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ISOLATION OF CELLULASE-PRODUCING MUTANTS FROM A *Penicillium* sp STRAIN DENOMINATED 3MUV24

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ABSTRACT

Three cellulase producing mutants were obtained from *Penicillium* sp strain 3MUV24. One of them arose spontaneously and the other two were obtained by treatment with UV-light (254 nm). Selection was performed on agar plates containing cellulose. Mutants were detected on the basis of production of large clear areas on the cellulose agar plates. The filter paper hydrolyzing activity and β-glucosidase production by one of the mutant strains were approximately two-fold and six-fold, respectively, when compared to the parental strain.

INTRODUCTION

Cellulase production at a reduced cost is one of the challenges faced by biotechnology. This has stimulated programs aiming at the genetic improvement of cellulase-producing strains (Montenecourt and Eveleigh, 1977; Bailey and Nevalainen, 1981; Shoemaker *et al.*, 1981; Joglekar and Karanth, 1984; Figueiredo *et al.*, 1985; Lachke *et al.*, 1986; Brown *et al.*, 1987; Goldman and Azevedo, 1989).

The present study was undertaken to obtain cellulolytic mutants of the 3MUV24 strain of *Penicillium* sp from the collection of wild and mutant cellulolytic strains of the Biotechnology Institute, University of Caxias do Sul.

MATERIAL AND METHODS

Strains

Strain 3MUV24 was used for the derivation of mutants 3MUV243, 3MUV2431 and 3MUV2434. Strain 3MUV24 is a mutant of the progeny of the 2HH strain of

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Penicillium sp, originally isolated from the gut of the coleopteran *Anobium punctatum* in Caxias do Sul, RS (Figure 1).

Culture and cellulase-production media

The media used for strain culture and storage (CM), for the selection of glucose-derepressed clones (HM) by the method of Montenecourt and Eveleigh (1977), and for cellulase production (PM), whose salt composition is identical to that of the medium of Mandel and Reese (1956), as described in Table I.

Table I - Composition of the media used.

Components	Media and amounts		
	CM	HM	PM
KH_2PO_4	2 g	2 g	2 g
$(NH_4)_2SO_4$	1.4 g	1.4 g	1.4 g
$CO(NH_2)_2$	0.3 g	0.3 g	0.3 g
$MgSO_4$-7 H_2O	0.3 g	0.3 g	0.3 g
$CaCl_2$	0.3 g	0.3 g	0.3 g
$FeSO_4$-7 H_2O	5 mg	5 mg	5 mg
$MnSO_4$-1 H_2O	1.56 mg	1.56 mg	1.56 mg
$ZnSO_4$-7 H_2O	1.4 mg	1.4 mg	1.4 mg
$CoCl_2$	2 mg	2 mg	2 mg
Peptone	1 g	1 g	1 g
Tween 80	2 ml	2 ml	2 ml
Oxgall	--	3.7 g	--
Agar	20 g	20 g	--
Filter paper (ground)	10 g	--	10 g
Swollen cellulose	--	10 g	--
Glucose	--	20 g	--
Distilled water	1 l	1 l	1 l

CM, Medium for conidium production and strain maintenance; HM, medium for the observation of hydrolysis haloes under conditions of catabolic repression, according to the method of Montenecourt and Eveleigh (1977), modified; PM, liquid medium for cellulase production.

The nutrient composition of the media was based on the medium used by Mandels and Reese (1957) for *Trichoderma viride*.

Mutagenesis

Seven-day conidia from colonies grown in test tubes containing CM slants at 28°C were used for mutagenic treatment. Suspensions of 1 x 10^7 conidia/ml were obtained by scraping cells from the tubes, followed by separation of conidia in 2 ml 0.1% Tween 90 solution and dissolving in 0.9% NaCl. Ten-ml volumes of conidial suspension were placed on Petri dishes and treated with UV-light (254 nm) from a Tohwalite-G15T8 lamp located at a distance of 30 cm from the cultures. Exposure times were 0, 30, 60, 90 and 120 seconds and a maximum mortality of 99% was obtained. After mutagenic treatment, the suspensions were appropriately diluted and spread on plates containing HM medium for the isolation of glucose-derepressed colonies, which were identified as those producing haloes under repressed conditions.

