Microbiology, Vol. 74, No. 2, 2005, pp. 141–146. Translated from Mikrobiologiya, Vol. 74, No. 2, 2005, pp. 172–178.

EXPERIMENTAL ARTICLES

The Selection and Properties of *Penicillium verruculosum* Mutants with Enhanced Production of Cellulases and Xylanases

I. V. Solov'eva*,[1], O. N. Okunev*, V. V. Vel'kov*, A. V. Koshelev*, T. V. Bubnova*, E. G. Kondrat'eva, A. A. Skomarovskii**, and A. P. Sinitsyn****

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

***Moscow State University, Vorob'evy gory, Moscow, 119992 Russia*

Received December 16, 2003; in final form, March 23, 2004

Abstract—The paper describes three *Penicillium verruculosum* 28K mutants with about threefold enhanced production of five industrially important carbohydrases. The two-stage fermentation process that we developed provided a further two- to threefold increase in the production of carbohydrases. Physiological and biochemical studies showed that the synthesis of all five carbohydrases is inducible. Carboxymethylcellulase, xylanase, and β-glucanase are synthesized under a common regulatory control, as is evident from the concurrent increase in the synthesis of these enzymes in the presence of microcrystalline cellulose. The synthesis of avicelase and β-glucosidase is evidently induced by other cellulose- and hemicellulose-containing compounds present in the fermentation medium and, hence, is regulated independently of the three aforementioned enzymes.

Key words: cellulase, xylanase, mutants, fed-batch cultivation.

Microbial carbohydrases (cellulases and hemicellulases) that catalyze the hydrolysis of plant polysaccharides are industrially important enzymes that are used to saccharify industrial and agricultural cellulose-containing residues, treat cellulose pulp and paper wastes in the paper industry, bleach textiles (specifically, to remove excess dye and fuzz), enhance the extraction of fermentable substances in the beer brewing and alcohol fermentation industries, raise the nutritive value of feed in agriculture, etc. [1].

Although the ability to synthesize cellulases and hemicellulases is widely spread among microorganisms, only some are able to synthesize these carbohydrases in industrially important amounts. These include representatives of the fungal genera *Trichoderma, Sporotrichum (Phanerochaete), Fusarium, Humicola, Talaromyces*, and *Penicillium* [2]. There is evidence that *Penicillium* fungi are efficient producers of carbohydrases. In particular, the mutant *Penicillium occitanis* Pol 6 produces cellulases and pectinases [2, 3], *Penicillium pinophilum* NTG III/6 produces cellulase, xylanase, and β-glucosidase [4], *Penicillium funiculosum* Cu-1 synthesizes cellulase and xylanase [5], and *Penicillium canescens* RTM-22 produces xylanase [6].

Unlike *Trichoderma* fungi, those of the genus *Penicillium* synthesize more balanced cellulolytic complexes, which efficiently degrade cellulose and cellulose-containing residues [7]. In addition, the enzymes produced by *Penicillium* fungi are technologically more stable [8]. For this reason, the selection of *Penicillium* sp. strains with enhanced production of cellulolytic enzymes is of great scientific and practical interest.

This work was undertaken to select *Penicillium verruculosum* 28K mutants with enhanced production of cellulolytic enzymes and study their physiological and biochemical properties.

MATERIALS AND METHODS

Microorganisms. The microorganisms studied in this work were the laboratory strain *Penicillium verruculosum* 28K and its mutants, which were derived by means of multistage mutagenesis and subsequent selection. The strains were maintained on glucose–potato agar slants at 4°C.

