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Screening Genus *Penicillium* for Producers of Cellulolytic and Xylanolytic Enzymes

Kristian B. R. Krogh, Astrid Mørkeberg, Henning Jørgensen,[†] Jens C. Frisvad, and Lisbeth Olsson[*]

Center for Process Biotechnology, BioCentrum-DTU, Technical University of Denmark, Building 223, DK-2800 Lyngby, Denmark, E-mail: lo@biocentrum.dtu.dk.

Abstract

For enzymatic hydrolysis of lignocellulosic material, cellulolytic enzymes from *Trichoderma reesei* are most commenly used, but, there is a need for more efficient enzyme cocktails. In this study, the production of cellulolytic and xylanolytic enzymes was investigated in 12 filamentous fungi from genus *Penicillium* and compared with that of *T. reesei*. Either Solka-Floc cellulose or oat spelt xylan was used as carbon source in shake flask cultivations. All the fungi investigated showed coinduction of cellulolytic and xylanolytic enzymes during growth on cellulose as well as on xylan. The highest filter paper activity was measured after cultivation of *Penicillium brasilianum* IBT 20888 on cellulose.

Index Entries: Cellulolytic enzymes; hemicellulolytic enzymes; enzymatic hydrolysis; coinduction.

Introduction

Today, an international awareness of the increasing CO_2 concentration in the atmosphere has resulted in the formation of the Kyoto Protocol, which has led many countries to make the commitment to decrease the emission of CO_2. One way of decreasing CO_2 emissions could be substitution of fossil fuels with renewable energy sources. The net production of CO_2 is significantly lower when bioethanol produced from plant materials is used as transportation fuel instead of fossil fuels, since CO_2 is assimilated

[†]Present address: Plant Fibre Laboratory, Department of Agricultural Sciences, The Royal Veterinary and Agricultural University, Højbakkegård Alle 1, DK-2630 Taastrup, Denmark.

*Author to whom all correspondence and reprint requests should be addressed.

during photosynthesis *(1)*. To meet the future demand for bioethanol, not only starch but also lignocellulosic materials need to be used as substrate.

The polysaccharides in the raw materials need to be hydrolyzed before the sugar monomers can be fermented to ethanol. Today, enzymatic hydrolysis is regarded as a method with great potential. One major obstacle to overcome is the high cost of cellulolytic enzymes. In 2001, the United States Department of Energy formed a contract with two commercial producers of cellulolytic enzymes in an attempt to achieve a 10-fold decrease in the cost of the cellulolytic enzymes (www.ott.doe.gov/biofuels/research_partnerships.html).

The decay of plant material in nature is partly owing to the production of cellulolytic and hemicellulolytic enzymes in microorganisms. Filamentous fungi, such as *Trichoderma reesei*, *Penicillium pinophilum*, and *Humicola insolens* have demonstrated the capability of secreting large amounts of cellulolytic enzymes *(2)*. The most extensively studied microorganism producing cellulolytic enzymes is the filamentous fungus *T. reesei* which is the preferred microorganism for industrial production of cellulolytic enzymes. One of the main limitations of the cellulolytic system from *T. reesei* is the low amount of β-glucosidase (BG) *(3)*. Low BG activity leads to a buildup of cellobiose during hydrolysis, which inhibits the activity of the cellobiohydrolases (CBHs) to a larger extent than glucose does *(4)*. Therefore extra BG needs to be added for an efficient hydrolysis of cellulosic materials *(5)*.

Microorganisms grow in various habitats in nature, and they have therefore adapted to various physical and chemical conditions. In the search for microorganisms that efficiently can degrade lignocellulose, several species from genus *Penicillium* were tested. In forest soil, where large amounts of plant materials are degraded, an abundance of *Penicillium* species is present *(6)*. Because of this fact and that enzyme mixtures from various *Penicillium* species have been shown to perform well in the hydrolysis of different kinds of lignocellulosic material *(7–9)*, we screened 12 different *Penicillium* species for their production of cellulolytic and xylanolytic enzymes.

Materials and Methods

Strains

The filamentous fungi screened were all from the genus *Penicillium* (Table 1) and were selected from the culture collection at BioCentrum-DTU, Technical University of Denmark. The filamentous fungi *T. reesei* Rut C30 was used as reference strain.