Cellulase production

To evaluate the cellulolytic potential of the selected clones, the strains were tested for cellulase excretion in shaken flasks. One-week old conidia grown in test tubes containing CM were used as inocula distributed among 500-ml flasks containing 100 ml PM medium, at a final concentration of 1 x 10^5 conidia/ml. The flasks were kept under constant shaking at 180 rpm at 28°C for eight days. Samples of the enzyme solutions were obtained by filtering the liquid culture through filter paper, 0.005% sodium azide was added and they were stored in the refrigerator.

Enzyme measurement

The enzyme filtrates were used for FPA determination according to the method of Mandels *et al.* (1976) and salicin was used for the determination of β-glucosidase activity (Wase and Raymahsay, 1986). For FPA determination, 0.5 ml enzyme solution, 1 ml sodium citrate buffer, pH 4.8, and 50 mg filter paper (Whatman no. 1) were maintained in a test tube at 50°C for sixty minutes. The reaction was stopped by the addition of 3 ml DNS reagent (Miller, 1959), followed by boiling in a water bath for five minutes. The colored solution was quantified after natural cooling, in a spectrophotometer at 545 nm. For the determination of salicinase activity, 0.5 ml of 1% salicin in 0.05 M sodium citrate buffer, pH 4.8, was placed in a test tube to which 0.5 ml enzyme solution was added. After 30 minutes of hydrolysis at 50°C, the reducing sugars released were measured as was done for FPA, using the DNS reagent. FPA units (U/ml) and salicinase units (U/ml), β-glucosidase which represents activity are defined as the amount of enzyme in 1 ml which releases 1 μmol of reducing sugars (RS)/minute. When the enzyme solutions liberated more than 2 mg RS from the filter paper or 0.6 mg from

salicin under the assay conditions, they were diluted and the dilution needed to release 2 mg RS from filter paper and 0.6 mg from salicin was estimated.

The results obtained for each mutant were compared statistically using the hypothesis test for a difference between two groups of means, using the Microsta program.

RESULTS AND DISCUSSION

In the present study, mutagenesis and screening were used to obtain variants of cellulase-producing clones (Figure 1), after isolation of a spontaneous mutants with pink conidia from one of the control plates in experiments of mutagenesis with UV-light irradiation using strain 3MUV24. From this spontaneous mutant, which was called 3MUV243, we obtained clones 3MUV2431 and 3MUV2434, using UV-light and HM. These clones were selected because of their ability to produce larger areas of clearing on HM when compared to the remaining colonies grown on the plates.

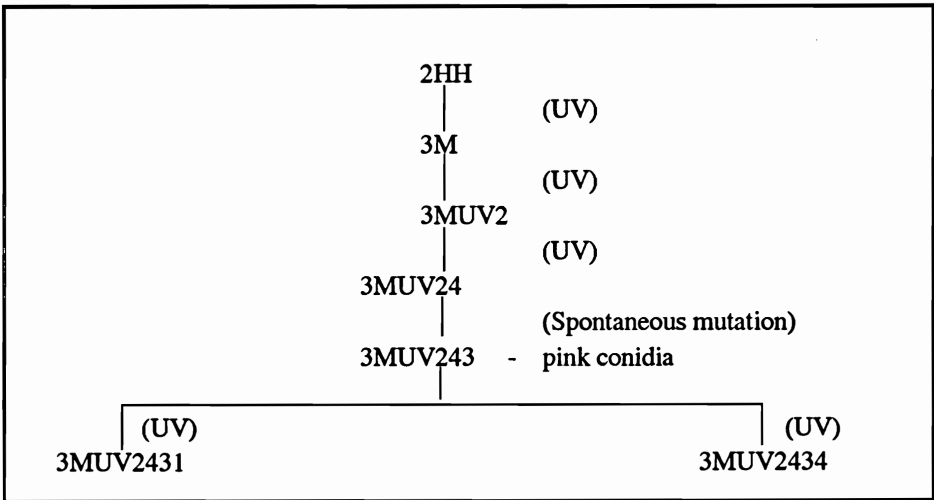

Figure 1 - Phylogeny of *Penicillium* sp 2HH mutants. UV-light (UV-light (254 nm).

After the flasks were shaken, it was possible to differentiate the strains in terms of the enzymatic activity of their filtrates on filter paper and salicin. As shown in Tables II and III, cellulase production by strain 3MUV24 and its mutant 3MUV243 (pink conidia) did not differ in terms of FPA or salicinase activity. The results obtained with strains 3MUV2431 and 3MUV2434 showed that these strains excreted more cellulases (FPU) than the parental strain, and that strain 3MUV2434 was the better FPA producer of the two.