Mutagenesis and selection. Fungal spores were suspended in distilled water containing 0.1% Tween-80 and exposed to ultraviolet radiation for 3 min. The appropriate dilutions of the control (unirradiated) and irradiated spores were plated onto glucose–potato agar. The plates were incubated at 28°C for 48 h and exam-

[1] Corresponding author. E-mail: solo@ibpm.pushchino.ru

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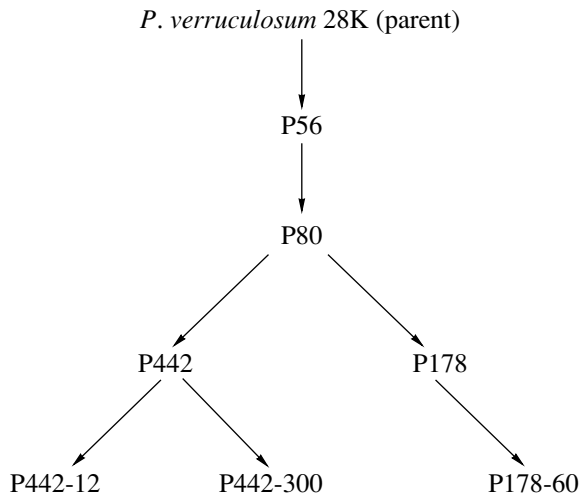

A scheme illustrating the selection of *P. verruculosum* 28K mutants.

ined for the number of grown colonies, after which the survival rate of mutants was calculated. A survival rate between 1 and 5% was considered to be sufficient for mutant selection. All surviving clones were transferred to selective agar plates, which contained either 0.1% Na carboxymethylcellulose (CMC) or 1% xylan, and incubated at 28°C for 48 h. CMC and xylan were purchased from Sigma (United States).

The cellulolytic activity of mutant clones was determined by their ability to grow and produce clear zones of CMC hydrolysis around the colonies. To estimate the diameter of the zone of CMC hydrolysis, plates with grown colonies were flooded with a 0.1% solution of Congo red and incubated at room temperature for 15 min. The staining solution was then decanted, and the plates were flooded with 1 M NaCl and incubated at room temperature for the next 10 min [9]. Mutants with enhanced synthesis of CMCase were selected based on the criterion of the maximum ratio of the CMC hydrolysis zone diameter (D2) to that of the colony (D1).

The xylanase activity of mutant clones was determined and hyperxylanolytic mutants were selected in essentially the same manner as in the previous case, except that the agar plates contained 1% xylan instead of 0.1% CMC.

Testing of strain sensitivity to catabolite repression. Clones were grown at 28°C for 48 h on agar plates containing 0.1% CMC and 2% glucose. Clones with enhanced resistance to catabolite repression were selected based on the criterion of the maximum ratio of the diameter of the CMC hydrolysis zone in the presence of glucose (D2) to that of the colony (D1).

Cultivation in flasks. The parent strain *P. verruculosum* 28K and its mutants were cultivated in 500-ml Erlenmeyer flasks with 100 ml of a complex medium containing (g/l) KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; yeast extract, 10.0; microcrystalline cellulose, 40.0; and wheat bran, 10.0. When required, the medium was supplemented with 20 g/l xylan and glucose. The flasks were inoculated with a vegetative mycelium, which was prepared by incubating fungal conidia for 48 h in the basal mineral medium supplemented with 10 g/l yeast extract and 5 g/l glucose. Cultivation was performed at 28°C on a shaker for 6 days. Then cells were removed by centrifugation, and the culture liquid was used to assay extracellular cellulolytic enzymes.

Strain cultivation in a fermenter. The parent and mutant strains were also cultivated in an ANKUM-2M fermenter with 7 l of the cellulose-containing complex medium. The cultivation temperature was 28°C. The pH of the medium was maintained within 4.5–5.0 by adding either HCl or NH_4OH; pO_2 was maintained at 25–30% of oxygen saturation by changing the rates of medium stirring and aeration intensity. During fed-batch cultivation, glucose was supplied to the fermenter at a specified rate. Culture samples were withdrawn at 12-h intervals, centrifuged, and the supernatants were analyzed for the activity of extracellular cellulolytic enzymes and protein content.