Preparation of Inoculum

Each strain was received on a Czapek yeast autolysate agar plate from the culture collection. Spores were transferred to a potato dextrose agar (PDA) (Difco, Detroit, MI) plate and the PDA plates were kept at the optimal temperature for growth (Table 1). After 2 wk the strains on the PDA

Table 1
Subgenus, Optimal Growth Temperature, and Origin for 12 *Penicillium* Strains Investigated

Strain	Subgenus	Temperature (°C)	Isolated from
P. allii IBT 3803	*Penicillium*	25	Garlic, Denmark
P. persicinum IBT 13226	*Furcatum*	25	Soil, USA
P. simplicissimum IBT 13237	*Furcatum*	30	Flannel bag, South Africa
P. simplicissimum IBT 15303	*Furcatum*	30	Feed, Norway
P. brasilianum IBT 20888	*Furcatum*	30	Seaweed, Denmark
P. pinophilum IBT 4186	*Biverticillium*	30	Maize, India
P. funiculosum IBT 5816	*Biverticillium*	30	Citric acid (10 %), Australia
P. pinophilum IBT 10872	*Biverticillium*	30	Maize, India
P. rubicundum IBT 10943	*Biverticillium*	30	Cultivated soil, United States
P. aculeatum IBT 18363	*Biverticillium*	30	Rhizosphere of bamboo, Taiwan
P. verruculosum IBT 18366	*Biverticillium*	30	Soybeen seed, Taiwan
P. minioluteum IBT 21486	*Biverticillium*	25	Fruit, Denmark

plates had produced spores, which were suspended in 0.1% (v/v) Tween-80 (P-1754; Sigma, St. Louis, MO).

Shake Flask Cultivations

An amount of each spore suspension was transferred to a 500-mL shake flask in order to obtain a spore concentration of 10^6 spores/mL. The medium was a modified Mandels and Weber medium *(10)*, in which the concentration of KH_2PO_4 was increased by 50% to improve buffer capacity. The initial volume in the shake flask was 150 mL. The carbon sources were either 2% (w/v) Solka-Floc cellulose (FCC200; Fiber Sales & Development) or 2% (w/v) oat spelt xylan (X-0627; Sigma). Cultivations were carried out aerobically at 150 rpm and at the optimal growth temperature for each strain (Table 1) and *T. reesei* Rut C30 was cultivated at 30°C. Samples were taken at regular time intervals during the cultivations, filtered through a 0.22-µm low-protein-binding filter (Cameo 25 GSS; Osmonics), and the filtrates were stored at –20°C.

Enzymatic Assays

Filter Paper Activity

Total cellulolytic activity was measured using the filter paper assay (FPA) according to Ghose *(11)* based on an estimation of the released reducing sugars by dinitrosalicylic (DNS) acid *(12)*.

Xylanase Activity

Xylanase (XA) activity was measured through the degradation of birch-wood xylan (7500.1; Roth, Karlsruhe, Germany) according to Bailey et al. *(13)* based on an estimation of the released reducing sugars by DNS acid *(12)*.

Endoglucanase and Endoxylanase Activities

Endoglucanase (EG) and endoxylanase (EX) activities were measured using azo-carboxymethyl cellulose (Megazyme, Bray, Ireland) and azo-xylan (Megazyme), respectively, as substrate as described by Jørgensen et al. *(14)*.

BG, β-Xylosidase, and α-L -Arabinofuranosidase Activities

BG, β-xylosidase (BX), α-L -arabinofuranosidase (AF), and β-galactosidase activities were measured using *p*-nitrophenyl-β-D-glucopyranoside (73676; Fluka), *p*-nitrophenyl-β-D-xylopyranoside (Sigma N-2132), and *p*-nitrophenyl-α-L-arabinofuranoside (N-3641; Sigma), respectively, as substrate as described by Jørgensen et al. *(14)*.

CBH Activity

CBH activity was measured in a 1 m*M* *p*-nitrophenyl-β-D-cellobioside (N-5759; Sigma) substrate solution at pH 4.8 with 50 m*M* sodium citrate. The substrate solution also contained 1.3 m*M* D-glucono-1,5-δ-lactone in

order to inhibit BG from hydrolyzing the substrate and thereby overestimating the CBH activity *(15)*. Otherwise, the procedure was as described for BG, BX, and AF activity.

Determination of Protein

Intracellular Protein

Mycelium was washed twice with 0.9% (w/v) NaCl followed by three extraction steps. In each extraction step the mycelium was boiled for 10 min with 1 *M* NaOH and the supernatant was collected after centrifugation *(16)*. The amount of protein in the pooled supernatant was measured based on the biuret method *(17)*.

Extracellular Protein

The concentration of extracellular protein was quantified using the "Bio-rad total protein" assay based on the Bradford *(18)* method with γ-globulin (G-7516; Sigma) as standard. The assay was performed using an analytical robot (Cobas Mira; Roche, Rotkreutz, Switzerland).

