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Mutation of *Aspergillus niger* for hyperproduction of citric acid from black strap molasses

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Summary

Spore suspensions of *Aspergillus niger* GCB 75, which produced 31.1 g/l citric acid from 15% sugars in molasses, were subjected to u.v.-induced mutagenesis. Among three variants, GCM 45 was found to be the best citric acid producer and was further improved by chemical mutagenesis using NTG. Out of 3 deoxy-D-glucose-resistant variants, GCM 7 was selected as the best mutant which produced 86.1 ± 1.5 g/l citric acid after 168 h of fermentation of potassium ferricyanide + H_2SO_4-pretreated black strap molasses (containing 150 g sugars/l) in Vogel's medium. On the basis of comparison of kinetic parameters, namely the volumetric substrate uptake rate (Q_s), and specific substrate uptake rate (q_s), the volumetric productivity, theoretical yield and specific product formation rate, it was observed that the mutants were faster growing organisms and had the ability to overproduce citric acid.

Introduction

Citric acid fermentation is one of the largest biotechnological industries (Rohr 1998). Citric acid is a primary product of *Aspergillus niger*'s metabolism. It is used in several industries (Bennett & Klich 1992). According to the latest estimates, citric acid produced through fermentation amounts to 700,000 tons/annum and its demand is ever increasing (Shoukat *et al.* 1997).

The techniques of u.v.-, gamma ray-induced or chemical (NTG) mutagenesis have long been accepted as routine methods to improve the yield of citric acid by *A. niger* (Gupta & Sharma 1995). The present work describes the isolation of mutants of *A. niger* for hyperproduction of citric acid through u.v. irradiation followed by NTG-mutagenesis. The best mutants were compared with the parental strain for production of citric acid following fermentation of clarified black strap molasses.

Materials and Methods

Organism

Aspergillus niger strain GCB 75, collected from Government College Culture Collection, was maintained on potato-dextrose agar slants and stored at 5 °C in a refrigerator. All the media, unless otherwise stated, were sterilized at 1.05 kg/cm^2 pressure at 121 °C for 15 min.

Inoculum preparation

Inocula were prepared in side-arm Erlenmeyer flasks using the following methods: 45 ml of Vogel's medium [containing 0.5% trisodium citrate, 0.5% KH_2PO_4, 0.2% NH_4NO_3, 0.4% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.1% peptone, 0.2% yeast extract (pH 5.50)] was dispensed into a 300 ml conical flask. Chromic acid washed marble chips (12–15 in number) were added in the flask (to breakup the mycelial pellets) and were autoclaved for fifteen min at 121 °C. Two ml of sterilized 50% (w/v) stock solution of glucose were aseptically added to the autoclaved Vogel's medium as a carbon source. The flasks were inoculated with a loopful of *A. niger* spores under aseptic conditions. The inoculum was allowed to grow at 30 °C on a rotary shaker (120 rev/min). The cells were harvested, centrifuged at 8331 ×*g* for 15 min, washed twice with saline and resuspended in saline solution. The optical density was measured calorimetrically (at 610 nm) and maintained at 0.5 × 10 dilution. The above fungal population was used for mutational work or to inoculate the citric acid fermentation medium.

Improvement of strain

Mutagenesis using u.v. irradiation

The spore suspension of 24 h culture of *A. niger* GCB 75 in saline was transferred in sterile 0.005% dioctyl

sulphosuccinate (Sigma Chemical Company, St. Louis, USA) solution. The colony forming units/ml (c.f.u./ml) on ox gall-potato-dextrose-agar medium were maintained at 0.5×10^9 cells/ml for u.v. irradiation and further studies. The dose of exposure to suspension was 1.2×10^6 J/m^2/s for different time intervals (15–180 min). The survival curve was prepared and time of exposure giving (0.5×10^6 c.f.u./ml) 3 log kill was selected for mutation of the organism. The mutant derivatives were selected, characterized in solid and liquid culture media as described previously (Parvez *et al.* 1998). The best mutant was selected from a number of variants and designated GCM 45 (Table 1).

