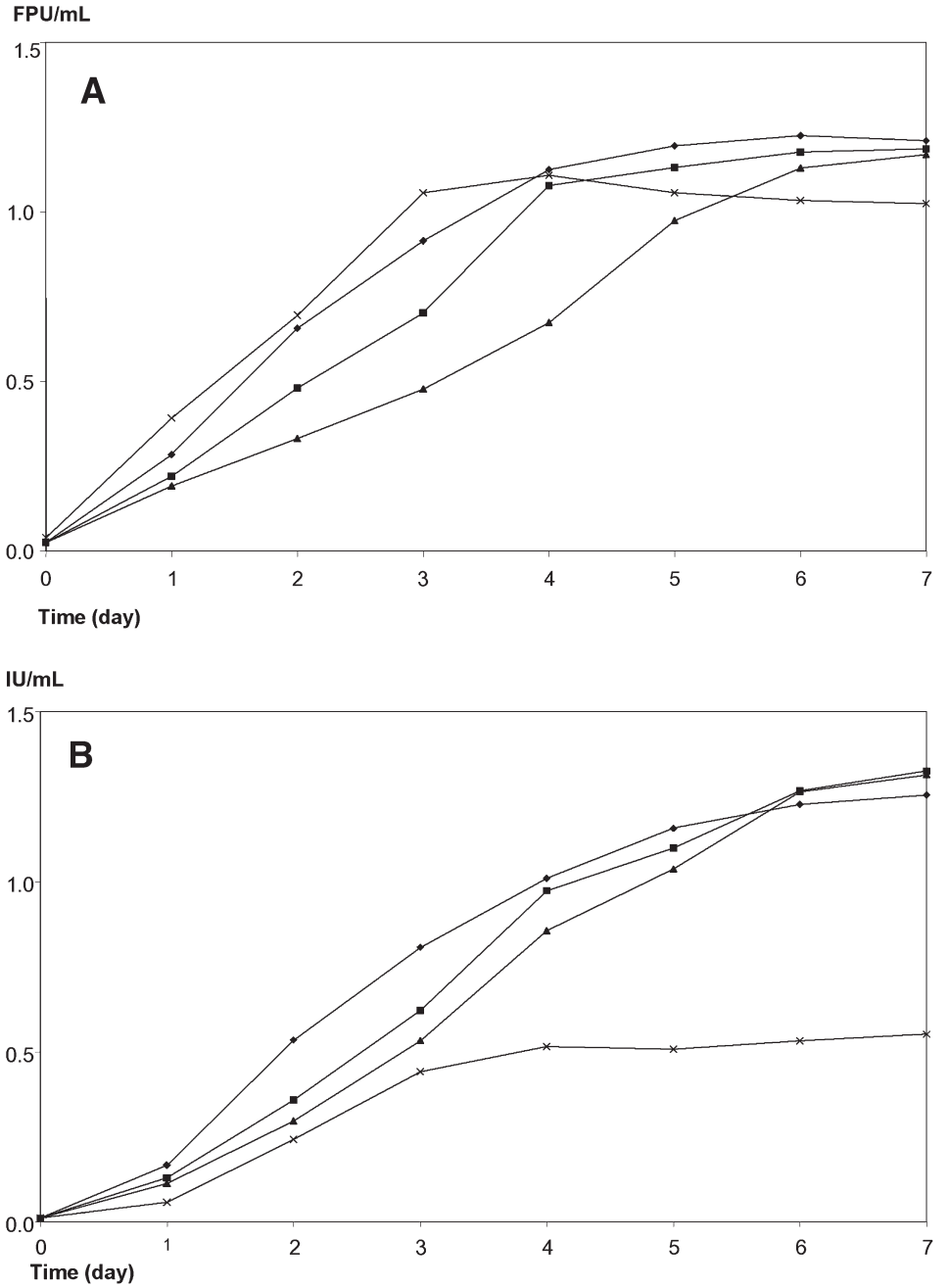

Fig. 3. (A) Time course of FPAs in experiments with various buffer concentrations (♦, 0.05 *M*; ■, 0.10 *M*; ▲, 0.15 *M*; x, without buffer); **(B)** time course of β-glucosidase activities in experiments with various buffer concentrations (♦, 0.05 *M*; ■, 0.10 M; ▲, 0.15 M; x, without buffer).

Table 2
Enzyme Production Rates in Experiments with Various Buffer Concentrations (d 3)

Buffer concentration (*m*)	FPU/(L·h)	β-Glucosidase activity/(L·h)
0.00	14.7	6.1
0.05	12.7	11.2
0.10	9.8	8.6
0.15	6.6	7.4

As shown in Table 1, average pH values were in the range of 5.6–5.7 in experiments with daily pH adjustment, and in buffered cultures the average pH values varied between 5.9 and 6.0. In cultivations without pH adjustment, the average pH values were in the range of 3.7–4.9.

The effect of the concentration of buffer components was investigated on Solka Floc 200 in the range of 0.05–0.15 *M* (see Table 1). The results are presented in Fig. 3A,B and in Table 2. The higher the concentration of buffer, the longer was the lag phase and the lower the enzyme production rate (see Table 2). However, buffer concentration had no influence on final FPA and β-glucosidase activity. Also note that the enzyme production rate of cellulases was the highest in cultivations without buffer. The average pH values were in the range of 5.6–6.0.