Results and Discussion

Production of cellulolytic and xylanolytic enzymes was investigated in 12 *Penicillium* species isolated from different habitats (Table 1), as well as in the well-characterized fungus *T. reesei* Rut C30. These filamentous fungi were cultivated aerobically for 220–240 h in shake flasks with 20 g/L of cellulose (Solka-Floc) or 20 g/L of xylan from oat spelts as carbon source. The cell mass concentration was estimated through measurements of intracellular protein. Cellulolytic enzyme production was characterized through measurements of BG, EG, and CBH activity, and total cellulolytic activity was determined as FPA. Xylanolytic enzyme production was investigated by measuring BX, EX, and AF activity, and the total XA activity was determined through the degradation of birchwood xylan.

Growth

To keep the production time as short as possible, it is important that the microorganism grows relatively fast. In samples containing insoluble substrates, the amount of cell mass cannot be determined by measurement of dry matter. The general trend in cell mass concentration, estimated indirectly from the amount of intracellular protein, was a faster initial growth on xylan compared to cellulose, but a higher final cell mass concentration when cellulose was used as carbon source compared to xylan (data not shown). Xylan is less ordered and has fewer hydrogen bonds than cellulose, and the higher initial growth rate on xylan may be owing to the structure of xylan, which is more accessible for the enzymes than cellulose. *P. verruculosum* IBT 18366, *P. brasilianum* IBT 20888, and *T. reesei* RUT C30 reached the highest cell mass concentration after cultivation on cellulose, and after cultivation on xylan, *P. persicinum* IBT 13226 and *P. verruculosum* IBT 18366 reached the highest cell mass concentration.

