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β-GLUCOSIDASE PRODUCTION OF TWO DIFFERENT *ASPERGILLUS* STRAINS

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β-Glucosidase has an important role in cellulose degradation by cleaving the cellobiose to glucose units. Supplementation of *Trichoderma* cellulase with exogenous β-glucosidase is needed to prevent the inhibition effect of cellobiose on exoglucanases and endoglucanases.

Production of β-glucosidase by *Aspergillus niger* and *Aspergillus phoenicis* has been investigated under different fermentations. Both strains were appropriate for enzyme production at high level under the applied conditions.

Cultivation of *A. phoenicis* on Mandels' medium containing a complex nitrogen source has resulted in a higher β-glucosidase activity than on Vogel's medium. In an air-lift fermenter *A. phoenicis* grew in the shape of beads with 1.41 IU g^{-1} cell-associated enzyme activity. The fungal pellets can be used as in situ immobilized enzyme preparation.

A. niger produced extracellular β-glucosidase at the level of 2.1 IU ml^{-1} in stirred-tank fermenter with a yield of 210 IU g^{-1} glucose and productivity of 21.8 IU l^{-1} h^{-1}.

Keywords: β-glucosidase, fermentation, *A. niger*, *A. phoenicis*

Enzymatic hydrolysis of lignocellulosic materials appears to be one of the most promising ways to produce renewable energy. In the hydrolysis of cellulose – the major component of plants – three main components of the cellulase enzyme system are acting simultaneously, i.e. 1,4-β-D-glucan-4-glucanohydrolases (endoglucanase, EC 3.2.1.4), 1,4-β-D-glucan-cellobiohydrolases (exoglucanase, EC 3.2.1.91) and β-1,4-glucosidase (β-glucosidase, EC 3.2.1.21). The hydrolysis of insoluble native cellulose carried out by the synergistic action of exoglucanases and endoglucanases results in soluble cellodextrins and cellobiose; then β-glucosidase cleaves the cellobiose units to glucose (ENARI, 1987).

However *Trichoderma reesei*, which is one of the best cellulase producing microorganisms (MANDELS, 1975) secretes β-glucosidase at a low level. The cellobiose accumulating during the hydrolysis inhibits the activity of endoglucanase and

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exoglucanase, decreasing the overall rate of hydrolysis (HOWELL & STUCK, 1975, HSU et al., 1980, MAGUIRE, 1977). One the other hand cellobiose is not fermentable by *Saccharomyces cerevisiae* (WOODWARD & WISEMAN, 1982). Therefor β-glucosidase activity should be kept at an optimal level related to cellulase in order to increase the saccharification rate of cellulose (STERNBERG et al., 1977, KHAN et al., 1985). One way to diminish the effect of cellobiose inhibition is the addition of exogenous β-glucosidase. Fungi belonging to the genus *Aspergillus* produce extracellular and cell-associated ß-glucosidase in high yield, which is compatible with *T. reesei* cellulases (ALLEN & STERNBERG, 1980, RÉCZEY et al., 1989, FLACHNER et al., 1999).

In this study *Aspergillus phoenicis* QM 329 and *Aspergillus niger* BKM F-1305 were examined, grown in shake flask and different kind of fermenter (air-lift, stirred-tank fermenter). The aim of the work was to compare the extracellular and intracellular β-glucosidase production of the above *Aspergilli* under different fermentation conditions.

1. Materials and methods

1.1. Microorganisms

Aspergillus niger BKM F-1305 and *Aspergillus phoenicis* QM 329 were maintained on agar slant containing 5% malt extract, 18 g l^{-1} bacto agar, at 30 °C. After 14 days the spores were used for inoculation. The strains were obtained from the Culture Collection of the Dept. of Agricultural Chemical Technology, Technical University of Budapest.

1.2. Media

Inoculum was prepared the following way. One percent of spore suspension, containing 5×10^5 conidia ml^{-1}, was used to initiate growth on 5% malt extract at 5.4–5.6 pH. The inoculum preparation was complete after 2 days at 31 °C and 350 r.p.m.

Two different nutrient media were used. The composition of Vogel's medium was (g l^{-1}): glucose 10, proteose peptone 1.0 and citric acid 0.5. After sterilization 2% Vogel's stock solution was added to the medium (VOGEL, 1964).

