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USE OF MUTATION STRATEGIES APPLIED TO *ASPERGILLUS TERREUS* ATCC 52430 TO OBTAIN MUTANTS WITH IMPROVED CELLULASE PRODUCTIVITY

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SUMMARY

Mutation studies were carried out on *Aspergillus terreus* ATCC 52430 to increase cellulase production. Optimal treatment conditions for increasing cellulase activity by UV mutagenesis were observed by exposure of spores to a UV germicidal lamp, wavelength 250 nm at a distance of 30 cm for 1 min. Nitrosoguanidine mutagenesis was optimal with respect to development of high cellulase mutants when spores were exposed to 500 ug/ml nitrosoguanidine for 1 h. Mutants were isolated on agar containing Walseth cellulose and the catabolite repressor, glycerol. Successive UV and nitrosoguanidine treatments resulted in isolation of strain UNG1-40 having 3.5, 4.6 and 3.3-fold increases in filter paper, ß-glucosidase and carboxymethyl cellulase activity, respectively, compared to *A. terreus* ATCC 52430.

INTRODUCTION

A major barrier to the application of cellulase for conversion of cellulose to glucose is the high cost of the enzyme (Wilke *et al.,* 1976). Although *Trichoderma* cellulases have been most effective in hydrolysing crystalline cellulose, the predominant end product is cellobiose and not glucose (Ward and Moo-Young, 1989). *Trichoderma* is not a strong producer of ß-glucosidase (ß-GLCase) which is susceptible to end-product inhibition by glucose (Ward, 1985). ß-GLCase is additionally important in the hydrolysis process in that it aids removal of cellobiose which inhibits the other cellulase components. *Aspergillus* species are better sources of ß-GLCase than *Trichoderma* and their ß-GLCases are less susceptible to end-product inhibition. *A. terreus* ATCC 52430, isolated from wood, exhibited an increased capacity to produce ß-GLCase as compared to known cellulase producers (Araujo and D'Souza, 1980). This organism and its cellulolytic enzymes have been extensively investigated (Araujo and D'Souza, 1986a,b; D'Souza and Volfova, 1982). In this paper, we report the results of a programme aimed at developing improved cellulase producers by mutation of this isolate.

MATERIALS AND METHODS

***Culture maintenance*:** The organism was maintained on modified *T. viride* medium (Mandels and Weber, 1969) containing 2% w/v agar and Whatman No.1 filter paper.

UV mutagenesis: A spore suspension from culture slants, stored at 4°C for 12-15 d, was prepared in distilled water containing 0.01% (v/v) Tween 80. Fragments of mycelia were removed by filtration through sterilised cotton wool filters. Numbers of spores were determined in a haemocytometer and adjusted to 10^6/ml. This suspension (5 ml) was exposed in a petri dish, with occasional shaking, to a UV lamp, wavelength 250 nm for 30-300s at distances of 15-60 cm from the lamp.

Nitrosoguanidine mutagenesis: A spore suspension was prepared in 0.02 M sodium acetate, pH 5.6, containing 0.01% (v/v) Tween 80 and mixed, filtered and counted as for UV mutagenesis. 10 ml of the spore suspension, diluted with acetate buffer to give a count of 10^7-10^8/ml, was added to a 100 ml Erlenmeyer flask and treated with nitrosoguanidine at 300-1000 µg/ml. Flasks were shaken and incubated at 30°C for 30-180 min. Samples (1 ml) were diluted with 9 ml acetate buffer, centrifuged at 10,000 rpm for 15 min. and washed to remove nitrosoguanidine. A control suspension containing no nitrosoguanidine was treated in a similar manner.

Isolation of cellulase producing mutants: Immediately following mutagenesis, spores were serially diluted up to ten fold in distilled water and plated on modified *T. viride* medium, with 2% agar, supplemented with 0.5% Walseth cellulose (Walseth, 1952) as carbon source and 5% glycerol as catabolite repressor. To determine the percentage of survivors, spores were plated on the isolation medium without glycerol. Petri dishes were wrapped in aluminium sheets to reduce photoreactivation and incubated for 2.5-3 d at 30°C. Plates were then incubated at 50°C for 18-20 h to accelerate development of clearing zones around cellulase producing colonies. Colonies with the greatest diameters of zone clearance were tested for cellulase productivity.