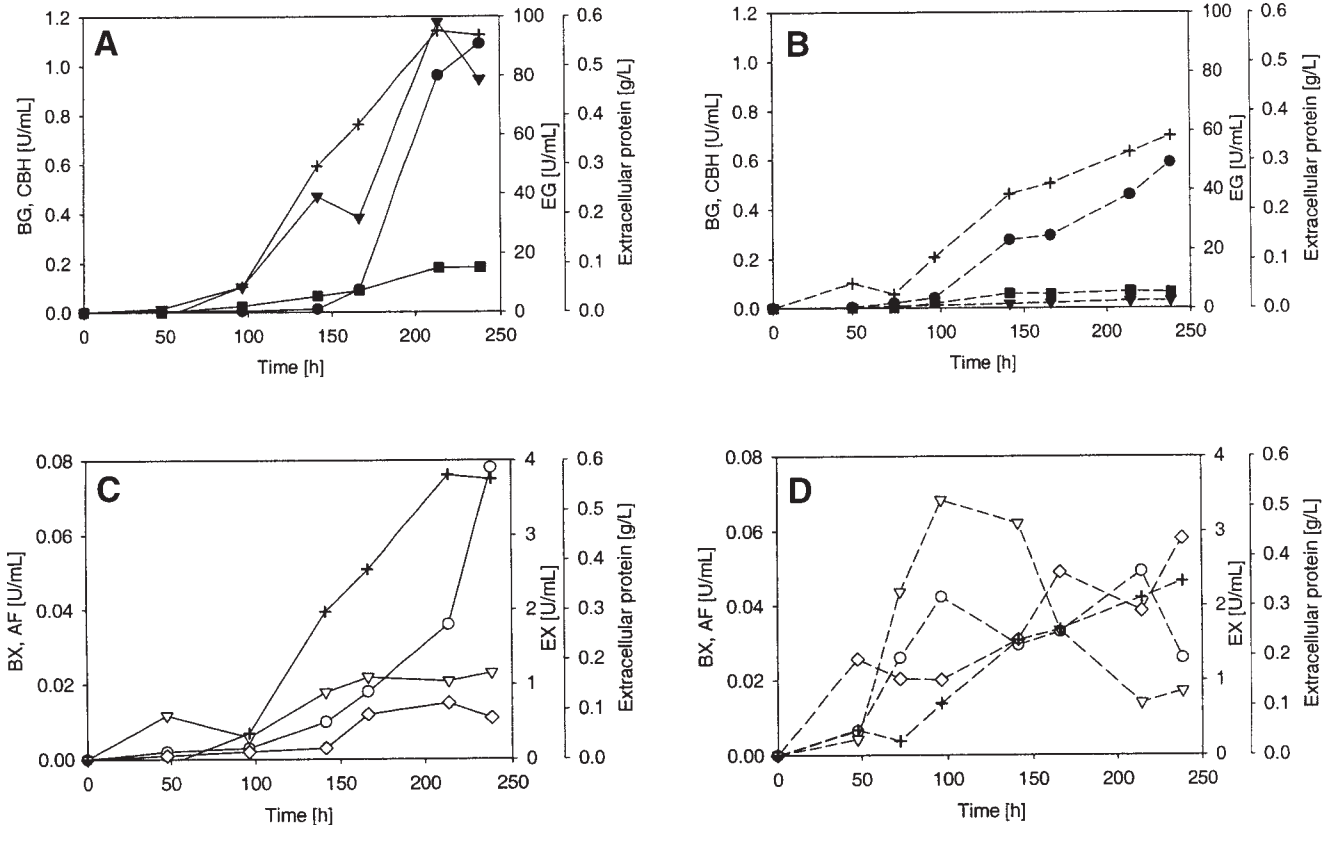

Fig. 1. Cellulolytic and xylanolytic activities and extracellular protein during time course of the cultivation of *P. brasilianum* IBT 20888. **(A)** Cellulolytic activities on cellulose; **(B)** cellulolytic activities on xylan; **(C)** xylanase activities on cellulose; and **(D)** xylanolytic activities on xylan. (●) BG; (■) CBH; (▼) EG; (○) BX; (◇) AF; (▽) EX; (+) extracellular protein; (—) cultivation on cellulose; and (- - -) cultivation on xylan.

Enzyme Production

In all cultivations, the general trend was a steady increase in cellulolytic and xylanolytic enzyme activities in the cultivation broth during the time course, as previously demonstrated by Schulz and Hirte *(19)* in a screening experiment of different *Penicillium* species cultivated on a plant hydrolysate. The same trend was observed during both cultivation on cellulose and on xylan (as exemplified in Fig. 1 A–D). Even though each measured enzyme activity increased during the time course of the cultivation, the specific activity of all enzymes was not constant in all cultivations. During cultivation of *P. brasilianum* IBT 20888 on cellulose, BG activity increased 10 times from 170 to 240 h, but CBH and EG activity only doubled (Fig. 1A). Increasing xylanolytic activity throughout cultivation on cellulose has been observed in *T. reesei (20)*, and increasing cellulolytic activity throughout cultivation on xylan has been demonstrated for an *Aspergillus* species *(21)*. Activities of cellulolytic enzymes in the supernatant were generally higher during growth on cellulose than during growth on xylan (Fig. 1A, 1B), whereas the activities of xylanolytic enzymes were higher during growth on xylan than during growth on cellulose (Fig. 1C, 1D). Our study demonstrated that there was a coinduction between cellulolytic and xylanolytic activities whether the substrate for cultivation of the fungus was cellulose or xylan. Furthermore, EX activity decreased in some cultivations when the substrate was xylan (data not shown).

Cellulolytic Enzymes

Cultivation of the different filamentous fungi for 230 h on cellulose resulted in different concentrations of extracellular protein and FPA, ranging from 0.01 to 0.78 g/L and from 0.02 up to 0.68 filter paper units (FPU)/mL, respectively. A relatively high protein concentration was shown to be correlated with a high FPA during growth on cellulose (Fig. 2). The specific FPA was found to be 0.77 FPU/mg of protein through linear regression with a regression coefficient of 0.77. A similar specific activity has been shown to result from a cultivation of *P. pinophilum (22)* on a substrate containing both cellulose and hemicellulose. Comparison of the specific FPA resulting from the growth of *P. occitanis* and *T. reesei* QM9414 on cellulose showed that the specific FPA was higher for the *Penicillium* species than for *T. reesei (23)*, as observed for *P. brasilianum* IBT 20888 and *T. reesei* Rut C30 in the present study. The *Penicillium* species with the highest FPA after growth on cellulose were *P. brasilianum* IBT 20888, *P. verruculosum* IBT 18366, *P. pinophilum* IBT 10872, and *P. minioluteum* IBT 21486. The highest FPA (0.68 FPU/mL) was measured after cultivation of *P. brasilianum* IBT 20888; this FPA was even higher than the 0.54 FPU/mL resulting from growth of *T. reesei* Rut C30 (Table 2). Fungi with low FPA might contain specific enzymes with interesting properties, such as higher specific activity for single enzymes or a lower product inhibition; thus, it might be interesting to also investigate these strains in further detail. If a fungus produces