The induction effect of Tris-maleate buffer was tested using sorbitol, which is a neutral carbon source with respect to cellulase production. The experiments were performed with and without buffer. Close to zero enzyme activities (data not shown) were reached in both experimental setups; however, the lag phase was longer in the buffered cultures.

Enzyme Localization

Localization of cellulase components was determined by measuring cell-bound and intracellular FPAs and β-glucosidase activities (see Table 3). Generally, intracellular and cell-bound FPAs were about 5% of the whole enzyme activities, and there were no considerable differences within applied pH strategies and carbon sources. By contrast, cell-bound β-glucosidase activities were considerably high, especially in the case of buffered cultures, where they varied in the range of 0.20–0.30 IU/mL. Moreover, intracellular β-glucosidase activities of buffered cultures were between 0.02 and 0.07 IU/mL, which were significantly, at least 2.5-fold, higher than obtained in nonbuffered cultures.

Determination of Biomass and Consumption of Maleic Acid

The biomass of cultures was determined. The same amount of biomass was produced in pH-adjusted and buffered cultivations (G and I), whereas

Table 3
Intracellular and Cell-Bound FPAs and β-Glucosidase Activities (d 7)

	FPA (FPU/mL)		β-Glucosidase activity (IU/mL)	
Experiment	Intracellular	Cellbound	Intracellular	Cellbound
A	0.05	0.04	0.02	0.26
B	0.05	0.03	0.01	0.10
C	0.06	0.05	0.05	0.30
D	0.03	0.03	0.01	0.17
E	0.05	0.04	0.01	0.05
F	0.05	0.04	0.02	0.20
G	0.04	0.04	0.01	0.13
H	0.05	0.04	0.01	0.13
I	0.07	0.04	0.07	0.26

30% more biomass was obtained in non-pH-adjusted cultures. According to determinations of maleic acid, the content of maleic acid of all buffered cultures remained constant.

Discussion

The obtained FPAs and endoglucanase activities are in good accordance with results published elsewhere *(5)*. Solka Floc consists of cellulose and xylan. Therefore, it is a rather good carbon source for cellulase production. Lactose can also induce the production of cellulases to a lesser extent *(23,24)*. However, the lactose induction mechanism is still not clear *(24)*. Glucose does not repress cellulase production in *T. reesei* RUT C30, because it is *acre 1* mutant strain *(25)*. However, only low levels of cellulases can be produced on glucose, because of the lack of inducers *(25)*. The applied pH-controlling strategies had no effect on the final FPAs and endoglucanase activities. However, there were major differences for enzyme formation rates. The highest activities could be reached in pH-adjusted cultures on d 4, whereas in cultivations with other pH-controlling strategies, the highest activities were obtained on d 7 of fermentations.

The final β-glucosidase activities were much higher in buffered cultures for intracellular, extracellular, and cell-bound activities. Therefore, it is tempting to speculate that a buffer component enhances β-glucosidase production. Strongly supporting this view is the fact that in buffered cultures major changes occurred in protein concentration between the d 4 and d 7, which were believed to be mainly owing to β-glucosidase production. In addition, it seems that applied pH strategies had a considerable effect on the production of β-glucosidases. Generally, in non-pH-adjusted cultures the activities of intracellular, extracellular, and cell-bound β-glucosidases were about two times lower compared with pH-adjusted cultivations.

It should also be noted that generally the average pH values of buffered cultures were slightly higher than those of nonbuffered cultivations. However, the average pH values in cultures with 0.05 *M* Tris-maleate buffer were lower than in nonbuffered cultivation (experiments A and K). These observations led us to conclude that not the pH but the buffer components themselves induce higher β-glucosidase activity. Moreover, previous results indicate that maleic acid is the buffer component that enhances cellulase production, because similar results could be obtained without Tris-hydroxymethylaminomethane by applying maleate buffer *(16)*.

It seems that because of the salts in buffered cultivations, *T. reesei* needs a longer adaptation time compared with nonbuffered cultures. Therefore, the higher the concentration of buffer, the lower the initial enzyme production rate. However, until d 7 of experiments, the differences in enzyme activities were equalized. Therefore, it can be concluded that the concentration of buffer components in the range of 0.05–0.15 *M* had no effect on final β-glucosidase and filter paper activities.

On neutral carbon source, i.e., on sorbitol, which is neither an inductor nor a repressor *(25)*, β-glucosidase production was not enhanced by buffer components. Therefore, it is probable that maleic acid is a coinductor. This view is supported by the observation that in the case of glucose, on which low levels of β-glucosidase were produced, there were smaller differences between buffered and nonbuffered cultures in terms of final β-glucosidase activities compared with cultivations on Solka Floc 200 and lactose.

According to the results of enzyme location measurements, it seems that only a few percent of endo- and exoglucanases were intracellular and cell-bound enzymes. By contrast, the amount of cell-bound β-glucosidase could be rather significant, up to 60% of the total activity. Therefore, it can be argued that a significant part of β-glucosidase remains bound to the cells. Intracellular β-glucosidase activities were only a few percent of the total activities.

Determination of dry mass and maleic acid measurements suggest that buffer components had no effect on biomass formation.

Acknowledgment

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