Cellulase productivity testing: The modified *T. viride* medium supplemented with 1% (w/v) microcrystalline cellulose, 0.01% (v/v) Tween 80 and 1 ml/l trace metal solutions (Araujo and D'Souza, 1980) was used for screening cellulase mutants. 500 ml Erlenmeyer flasks containing 100 ml of this medium were inoculated with 3-3.5 x 10^8 spores and cultivated at 30 °C on a rotary shaker set at 180 rpm for 5 d. Cellulase assays were then carried out on culture cell free supernatants using the filter paper (FPase) and carboxymethyl cellulase (CMCase) assays (Mandels *et al.*, 1976) and a ß-GLCase assay (Andreotti *et al.*, 1977).

RESULTS

The effect of exposure time and distance from the UV source on spore survival was investigated. The data were plotted to give a logarithmic regression and the best fit curves were computed. The percentages of viable spores after 30 and 60 s were 16.4% and 1.7% respectively (Fig. 1A). Percentages of surviving spores after a 60s exposure at a distance of 30 and 60 cm from the UV source were 0.15% and 18% respectively (Fig. 1B). Preliminary selection of mutants was from treated samples which gave up to 10% survival., Four UV mutants from each treatment were selected and evaluated for cellulase productivity in shake flasks (Table 1). A 30 cm distance and 60 s exposure to UV gave higher frequencies of mutations and isolated mutants manifested highest average cellulase activities. Subsequent UV mutations were carried out at 30 cm distance from the UV lamp for 60 s. Survival curves of spores after treatment with 300-1000 µg/ml NTG for 0-4 h were also prepared (Fig. 2). As the concentration of NTG or treatment time was increased, surviving spore numbers were reduced. NTG mutants, which gave up to 10% survival after a 1 h treatment, were tested for cellulase activity in shake flasks (Table 2). An NTG concentration of 500 µg/ml (1h treatment) produced strains with higher cellulase activity and these conditions were used for subsequent NTG mutations.

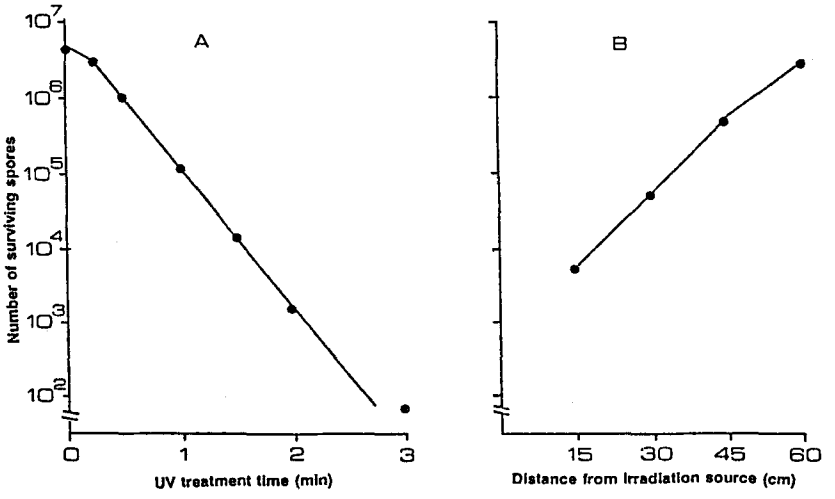

Figure 1 Survival curve of *A. terreus* ATCC 52430 spores with UV treatment at
A. Fixed distance from the UV source and different time intervals
B. At a constant time interval and at different distances from the UV source

Table 1 Quantitative evaluation of mutants prepared from *A. terreus* ATCC 52430 by UV irradiation at different time intervals and at various distances from the UV source

Distance from the UV lamp (cm)	Time of irradiation (s)	Cellulase activity (u/ml) Avg activities of 4 isolated mutants		
		FPase	ß-GLCase	CMCase
30	30	0.52	0.84	8.7
30	60	0.61	1.02	9.5
30	90	0.46	0.90	9.4
15	60	0.47	0.93	8.0
45	60	0.52	0.96	9.2
Control *A. terreus* ATCC 52430	60	0.28	0.33	5.2