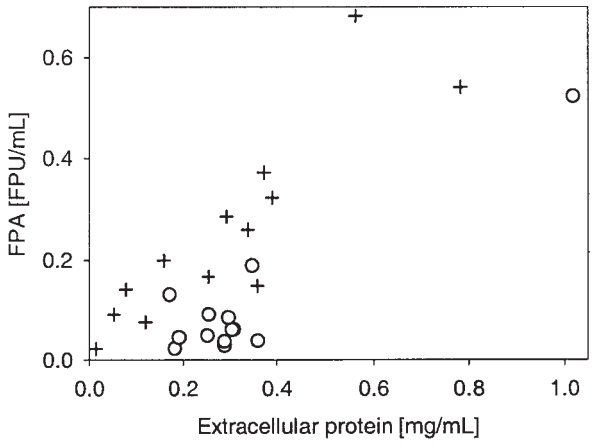

Fig. 2. FPA vs protein concentration in cultivation broth after cultivation of fungi on cellulose (+) and xylan (○). Each point in the plot represents the final protein concentration and the corresponding measured FPA in the cultivation broth for each fungus on each substrate.

Table 2
Cellulolytic Activities for *T. reesei* Rut C30 and for Four *Penicillium* Species with Highest FPA After Cultivation in Shake Flasks[a]

	BG (U/mL)	EG (U/mL)	CBH (U/mL)	FPA (FPU/mL)
T. reesei Rut C30	0.03 (0.31)	87 (44)	0.16 (0.07)	0.54 (0.52)
P. brasilianum IBT 20888	1.09 (0.59)	98 (2.6)	0.18 (0.07)	0.68 (0.19)
P. verruculosum IBT 18366	0.97 (0.33)	12 (0.5)	0.08 (0.02)	0.37 (0.13)
P. pinophilum IBT 10872	2.45 (0.80)	6 (1.3)	0.07 (0.07)	0.32 (0.06)
P. minioluteum IBT 21486	1.70 (0.78)	9 (1.2)	0.11 (0.06)	0.29 (0.05)

[a]Numbers not in parenthesis are activities after cultivation on cellulose, and numbers in parenthesis are activities after cultivation on xylan. Owing to assay limitations, the measured CBH activity should be seen as a quantitative indication.

a single enzyme with a desirable property, it is possible to increase the secretion of this enzyme through mutagenesis. It has been demonstrated that FPA in a *P. pinophilum* strain can be increased four times through three rounds of mutation and selection of the best-producing strain *(24)*.

BG activity was more than one order of magnitude higher for the four *Penicillium* species than BG activity resulting from cultivating *T. reesei* Rut C30. EG activity was one order of magnitude higher for *T. reesei* Rut C30 and *P. brasilianum* IBT 20888 than for the other three *Penicillium* species, which may provide an explanation for the lower FPA obtained from *P. verruculosum* IBT 18366, *P. pinophilum* IBT 10872, and *P. minioluteum* IBT 21486.

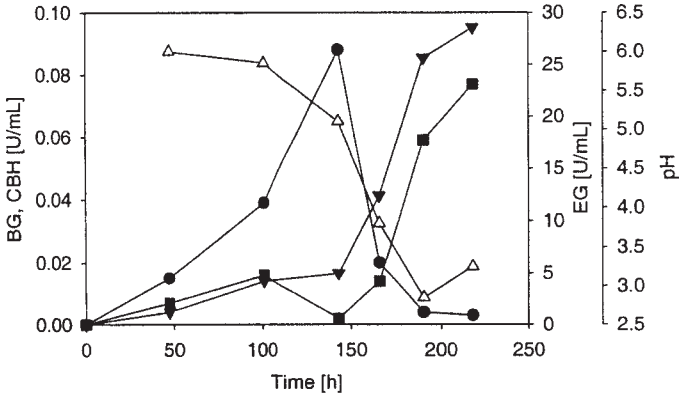

Fig. 3. Cellulolytic activities and pH during cultivation of *P. persicinum* IBT 13226 on cellulose (●) BG; (■) CBH; (▼) EG; and (△) pH.

When considering the results for FPA after cultivation of the fungi on xylan, the filter paper activity showed less correlation to the concentration of extracellular protein than when the fungi were grown on cellulose (Fig. 2). *T. reesei* Rut C30 had a much higher FPA, 0.52 FPU/mL, than any of the other fungi when grown on xylan (Table 2). The FPA for *T. reesei* Rut C30 was one order of magnitude higher than the FPA for *P. pinophilum* IBT 10872, although the only activity that was higher for *T. reesei* Rut C30 was the EG activity. The FPA could not be fully explained by the measured cellulolytic activities, for instance after growth on xylan, the cellulolytic activities were all lower for *P. verruculosum* IBT 18366 than for *P. pinophilum* IBT 10872, and yet *P. verruculosum* IBT 18366 yielded higher FPA (Table 2).