The composition of Mandels medium was (g l^{-1}): urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4$ 7 H_2O, 0.3, proteose peptone 0.75, yeast extract 0.25, and trace elements were added to the medium in the following concentrations from 1% solutions: 3.3 ml l^{-1} $FeSO_4$. 7 H_2O, 1.1 ml l^{-1} $MnSO_4$, 0.9 ml l^{-1} $ZnSO_4$ and 1.3 ml l^{-1} $CoCl_2$ together with 10 g l^{-1} glucose as carbon source (MANDELS & WEBER, 1969).

1.3. β-Glucosidase production

Shake flask cultures were made in 750 ml Erlenmeyer flasks containing 150 ml medium at 30 °C and 350 r.p.m. Inoculation was made with 15 ml inoculum, described above. Samples were taken once a day to observe consumption of reducing sugar and production of β-glucosidase; pH was adjusted daily to 5.8–6.0. Three parallel flasks were run with both fungi.

The stirred-tank reactor was a 24 l BIOSTAT U (Braun, Germany) fermenter with working volume of 18 l, 0.5–1.0 v/v min^{-1} aeration and 250–300 r.p.m. stirring. The inoculum constituted 10% of the total volume. The initial pH was 5.8–6.0 and it was adjusted to 6.0 after the first day, when it was let to go down to pH 3.0, by addition of 10% NaOH and 10% H_2SO_4. Enzyme production was continuing on for 4 days, samples were regularly withdrawn and analysed for reducing sugar content and β-glucosidase activity. The calculated yield and productivity were based on extra- and intracellular β-glucosidase activity.

The air-lift fermenter was a Pyrex glass cylinder with a diameter of 70 mm and a height of 500 mm with 1200 ml working volume. The bottom of reactor was a porous glass filter with a pore size of 40–90 μm. Agitation and aeration were performed by letting sterile air through the glass filter at a rate of 450–500 l h^{-1}. Temperature was maintained at 30 °C, the initial pH was 5.2, not adjusted. The air-lift fermenter was directly inoculated with conidia resulting in 10^5–10^6 conidia ml^{-1} broth, determined with Bürker counting chamber.

One experiment was run with each *Aspergillus* strain in stirred-tank fermenter and in air-lift fermenter as well.

1.4. Assays

The substrate consumption was determined colorimetrically using dinitrosalicylic acid (DNS) reagent described by MILLER (1959). Samples of 0.1–0.5 ml were made up to 1.5 ml with distilled water and 3 ml of DNS reagent was added. The colour obtained after boiling the mixture for 5 min and diluting with 16 ml of distilled water was measured at 550 nm. The relative standard deviation of this method was ≤2.8%.

Extracellular β-glucosidase activity was measured with 5 mmol l^{-1} pNPG in 50 mmol l^{-1} citrate-buffer, pH 4.8. One ml of substrate and 0.1 ml of correspondingly diluted culture filtrate were incubated for 10 min at 50 °C (NORKRANS, 1957). The reaction was stopped by addition of 2 ml 1 mol l^{-1} Na_2CO_3. After cooling, 10 ml of distilled water was added and the absorbance was read at 400 nm. The activity was calculated as IU ml^{-1}. One international unit is defined as one μmol p-nitrophenol produced by 1.0 ml of enzyme per minute under the assay conditions. The assays were

done in duplicate on each sample and the mean of the obtained values was used. Relative standard deviation was ≤2.0%.

For cell-associated, intracellular β-glucosidase activity measurement the pellets were separated from the fermentation broth by filtration through a Büchner funnel. Wet pellets were directly used instead of 0.1 ml of culture filtrate and treated as above. Intracellular activity was calculated as IU g^{-1} wet pellet. The determinations were done in triplicate on each sample and the mean was used. Relative standard deviation was ≤4.5%.

2. Results and discussion

2.1. Extracellular β-glucosidase production on two different nutrient media

The comparison of β-glucosidase production of *Aspergillus phoenicis* on two different nutrient media was carried out in shake flasks with 10 g l^{-1} glucose as carbon source, inoculated by spore suspension. The results on Fig. 1A and 1B show that the reducing sugar concentration decreased in two days to zero on Mandels' medium while on Vogel's medium it lasted for three days. The starting pH 5.5–6.0 decreased to about 3 on both media but the reduction was much quicker on Mandels' medium. In both cultivations pH were adjusted to 6.0, but only on Vogel's medium occurred a drastic pH drop again. The greatest difference between the two media was in extracellular β-glucosidase production. On Vogel's medium enzyme activity appeared in the fermentation broth after a rather long lag phase of 3 days, while on Mandels' medium it reached 0.9 IU ml^{-1} already on the first day of cultivation. Maximum activity, yield and productivity were five times higher on Mandels' medium than on Vogel's after 7 days of fermentation (Table 1).