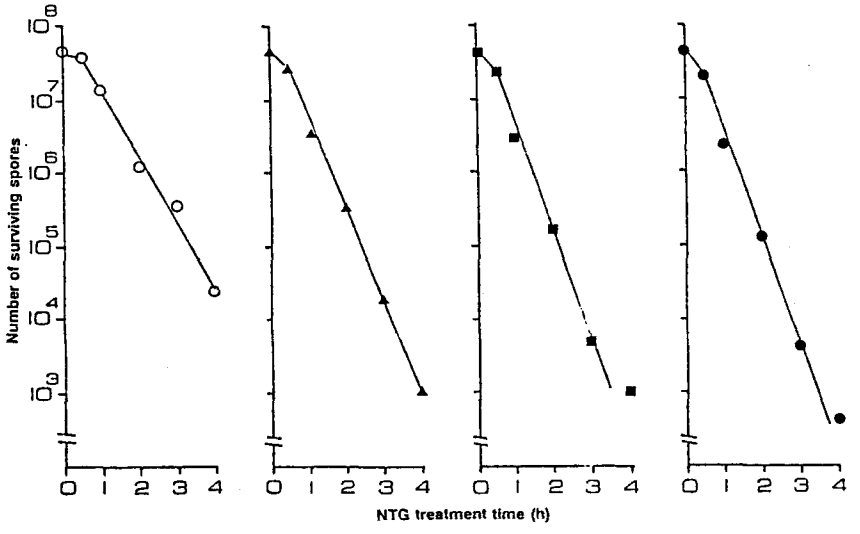

Figure 2 Survival curves of *A. terreus* ATCC 52430 spores treated with various NTG concentrations ○, 300 µg/l; ▲, 500 µg/l; ■, 750 µg/l; ●, 1000 µg/l.

Table 2 Quantitative evaluation of mutants prepared from *A. terreus* ATCC 52430 using various concentrations of NTG (treatment time 1 h)

NTG concentration (μg/ml)	Cellulase activity (U/ml) Avg activities of 5 isolated mutants		
	FPase	ß-GLCase	CMCase
300	0.50	0.90	9.3
500	0.61	1.14	11.0
750	0.50	0.98	9.3
1000	0.47	0.89	8.8
Control *A. terreus* ATCC 52430	0.28	0.33	5.2

A combination of mutation steps with selection of catabolite derepressed mutants was used to develop higher cellulase producing strains (Table 3). Following three UV irradiation and mutant isolation steps, one strain, UV3-25, showed a 2.4, 3.4 and 2.3-fold increase in FPase, ß-GLCase and CMCase respectively when compared with *A. terreus* ATCC 52430. Further UV mutation did not increase activity. Mutation of UV3-25 with NTG produced an isolate, UNG1-40, which exhibited 3.5, 4.6 and 3.3-fold increases in FPase, ß-GLCase and CMCase respectively, compared to *A. terreus* ATCC 52430. Further NTG treatment did not increase activity. In another series, mutant NG1-8, prepared by NTG treatment of *A. terreus* ATCC 52430, exhibited increases of 2.3, 3.6 and 2.3-fold in FPase, ß-GLCase and CMCase respectively. Further mutation of NG1-8 with NTG did not increase activity.

Table 3 Summary of the mutation programme

	Enzyme activity (U/ml)			
	FPase	ß-GLCase	CMCase	
Avg activities of 27 isolates (range)	0.46 (0.25-0.66)	0.73 (0.30-1.2)	9.7 (5.4-12.2)	
Mutant NG1-8	0.66	1.20	12.2	
↑ NTG				
***A. terreus* ATCC 52430**	**0.28**	**0.33**	**5.2**	**Starting strain**
↓ UV				
Avg activities of 26 isolates (range)	0.36 (0.24-0.55)	0.49 (0.30-0.85)	7.7 (4.3-9.3)	
Mutant UV1-12	0.55	0.85	9.3	
↓ UV				
Avg activities of 18 isolates (range)	0.54 (0.30-0.64)	0.89 (0.50-1.06)	9.2 (7.2-10.6)	
Mutant UV2-17	0.62	1.06	10.6	
↓ UV				
Avg activities of 30 isolates (range)	0.61 (0.48-0.68)	0.99 (0.6-1.12)	11.0 (8.6-12.6)	
Mutant UV3-25	0.68	1.12	12.6	
↓ NTG				
Avg activities of 42 isolates (range)	0.81 (0.40-0.97)	1.17 (0.86-1.55)	14.0 (8.1-17.0)	
Mutant UNG1-40	**0.97**	**1.55**	**17.0**	

The plate clearing assay proved useful for mutant selection. From 680 colonies screened following UV treatment, and 450 screened following NTG treatment, 95 and 57 colonies were isolated, respectively, which exhibited markedly wider zones of clearance on the isolation medium than the parent strain. Colonies of the parent strain and UNG1-40, illustrate these differences. In Figure 4, zones of cellulose clearing by *A. terreus* ATCC 52430 grown on Walseth cellulose medium without repression are illustrated. Glycerol, which acts as a catabolite repressor of cellulase production by the wild-type strain, was used to isolate derepressed mutants. The larger zones observed with UNG1-40, grown on glycerol-supplemented medium are illustrated in Figure 5.