BG activity seemed to deviate from the general trend in that the activity of the individual cellulolytic enzymes increased throughout the cultivation (Fig. 1). In some cultivations, the measured BG activity decreased in the middle or toward the end of the cultivation, as exemplified in Fig. 3. After growing *P. persicinum* IBT 13226 on cellulose for 140 h, BG activity started to decrease, and after 191 h no BG activity could be measured in the cultivation broth. In this time period, the pH dropped concurrently to a value of 2.85. For *T. reesei* Rut C30 and *P. minioluteum* IBT 21486, BG activity also decreased as the pH dropped to a value of 3.0. The pH instability of BG has been reported for other microorganisms: several species of *Aspergillus (25)*, *Thermomyces lanuginosus (26)*, and also *Trichoderma harzianum (27)*. Other experiments (data not shown) have demonstrated that during cultivation of *P. persicinum* IBT 13226 on cellulose in well-controlled bioreactors with pH control, BG activity reached a significantly higher value, 1.3 U/mL, compared to the present experiments, in which no activity was detected.

Table 3
Xylanase Activities for *T. reesei* Rut C30 and Four *Penicillium* Species with Highest Xylanase Activity After Cultivation in Shake Flasks[a]

	BX (U/mL)	EX (U/mL)	AF (U/mL)	XA (U/mL)
T. reesei Rut C30	1.67 (0.07)	3.9 (59)	0.17 (0.42)	16 (176)
P. persicinum IBT13226	ND (0.19)	3.8 (30)	ND (0.15)	2 (105)
P. funiculosum IBT 5816	0.08 (0.33)	0.39 (10)	0.02 (0.20)	2 (42)
P. simplicissimum IBT 13237	ND (ND)	0.30 (1.6)	0.01 (0.21)	7 (31)
P. simplicissimum IBT 15303	ND (ND)	3.4 (2.7)	ND (0.21)	19 (23)

[a]ND, not detected. Numbers not in parenthesis are activities after cultivation on cellulose, and numbers in parenthesis are activities after cultivation on xylan.

Xylanolytic Enzymes

Cultivation of filamentous fungi on xylan resulted in extracellular protein concentrations for the *Penicillium* species in the range of 0.17–0.36 mg/mL and 1.02 mg/mL for *T. reesei* Rut C30. The XA activity was measured to be between 1.5 and 105 U/mL for the *Penicillium* species and 176 U/mL for *T. reesei* Rut C30. No linear correlation was observed between the XA activity and the amount of secreted protein, although the highest XA activities were measured in cultivation broths with relatively high protein concentrations (data not shown). Among the *Penicillium* species examined, *P. persicinum* IBT 13226 had the highest XA activity (105 U/mL) after growth on xylan. *P. funiculosum* IBT 5816 had the second highest XA activity of 42 U/mL (Table 3). XA activity was in the range of 1.5–19 U/mL after the fungi were grown on cellulose, and like on xylan, no linear correlation between XA activity and the amount of secreted protein was observed (data not shown). A possible explanation for the missing linear correlation could be the heterogeneity of hemicellulose. Owing to adaptation to the habitat, each fungus may have changed the regulation of individual hemicellulolytic enzymes to the given conditions, but during growth on xylan from oat spelts in the present experiment, the fungus might still produce enzymes needed to hydrolyze the "original" substrate. Therefore, the best producers found in a screening experiment heavily depend on the chosen substrate.

The highest EX and AF activities were measured after cultivation of *T. reesei* Rut C30 on xylan, (59 and 0.42 U/mL, respectively) (Table 3). These activities were with a few exceptions almost twice as high as any of the activities obtained after cultivation of the *Penicillium* species. BX activity reached by far the highest activity after cultivation of *T. reesei* Rut C30 on cellulose. This was not the case for the *Penicillium* species that had the highest BX activity when they were cultivated on xylan. XA activity could not, like FPA, be fully explained by the individual xylanolytic enzymes, as demonstrated in a comparison of *T. reesei* Rut C30 and *P. simplicissimum* IBT 15303 after growth on cellulose.