Enzyme assay. Cellulolytic enzymes were assayed as described by Sinitsyn *et al.* [10]. Reducing sugars were determined by the Somogy–Nelson method [11, 12]. CMCase, xylanase, β-1,3-glucanase, avicelase, and mannanase activities were determined with medium-viscosity CMC, birchwood xylan, barley β-glucan, Avicel, and mannan, respectively. One unit of the activity of these enzymes was defined as the amount of enzyme releasing 1 μmole of reducing sugars (expressed as glucose) from 0.5% substrate at 50°C at pH 5.0 per minute. α-Galactosidase and β-glucosidase were assayed with *p*-nitrophenyl-α-galactopyranoside and *p*-nitrophenyl-β-glucopyranoside, respectively [10]. One unit of the activity of these enzymes was defined as the amount of enzyme releasing 1 μmole of *p*-nitrophenol per 1 min at 40°C.

All the substrates were purchased from Sigma. The enzymes were assayed at least in triplicate. The data presented in the tables are the mean values.

RESULTS AND DISCUSSION

Selection of mutants with enhanced production of cellulases and xylanases. The cellulase- and xylanase-producing strain *P. verruculosum* 28K was selected from soil. To enhance the production of these enzymes, the strain was subjected to four cycles of UV mutagenesis and subsequent selection (figure), as a result of which several strains with enhanced production of cellulolytic enzymes were selected (Table 1).

The best mutants (P442-300, P442-12, and P178-60) produced three to four times more CMCase and xylanase than did the parent strain. The concurrent increase in the activity of these enzymes upon cultivation in the

presence of microcrystalline cellulose as the inducer suggests a common mechanism of their induction, as in the case of *Trichoderma reesei, Sclerotium rolfsii* [13], and *P. funiculosum* [14]. It should, however, be noted that the cellulases and xylanases of *Trichoderma harzianum* [15], *Aspergillus terreus* [16], and *Aspergillus foetidus* [13] are regulated independently.

The study and optimization of carbohydrase production by *P. verruculosum* 28K and its mutants. The cellulolytic activity of *P. verruculosum* 28K and its mutants was first studied upon their batch cultivation in an ANKUM-2M fermenter in the cellulose-containing complex medium. The cultivation temperature was 28°C, the pH of the medium was maintained within 4.5–5.0, and pO_2 was not less than 30% of oxygen saturation. After the cultivation was completed (144 h), the culture liquid was analyzed for extracellular cellulase and xylanase activities and protein content (Table 2).

As can be seen from this table, the production of cellulases and xylanases by the mutant strains grown in the medium containing cellulose and wheat bran increased correspondingly. In this case, the enhanced production of these enzymes was associated with the rise in the content of extracellular protein, which suggests an enhanced secretion of extracellular proteins, including cellulolytic enzymes, by the mutants.

The cellulase and xylanase activities of the mutants grown in a batch mode in the fermenter and Erlenmeyer flasks virtually did not differ. For general reasons, the production of cellulolytic enzymes by a fungus can be improved by increasing the biomass yield. This might be accomplished by adding an easily metabolizable carbon source (such as glucose) to the medium. However, this approach proves to be problematic, since the synthesis of cellulases and xylanases in mycelial fungi (including those of the genus *Penicillium*) is subject to catabolite repression by glucose [14, 17]. This difficulty can be surmounted by selecting mutants that are relatively resistant to catabolite repression.

Mutant clones of such a kind were selected based on the criterion of an increased ratio of the diameter of the clear zone of CMC (or xylan) hydrolysis (D2) around a colony to the diameter of this colony (D1), when clones were grown on agar plates containing 0.1% CMC (or 1% xylan) and 2% glucose. Analysis showed that the most active mutant strains, P442-300, P442-12, and P178-60, produced the maximum zones of CMC and xylan hydrolysis around the colonies (data not presented), suggesting that they are rather insensitive to catabolite repression by glucose.

Bearing this in mind, we attempted to improve the production of cellulases and xylanases by the mutants by cultivating them in the fermenter in a two-stage mode, the first stage being batch cultivation in the cellulose-containing complex medium and the second stage being fed-batch cultivation in the same medium with glucose. The total cultivation time was 144 h. The culture liquid was sampled at 12-h intervals and analyzed for protein content and the activities of cellulase, xylanase, and some other enzymes involved in the degradation of plant polysaccharides (Table 3).