Table 1

Comparison of extracellular β-glucosidase activity, yield and productivity on two different culture media

Culture medium[a]	Vogel's medium	Mandels' medium
Activity (IU ml^{-1})	0.46	2.38
Yield (IU g^{-1} glucose)	46	238
Productivity (IU l^{-1} h^{-1}	2.7	14.2

[a] Cultivation was performed in shake flasks with *A. phoenicis*.

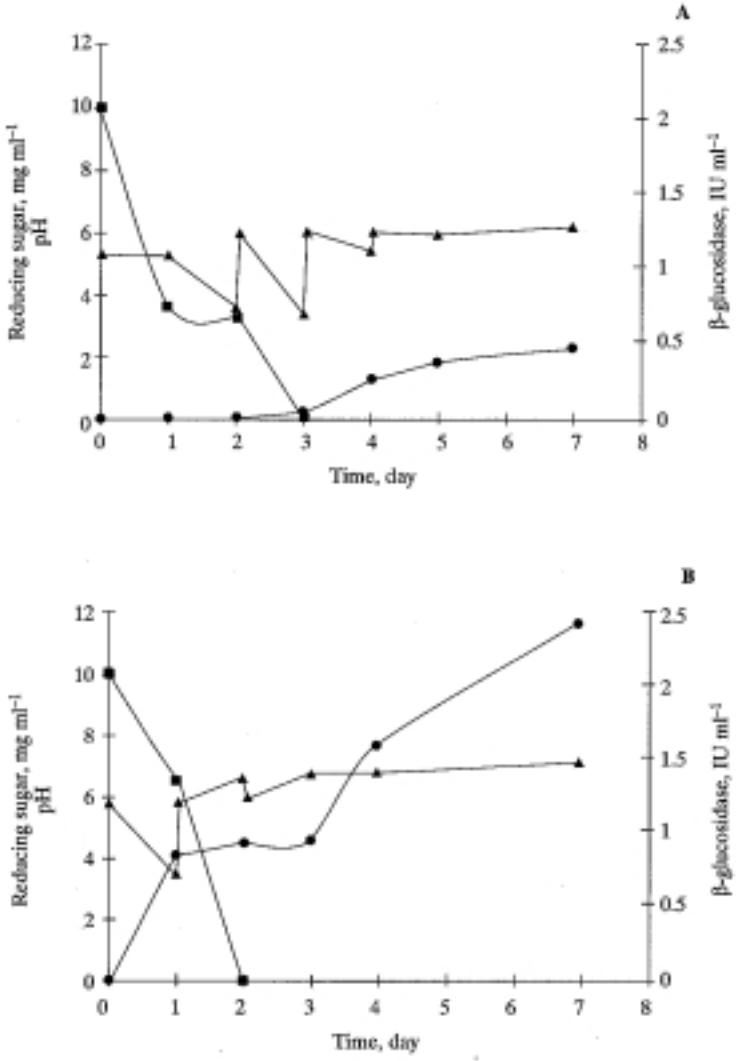

Fig. 1. Comparison of extracellular β-glucosidase production of *A. phoenicis* on different nutrient media. The cultivations were carried out in shake flasks with 10 g l^{-1} carbon source. A: Cultivation on Vogel's medium. B: Cultivation on Mandels' medium. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1})

2.2. β-Glucosidase production in shake flask and stirred-tank fermenter

Strains, *Aspergillus niger* and *Aspergillus phoenicis* were cultivated in shake flasks and stirred-tank fermenter in order to compare the extracellular and intracellular ß-glucosidase production under the same circumstances. Mandels' medium was used with 10 g l^{-1} glucose as carbon source. Both strains showed the same behaviour in shake flasks resulting in 2.80 IU ml^{-1} extracellular enzyme activity. The yield of both cultivation was 280 IU g^{-1} based on the added amount of glucose and 19.5 IU l^{-1} h^{-1} productivity was reached after a 6-day cultivation.

Whereas experiments performed in stirred-tank fermenter have shown different pH profile and carbon source utilization (Fig. 2A and 2B). *A. phoenicis* has consumed the carbon source somewhat slower but on the second day the reducing sugar concentration was zero in both experiments. In *A. niger* fermentation the pH dropped more rapidly and reached a minimum value of pH 1.6 at 22 h of fermentation. Change in pH was not so drastic in *A. phoenicis* cultivation, where minimum was pH 2.4 at 41 h of fermentation. Appearance of the β-glucosidase happened at about the same time, when pH has dropped under 3.0 and the carbon source was consumed. Final extracellular and intracellular β-glucosidase activity were 2.4-fold and 2.7-fold higher in *A. niger* cultivation than in *A. phoenicis* culture. The yield and productivity of *A. phoenicis* based on extra- and intracellular activity were only 38 and 39%, respectively, related to *A. niger* (Table 2).