Table II - Cellulase production (filter paper hydrolyzing activity - FPA) in liquid culture[a] by *Penicillium* sp 2HH mutants.

Strain	Filter paper hydrolyzing activity[b] (U/ml)			
	Flask 1	Flask 2	Mean ± SD	P[c]
3MUV24	0.67	0.51	0.590 ± 0.113	
3MUV243	0.56	0.60	0.580 ± 0.028	> 0.05
3MUV2431	1.00	1.03	1.015 ± 0.021	< 0.05
3MUV2434	1.37	1.23	1.300 ± 0.098	< 0.05

[a] Liquid cultures were maitnained at 28°C for eight days with shaking at 180 rpm in 500-ml flasks containing 100 ml PM.

[b] See text for details about FPU determination.

[c] Probability that the values obtained for the mutant belong to the same distribution as the values obtained for strain 3MUV24 (see text).

Table III - Salicinase production in liquid culture[a] by *Penicillium* sp 2HH mutants.

Strain	Enzymatic activity towards salicinase (U/ml)[b]			
	Flask 1	Flask 2	Mean ± SD	P[c]
3MUV24	0.07	0.04	0.050 ± 0.021	
3MUV243	0.10	0.09	0.095 ± 0.007	> 0.05
3MUV2431	0.09	0.10	0.095 ± 0.007	> 0.05
3MUV2434	0.62	0.58	0.600 ± 0.028	< 0.01

[a] Liquid cultures were maintained at 28°C for eight days with shaking at 180 rpm in 500-ml flasks containing 100 ml PM.

[b] See text for details about salicinase activity determination.

[c] Probability that the values obtained for the mutant belong to the same distribution as the values obtained for strain 3MUV24 (see text).

The salicinase values listed in Table III show that there was no difference in enzymatic activity towards salicin between strains 3MUV24 and 3MUV243 or between strains 3MUV24 and 3MUV2431. However, salicinase values were six times greater for strain 3MUV2434 than for the parental strain MUV243 ($T = -21.8000$; $P < 0.01$).

As shown in Table IV, cellulase production (FPA) by mutant 3MUV2434 was comparable to that observed in other cellulolytic strains (Mandels *et al.*, 1974; Tangnu *et al.*, 1981; Joglekar and Karanth, 1984; Duff *et al.*, 1985; Saddler *et al.*, 1985; Brown *et al.*, 1987).

Table IV - Enzyme production (FPU)* by strain 3MUV2434 and other cellulolytic strains.

Strain	Filter paper hydrolyzing activity (FPA, IU/ml)** Carbon sources (10 g/l)				
	Barley chaff	Solka floc	Cellulose CF11	Ground filter paper	Avicel
Trichoderma reesei					
Qm6a	0.44[a]	---	---	---	---
QM9414	1.28[a]	1.48[f]	---	---	2.04[f]
RUT-NG14	0.98[a]	---	---	---	---
RUT-C30	1.14[a]	1.70[b] 3.10[d]	2.50[e]	---	2.40[d]
Trichoderma harzianum					
E58	---	2.20[b]	---	---	---
Penicillium pinophilum					
JAB/3B/15	2.08[a]	0.98[a]	---	---	---
JAB/3C/16	2.00[a]	0.70[a]	---	---	---
Penicillium funiculosum					
UV-49	---	1.20[c]	---	---	0.80[c]
Penicillium sp					
3MUV2434	---	---	---	1.30[g]	---

* Values obtained in different studies in which the medium of Mandels and Reese (1961) was used.

**FPU was measured by the method of Mandels *et al.* (1976).

[a] Brown *et al.* (1987); [b]Saddler *et al.* (1985); [c]Joglekar and Karanth (1984), who used induction with 2.5% Solka Floc; [d]fermentation value obtained by Tangnu *et al.* (1981) with pH and temperature control; [e]Duff *et al.* (1985); [f]Mandels *et al.* (1974); [g]present study. ---, Not used in the paper consulted.

The present data show that mutagenesis and screening can be applied to increase cellulase production by *Penicillium* sp strains maintained at the Biotechnology Institute of Caxias do Sul. We did not use the high substrate concentrations common to other studies (Ryu and Mandels, 1980; Mandels, 1982; Warzywoda *et al.*, 1983; Lanchke *et al.*, 1986; Brown *et al.*, 1987). Thus, the filter paper hydrolyzing activity and salicinase activity obtained for enzymatic filtrates of mutant 3MUV2434 place this strain in a position comparable to that of other currently available cellulolytic mutants, with a good potential for industrial use in cellulase production.