Table 1. The extracellular CMCase and xylanase activities of *P. verruculosum* 28K and its mutants cultivated in flasks in cellulose-containing complex medium

P. verruculosum strain	CMCase		Xylanase	
	U/ml	%	U/ml	%
28K	56	100	105	100
P56	71	126	126	120
P80	125	223	172	163
P442	136	248	314	299
P442-12	187	335	287	273
P442-300	236	421	348	331
P178	141	251	234	223
P178-60	175	312	445	424

Table 2. Protein content and activities of various carbohydrases in culture liquids of *P. verruculosum* 28K and its mutants cultivated in a batch mode in a 10-l fermenter

P. verruculosum strain	CMCase, U/ml	CMCase, U/mg protein	Xylanase, U/ml	Xylanase, U/mg protein	Protein, mg/ml
28K	65	10.0	118	18.1	6.5
P56	86	11.3	135	17.7	7.6
P80	92	10.5	212	24.3	8.7
P442-12	283	13.4	480	22.8	21.0
P442-300	197	16.4	280	23.3	12.0
P178-60	228	13.9	429	26.1	16.4

A comparison of the data presented in Tables 2 and 3 showed that the two-stage fermentation process improved the production of CMCase, xylanase, and β-glucanase by the mutants by two- to threefold. The production of α-galactosidase did not increase. The mutant P442-12 produced 2.5 times more extracellular protein than did the parent strain (Table 3).

The specific activities of avicelase, α-galactosidase, and β-glucosidase in the parent and mutant strains were almost the same (Table 4), suggesting that the enhanced production of these cellulolytic enzymes during the two-stage fermentation process was due to the increased yield of biomass and extracellular proteins in the mutants. As for the specific activities of CMCase, xylanase, and β-glucanase, they were found to increase in all three active mutants (except for the CMCase of mutant P442-12) by 1.5–2.5 times, suggesting that the production of these enzymes by the mutants is induced by the microcrystalline cellulose present in the medium.

Table 3. Protein content and activities of various carbohydrases in culture liquids of *P. verruculosum* 28K and its mutants cultivated in a fermenter in a fed-batch mode with glucose

P. verruculosum strain	Protein, mg/ml	CMCase, U/ml	Xylanase, U/ml	Avicelase, U/ml	β-Glucanase, U/ml	α-Gal, U/ml	β-Gl, U/ml
28K	18.1	216	192	15	214	12	17
P442-12	47.0	496	844	37	742	10	61
P442-300	31.0	528	791	20	886	7	57
P178-60	25.0	523	637	27	697	7	47

Note: α-Gal and β-Gl are α-galactosidase and β-glucosidase, respectively.

Table 4. Protein content and specific activities of various carbohydrases in culture liquids of *P. verruculosum* 28K and its mutants cultivated in a fermenter in a fed-batch mode with glucose

P. verruculosum strain	Protein, mg/ml	CMCase, U/mg protein	Xylanase, U/mg protein	Avicelase, U/mg protein	β-Glucanase, U/mg protein	α-Gal, U/mg protein	β-Gl, U/mg protein
28K	18.1	11.9	10.6	0.82	11.8	0.66	0.93
P442-12	47.0	10.5	18.0	0.78	15.8	0.21	1.30
P442-300	31.0	17.0	25.5	0.64	28.5	0.22	1.83
P178-60	25.0	20.9	25.4	1.08	27.8	0.28	1.88

Note: α-Gal and β-Gl are α-galactosidase and β-glucosidase, respectively.