Table 2

β-Glucosidase production on Mandels' medium in stirred-tank fermenter

Strains	*A. niger*	*A. phoenicis*
Extracellular enzyme activity (IU ml^{-1})	2.1	0.86
Inracellular enzyme activity (IU g^{-1} wet pellet)	12.5	4.68
Yield[a] (IU g^{-1} glucose)	185.8	70
Productivity[a] (IU l^{-1} h^{-1})	29.9	11.7

[a] Yield and productivity were based on extra- and intracellular enzyme activities

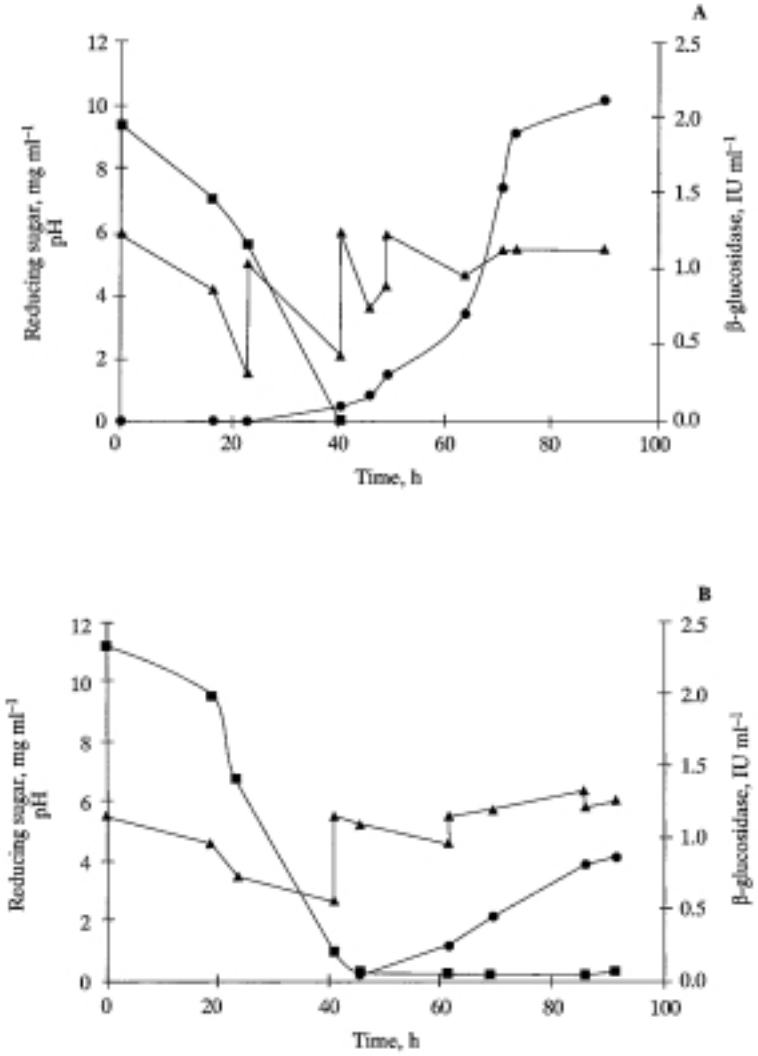

Fig. 2. Comparison of cultivation of *A. niger* and *A. phoenicis* in a 24-l stirred-tank fermenter on Mandels' medium. The working volume was 18 l with 10% of inoculation. The carbon source was glucose at 10 g l^{-1}. A: Cultivation of *A. niger* BKM F-1305. B: Cultivation of *A. phoenicis* QM 329. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1})