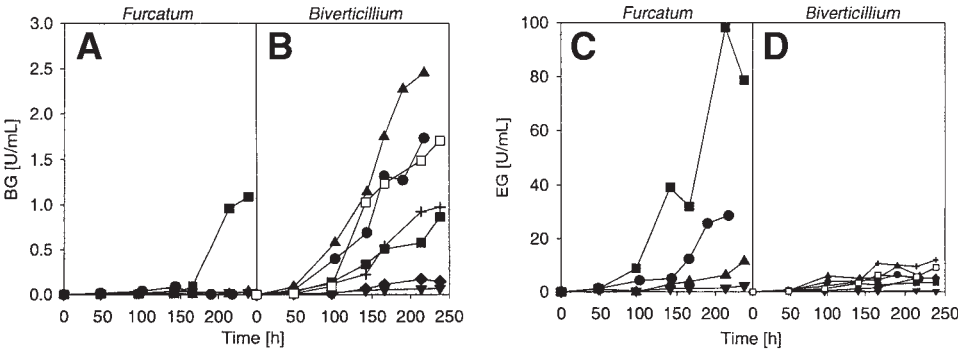

Fig. 4 BG activity during cultivation on cellulose for **(A)** subgenus *Furcatum* and **(B)** subgenus *Biverticillium*; and EG activity during cultivation on cellulose for **(C)** subgenus *Furcatum* and **(D)** subgenus *Biverticillium*. Subgenus *Furcatum*: (●) *P. persicinum* IBT 13226; (▼) *P. simplicissimum* IBT 13237; (▲) *P. simplicissimum* IBT 15303; (■) *P. brasilianum* IBT 20888. Subgenus *Biverticillium*: (●) *P. pinophilum* IBT 4186; (■) *P. funiculosum* IBT 5816; (▲) *P. pinophilum* IBT 10872; (▼) *P. rubicundum* IBT 10943; (◆) *P. aculeatum* IBT 18363; (+) *P. verruculosum* IBT 18366; (□) *P. minioluteum* IBT 21486.

Comparison of Enzyme Profiles in Subgenera Within Genus Penicillium

The genus *Penicillium* can be classified into different subgenera according to differences in morphology, physiology, and secondary metabolite production (Table 1). The *Penicillium* subgenus *Biverticillium* often occurs on wood, paper, and textile-related plant products *(28)*. By contrast, *P. brasilianum*, *P. simplicissimum*, and related species belong to the subgenus *Furcatum*, which often occurs in grassland soils *(6)*.

Comparisons of the cellulolytic activities resulting from growth on cellulose and xylanolytic activities resulting from growth on xylan for each subgenus showed that BG activity was higher for the subgenus *Biverticillium* than for the subgenus *Furcatum* during cultivation on cellulose (Fig. 4A, B). EG activity tended to be highest for the subgenus *Furcatum* during growth on cellulose (Fig. 4C, D). An investigation of the xylanolytic activities showed that six of seven fungi from the subgenus *Biverticillium* reached a higher BX activity than the maximum BX activity for the subgenus *Furcatum*. EX activity did not reveal any difference between the two subgenera (data not shown).

In the future, when a larger number of filamentous fungi in each subgenus have been screened for their production of cellulolytic and xylanolytic enzymes, a powerful tool to search for characteristics in the subgenera will be multivariate data analysis. In the search for an enzyme mixture with desirable properties for a given application, knowledge of enzyme activities characterizing each subgenus during growth on a given substrate can be valuable.

Other Screening Experiments

In the present study, the fungi were cultivated in submerged cultures, which can be habitats that are far from the conditions that each fungus has adapted to during evolution. Solid-state fermentation could very well be another interesting way to cultivate the microorganisms of interest for cellulase and hemicellulase production. It has earlier been shown that when *P. citrinum* was grown on rice husks, cellulolytic activity was three times higher in the solid-state fermentation than in the submerged culture *(29)*.

Conclusion

The filamentous fungi investigated showed coinduction of cellulolytic and xylanolytic enzymes. During growth on cellulose, products from the hydrolysis of cellulose also induced production of xylanolytic enzymes, and during growth on xylan, products from the hydrolysis of xylan also induced the production of cellulolytic enzymes.

FPA was used to evaluate how well suited filamentous fungi could be as a producer of cellulolytic enzymes. *P. brasilianum* IBT 20888 cultivated on cellulose resulted in the highest FPA, an activity that was even higher than the FPA resulting from growth of *T. reesei* Rut C30. *P. brasilianum* IBT 20888 was different from the other *Penicillium* species in the way that it produced almost an order of magnitude higher EG activity than any of the other species. Even though differences in single cellulolytic activities among the fungi were found, a linear correlation was observed between the amount of extracellular protein and the FPA measured after cultivation of each fungus on cellulose. The screening among the different filamentous fungi belonging to *Penicillium* showed that many species are interesting as producers of cellulolytic and xylanolytic enzymes, but further cultivation experiments need to be performed under more controlled conditions. Cultivations in shake flasks can be used as a relatively fast approach when screening many microorganisms for their production of cellulolytic and xylanolytic enzymes, but certain characteristics may not be apparent owing to the nature of shake flask cultivations.