Table 5. Protein content and activities of various carbohydrases in culture liquids of *P. verruculosum* 28K and its mutants grown on different substrates

P. verruculosum strain	Medium or substrate	Protein, mg/ml	CMCase, U/ml	Xylanase, U/ml	Avicelase, U/ml	β-Glucanase, U/ml	Mannanase, U/ml	α-Gal, U/ml	β-Gl, U/ml
28K	Glucose, 2%	0.4	0	0	0.01	0.3	0	12	0.02
	CMC, 2%	2.0	34	79	0.3	26	2.1	10	0.007
	Xylan, 2%	0.6	0.2	15	0.15	0.3	0	22	0.49
	Complex medium	1.3	15	18	0.51	7.2	2.9	4.6	2.12
P442-12	Glucose, 2%	0.5	0.5	0.2	0.01	0.2	0.3	18	0.10
	CMC, 2%	2.8	52	101	0.47	48	0.6	13	0.006
	Xylan, 2%	1.0	2.9	22	0.15	15	0	25	0.56
	Complex medium	7.2	143	282	4.37	163	14.0	15	1.70
P178-60	Glucose, 2%	0.5	0	0.2	0.29	0.7	0	15	0.09
	CMC, 2%	2.3	35	97	0.50	50	1.7	13	0.01
	Xylan, 2%	0.9	2.5	22	0.30	3.5	0.7	26	0.78
	Complex medium	8.6	123	263	4.46	178	11.7	7.5	1.34
β442-300	Glucose, 2%	0.4	0.6	0.2	0.33	0.2	0.2	20	0.09
	CMC, 2%	2.2	51	89	0.37	48	0	14	0.02
	Xylan, 2%	1.2	3.9	23	0.35	2.1	0	28	0.5
	Complex medium	8.3	135	384	5.30	202	12.0	20	1.03

Note: α-Gal and β-Gl are α-galactosidase and β-glucosidase, respectively.

Table 6. Substrates capable of inducing synthesis of carbohydrases by *P. verruculosum* 28K and its mutants

Substrate	Enzymes induced
Glucose	α-Galactosidase
Microcrystalline cellulose	CMCase, xylanase, β-glucanase
Xylan	Xylanase, β-glucosidase
Wheat bran	Avicelase, mannanase, β-glucosidase

As mentioned above, the two-stage fermentation process in the medium with wheat bran, yeast extract, and microcrystalline cellulose as the inducer improved the production of extracellular CMCase, xylanase, β-glucanase, avicelase, and β-glucosidase by the mutants. An important question that arises here is whether these enzymes have a common or different mechanisms of regulation or, to be more specific, whether these enzymes have a common inducer or different inducers in the cellulose-containing complex medium.

To clarify this, the parent and three mutant strains (P442-300, P442-12, and P178-60) were grown in the basal mineral medium supplemented with glucose, microcrystalline cellulose, or oat xylan at a concentration of 2%. The control medium contained microcrystalline cellulose and wheat bran. After cultivation at 28°C for 144 h, the culture liquids of the strains were analyzed for seven carbohydrases (Table 5).

When the strains were grown in the medium with 2% glucose, the activities of six carbohydrases (except for α-galactosidase) were very low, presumably due to the absence of an exogenous inducer. Microcrystalline cellulose was found to be a common inducer for CMCase, xylanase, and β-glucanase. Xylan served as an inducer for xylanase (though less efficient than microcrystalline cellulose) and β-glucosidase. Consequently, the synthesis of CMCase, xylanase, and β-glucanase in the strains studied is under a common regulatory control, which explains the concurrent increase in their production in the presence of microcrystalline cellulose.

The synthesis of avicelase, mannanase, and β-glucosidase was regulated independently of the above enzymes, as is evident from the low production of these three enzymes in the mineral medium with microcrystalline cellulose. At the same time, the production of avicelase, mannanase, and β-glucosidase was high in the complex medium. All this suggests that the synthesis of these three enzymes is induced by the cellulose- and hemicellulose-containing substances present in wheat bran.

The synthesis of α-galactosidase was almost independent of the substrate used, except that the activity of this enzyme was approximately twice as high during the cultivation of all the strains on 2% xylan. It should be noted, however, that the specific activity of α-galactosidase did not increase. These findings indicate that the synthesis of α-galactosidase in the parent and mutant strains under study is constitutive.

The substrates that induce the synthesis of particular carbohydrases in the strains studied are listed in Table 6. In general, the synthesis of almost all carbohydrases (except for α-galactosidase) is inducible, which agrees with the data available in the literature [14, 17, 18].

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