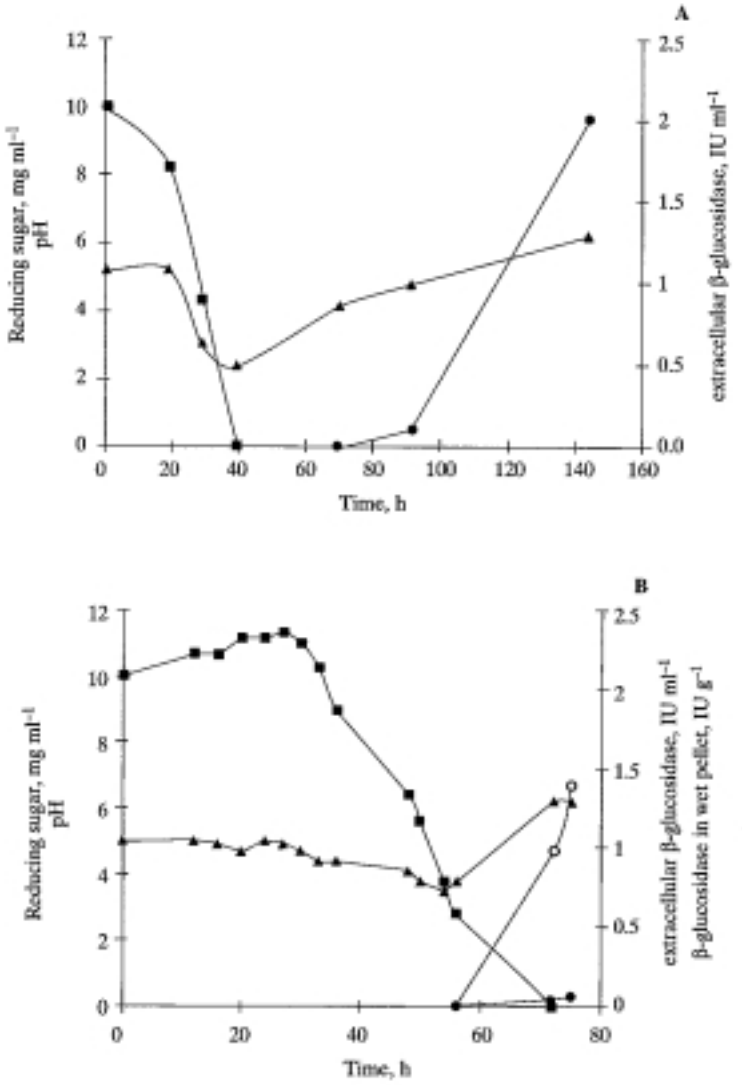

Fig. 3. Comparison of cultivation of *A. niger* and *A. phoenicis* in air-lift fermenter. The media were Mandels' and Vogel's medium, respectively. The working volume was 1200 ml with 10 g l^{-1} glucose as carbon source. A: Cultivation of *A. niger* BKM F-1305. B: Cultivation of *A. phoenicis* QM 329. —■—: reducing sugar (mg ml^{-1}); —▲—: pH; —●—: extracellular β-glucosidase activity (IU ml^{-1}); —○—: wet pellet activity (IU g^{-1})

2.3. Enzyme production in air-lift fermenter

Both *A. niger* and *A. phoenicis* were cultivated in air-lift fermenter using Mandels' and Vogel's media, respectively (Fig. 3.). *A. niger* consumed glucose in 39 h while there was a rapid decrease in pH to 2.4. In the *A. phoenicis* cultivation glucose was exhausted after 72 h. During this cultivation pH dropped quite slowly and reached a minimum, pH 3.5 in 54 h. *A niger* has grown in filamentous and micropellet shapes producing 2.01 IU ml^{-1} extracellular β-glucosidase activity after 6 days of cultivation. It was not possible to detect intracellular enzyme activity owing to these growing forms. Under the same circumstances *A. phoenicis* has grown in the shape of beads with 1.41 IU g^{-1} wet pellet activity and only 0.08 IU ml^{-1} enzyme was detectable in the supernatant (Fig. 3A, Fig. 3B).

3. Conclusions

Both *Aspergillus* strains can produce β-glucosidase at high level to supplement cellulase of *Trichoderma*, corresponding to earlier studies (ALLEN & STERNBERG, 1980, KERNS et al., 1987). However our results show that while *A. phoenicis* is recommended for immobilized enzyme production using air-lift fermenter, *A. niger* is recommended for soluble enzyme production in stirred-tank fermenter.

Cultivating *A. niger* in stirred-tank fermenter 12% and 56% higher extracellular enzyme productivity can be achieved than in shake flasks and in air-lift fermenter, respectively (Table 3.).

Comparing the inoculation method of *Aspergillus phoenicis*, using the 2-day-inoculum on malt extract 37% higher productivity was achieved than by using a direct inoculation with conidia (Table 1 and 2.2. Chapter).

Table 3

Productivity of extracellular β-glucosidase activity with Aspergilli *in different type of fermenters (IU l^{-1} h^{-1})*

Strains	*A. phoenicis*	*A. niger*
Shake flasks	19.5	19.5
Stirred-tank fermenter	9.3	21.8
Air-lift fermenter	0.56	13.9

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