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RESUMO

Três mutantes celulolíticos foram obtidos de uma linhagem de *Penicillium* sp denominada 3MUV24, um espontâneo e dois após mutagênese com luz ultravioleta (254 nm). A seleção dos mutantes foi desenvolvida em placas contendo meio sólido com celulose. Os mutantes foram detectados como aqueles que produziram áreas maiores de clareamento nas placas de celulose. A produção de FPA (atividade de hidrólise em papel de filtro) e β-glicosidase por uma das linhagens mutantes, desenvolvida a 28°C em frascos agitados de 500 ml com 100 ml de meio, foi aproximadamente 2 vezes e 6 vezes mais alta respectivamente, do que a linhagem parental.

REFERENCES

Bailey, M.J. and Nevalainen, K.M.H. (1981). Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microb. Technol. 3*: 153-157.

Brown, J.A., Falconer, D.J. and Wood, T.M. (1987). Isolation and properties of mutants of the fungus *Penicillium pinophilum* with enhanced cellulase and β-glucosidase production. *Enzyme Microb. Technol. 9*: 169-175.

Duff, s.J.B., Cooper, D.V. and Fuller, O.M. (1987). Effect of media composition and growth conditions on production of cellulase by a mixed fungal fermentation. *Enzyme Microb. Technol. 9*: 47-52.

Goldman, G.H. and Azevedo, J.L. (1989). Melhoramento genético de microrganismos produtores de celulase. *Cienc. Cult. 41*: 229-240.

Figueiredo, D.M.M., Pavani, B.S.C. and Thiemann, J.E. (1985). Método em placa para seleção de linhagens celulolíticas alto-produtoras. *XII Reunião Anual de Genética de Microrganismos*, Ouro Preto, MG, Brasil.

Joglekar, A.N. and Karanth, N.G. (1984). Studies on cellulase production by a mutant *Penicillium funiculosum* UV-49. *Biotechnol. and Bioeng. 26*: 1079-1084.

Lachke, A.H., Bastawde, K.B., Powar, V.K. and Srinivasan, M.C. (1986). Isolation of hypercellulolytic mutant (CU-1) of *Penicillium funiculosum. Enzyme Microb. Technol. 8*: 105-108.

Mandels, M. (1982). *Cellulases*. In: *Anual reports on fermentation processes* (Tsao, G.T., ed.). Academic Press, pp. 35-78.

Mandels, M., Hontz, L. and Nystrom, J. (1974). Enzymatic hydrolysis of waste cellulose. *Biotechnol. and Bioeng. 26*: 1471-1493.

Mandels, M., Andreotti, R. and Roche, C. (1976). Measurement of saccharifying cellulase. *Biotechnol. and Bioeng. Symp. 6*: 21-33.

Mandels, M. and Reese, E.T. (1957). Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. *J. Bacteriol. 73*: 268-278.

Montenecourt, B.S. and Eveleigh, D.E. (1977). Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol. 34*: 77-782.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry 31*: 426-428.

Ryu, D.D.Y. and Mandels, M. (1980). Cellulases: Biosynthesis and applications. *Enzyme Microb. Technol. 2*: 91-102.

Saddler, J.N., Hogan, C.M. and Louis-Seize, G. (1985). A comparison between the cellulase systems of *Trichoderma harzianum* E58 and *Trichoderma reesei* C30. *Appl. Microbiol. Biotechnol. 22*: 139-145.

Tangnu, S.K., Blanch, H.W. and Wilke, C.R. (1981). Enhanced production of cellulases, hemicellulase, and β-glucosidase by *Trichoderma reesei* (Rut C-30). *Biotechnol. and Bioeng. 23*: 1837-1849.

Shoemaker, S.P., Raymond, J.C. and Bruner, R. (1981). Cellulase: Diversity amongst improved *Trichoderma* strains. In: *Trends in the biology of fermentations for fuels and chemicals* (Hollander, A., ed.). Basic life Science. Plenum Publishing Company, 18 pp. 89-109.

Warzywoda, M., Vandecasteele, J.P. and Pouquié, J. (1983). A comparison of genetically improved strains of the cellulytic fungus *Trichoderma reesei. Biotechnol. Lett. 5*: 243-246.

Wase, D.A., Raymahasay, S. and Green, S. (1986). Inhibitor of B-D-glucosidase and endo-1,4-B-D-glucanase produced by *Aspergillus fumigatus* IMI255091. *Enzyme Microb. Technol. 8*: 48-52.

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