References

1. Kheshgi, H. S., Prince, R. G., and Marland, G. (2000), *Annu. Rev. Energy Environ.* **25,** 199–244.
2. Wood, T. M. (1985), *Biochem. Soc. Trans.* **13,** 407–410.
3. Ryu, D. D. Y. and Mandels, M. (1980), *Enzyme Microb. Technol.* **2,** 91–102.
4. Holtzapple, M., Cognata, M., Shu, Y., and Hendrickson, C. (1990), *Biotechnol. Bioeng.* **36,** 275–287.
5. Tengborg, C., Galbe, M., and Zacchi, G. (2001), *Enzyme Microb. Technol.* **28,** 835–844.
6. Christensen, M., Frisvad, J. C., and Tuthill, D. E. (2000), in *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*, Samson, R. A. and Pitt, J. I., eds., Harwood Academic Publishers, London, UK, pp. 309–320.
7. van Wyk, J. P. H. (1999), *Biomass Bioenergy* **16,** 239–242.
8. Thygesen, A., Thomsen, A. B., Schmidt, A. S., Jørgensen, H., Ahring, B. K., and Olsson, L. (2003), *Enzyme Microb. Technol.* **32,** 606–615.

9 Castellanos, O. F., Sinitsyn, A. P., and Vlasenko, E.Y. (1995), *Bioresour. Technol.* **52,** 119–124.
10 Mandels, M. and Weber, J. (1969), *Adv. Chem. Ser*. **95,** 394–414.
11. Ghose, T. (1984), in *Measurement of Cellulase Activities*, Commission on Biotechnology, International Union of Pure and Applied Chemistry, Delhi, India, pp. 1–13.
12 Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
13 Bailey, M. J., Biely, P., and Poutanen, K. (1992), *J. Biotechnol.* **23,** 257–270.
14. Jørgensen, H., Eriksson, T., Börjesson, J., Tjerneld, F., and Olsson, L. (2003), *Enzyme Microb. Technol.* **32,** 851–861.
15 Deshpande, M. V., Eriksson, K.-E., and Pettersson, L. G. (1984), *Anal. Biochem.* **138,** 481–487.
16. Herbert, D., Phipps, P. J., and Strange, R. E. (1971), in *Methods in Microbiology*, vol. 5B, Norris, J.R. and Ribbons, D.W., eds., Academic Press, London, UK, pp 242–248.
17 Gronall, A. G., Bardawill, D. J., and David, M. M. (1949), *J. Biol. Chem.* **177,** 751–766.
18 Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
19 Schulz, G. and Hirte, W. F. (1989), *Zentralbl. Mikrobiol.* **144,** 81–96.
20 Margolles-Clark, E., Ilmén, M., and Penttilä, M. (1997), *J. Biotechnol.* **57,** 167–179.
21 van Peij, N. N. M. E., Gielkens, M. M. C., de Vries, R. P., Visser, J., and de Graaff, L. H. (1998), *Appl. Environ. Microbiol.* **64,** 3615–3619.
22 Brown, J. A., Collin, S. A., and Wood, T. M. (1987), *Enzyme Microb. Technol.* **9,** 176–180.
23 Chaabouni, S. E., Hadj-Taieb, N., Mosrati, R., and Ellouz, R. (1994), *Enzyme Microb. Technol.* **16,** 538–542.
24 Brown, J. A., Falconer, D. J., and Wood, T. M. (1987), *Enzyme Microb. Technol.* **9,** 169–175.
25 Decker, C. H., Visser, J., and Schreier, P. (2000), *J. Agric. Food. Chem.* **48,** 4929–4936.
26 Lin, J., Pillay, B., and Singh, S. (1999), *Biotechnol. Appl. Biochem*. **30,** 81–87.
27 Yun, S. I., Jeong, C. S., Chung, D. K., and Choi, H. S. (2001), *Biosci. Biotechnol. Biochem.* **65,** 2028–2032.
28. Pitt, J. I. (1979), *The genus* Penicillium *and its teleomorphic states* Eupenicillium *and* Talaromyces, Academic Press, London, UK.
29. Kuhad, R. C. and Singh, A. (1993), *World J. Microbiol. Biotechnol.* **1,** 100–101.