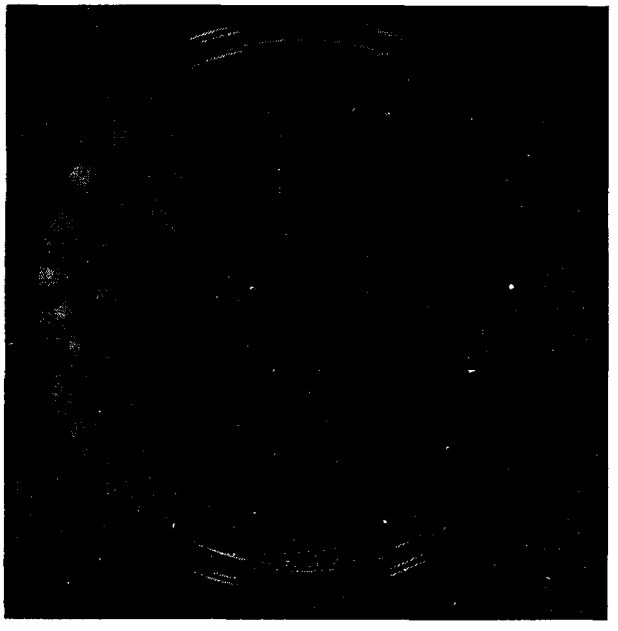

Figure 4 Zones of cellulose clearing by *A. terreus* ATCC 52430 wild-type grown on Walseth cellulose medium without repression

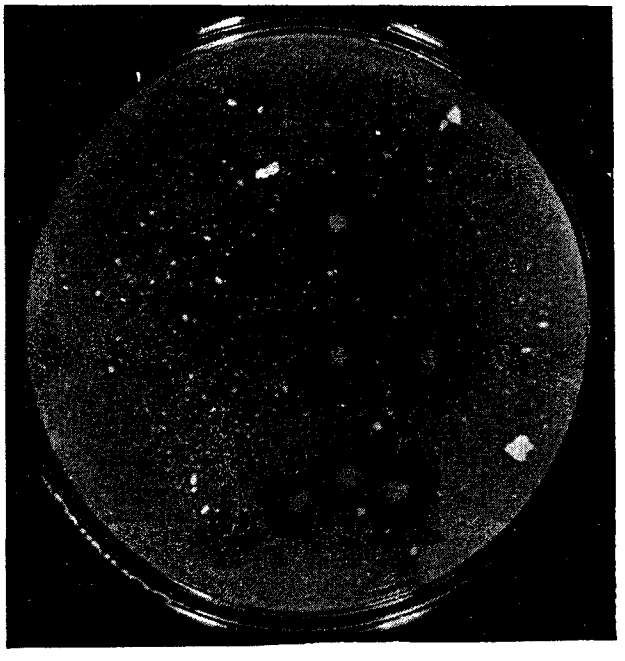

Figure 5 Zones of cellulose clearing by *A. terreus* mutant UNG1-40 grown on glycer supplemented Walseth cellulose medium

DISCUSSION

Many microbial cellulases are induced by cellulose but catabolite repressed by glucose and glycerol or their analogues (Ryu and Mandels, 1980). In general, high yielding constitutive cellulase mutants have not been reported (Allen and Roche, 1989). We found glycerol incorporation into cellulose-containing screening media to be an effective means of isolating catabolite repression resistant, overproducing cellulase mutants of *A. terreus*. High cellulase producing mutants of *Trichoderma* have been prepared by UV irradiation and/or NTG treatment using cellulose plate clearing isolation procedures (Montenecourt and Eveleigh, 1977; Nevalainen *et al.*, 1980). A visual plate detection system using esculin and ferric ions was used to identify *T. reesei* mutants with increased ß-GLCase activity (Szczodrak, 1989). Similar methods were used to obtain high cellulase producing mutants of *Penicillium funiculosum* (Joglekar and Karanth, 1984), *Myceliophtora thermophila* (Sen and Chakrabarty, 1984), *Sporotrichum pulverulentum* (Eriksson and Johnsrud, 1983) and *Talaromyces emersonii* (Moloney *et al.*, 1983). Fennington *et al.*, (1984) employed ethylmethane sulphonate in combination with UV light to develop mutants of *Thermomonospora curvata* while Macris (1984) used gamma irradiation to produce high CMCase and ß-GLCase producing mutants from *Alternaria alternata*. The non-metabolizable catabolite repressor, 2-deoxy-D-glucose, has been incorporated into some cellulase mutant isolation media (Coughlan, 1985).

The cellulases of *A. terreus* efficiently saccharify pretreated rice straw (Araujo and D'Souza 1986a). Application studies indicate that these strains may be good candidates as future sources of industrial cellulases. Further use of genetic techniques, combined with culture studies to maximise cellulase production, should facilitate a proper evaluation of the economic potential of this cellulase source.

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