Chemical mutagenesis

The chemical mutagenesis was carried out by using NTG, following the method of Roy & Das (1978). The best mutant, which exhibited enhanced production of citric acid in plate and liquid culture screening, was designated GCM 7 (Table 2).

Citric acid production

All studies of citric acid fermentation were carried out in medium composed of molasses clarified with potassium ferricyanide + H_2SO_4 in Vogel's medium (Parvez *et al.* 1998). The pH of the medium was adjusted to 5.5. For submerged fermentation, 190 ml of medium were dispensed in 1000 ml conical flasks, autoclaved at 121 °C for 15 min at 1.05 kg/cm^2. After cooling at room temperature, the flasks were inoculated with 10% inoculum containing 0.5×10^9 c.f.u./ml in triplicate and incubated on an orbital shaker (120 rev/min) at 30 °C for different time intervals (0, 8, 16, 24, 48, 72, 96, 120 h).

Methods of analysis

Fungus cell mass was determined gravimetrically using dry weight method after Pirt (1975). Citric acid, succinic acid, fumaric acid, malic acid and sugars in molasses or fermentation broth were determined using high performance liquid chromatography using a u.v. detector for acids as described earlier (Parvez *et al.* 1998) and a refractive index detector for sugars. Kinetic parameters for batch fermentation process were determined after Pirt (1975).

Statistical analysis

Treatment effects were compared by the method of Snedecor & Cochrane (1980). Significance has been presented as Duncan multiple range test in the form of probability (P) values.

Results and Discussion

In the present investigations, a strain of *A. niger* GBCB 75 and its mutants derivatives, GCM 45 and GCM 7 were examined for production of citric acid from 15% sugars in molasses in 1 l shake flasks as described under Methods. The parental strain and both mutants consumed 36.0 ± 2.5, 110.0 ± 1.5 and 120.0 ± 2.3 g sugars/l and synthesized 31.1 ± 2.0, 50.0 ± 2.0, 86.1 ± 1.5 g citric acid/l respectively (Tables 1 and 2). They also synthesized 16.0 ± 1.4, 28.9 ± 0.4 and 28.9 ± 0.4 g dry cell mass/l. The enhancement in citric acid production was substantial. All mutants were significantly improved for the values of $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$ over those of their parental strains.

Table 1. Citric acid production by u.v.-induced mutant strains of *A. niger* following growth on molasses pretreated with potassium ferricyanide in Vogel's medium (pH 5.5).

GCB/mutant strains tested	$Y_{x/s}$ (g/g)	Citric acid concentration (g/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$ (g/g)
Parental strain	0.39 ± 0.03c	31.1 ± 2d	0.45 ± 0.04a	1.9 ± 0.1d
GCM 35	0.42 ± 0.02b	42.0 ± 3c	0.40 ± 0.032b	2.1 ± 0.20c
GCM 45	0.44 ± 0.04ab	50.0 ± 2a	0.44 ± 0.03a	2.6 ± 0.20a
GCM 55	0.45 ± 0.03a	45.0 ± 3b	0.45 ± 0.03a	2.4 ± 0.2b

Each value is an average of three replicates. ± denotes standard deviation among the replicates. Numbers followed by different letters differ significantly at $P \geq 0.05$.

Table 2. Production of citric acid by mutants of *A. niger* obtained following mutagenesis with NTG.

Strains tested	$Y_{x/s}$ (g/g)	Citric acid (g/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$ (g/g)
Parental strain	0.39 ± 0.03c	31.1 ± 2e	0.45 ± 0.04e	1.9 ± 0.1e
u.v.-derived best mutant				
GCM 45	0.44 ± 0.04b	50.0 ± 2d	0.65 ± 0.04d	2.6 ± 0.20d
NTG-derived mutants				
GCM 2	0.45 ± 0.03b	65.0 ± 4c	0.75 ± 0.04c	2.8 ± 0.20b
GCM 4	0.46 ± 0.03b	75.0 ± 5b	0.80 ± 0.05b	2.7 ± 0.20c
GCM 7	0.49 ± 0.04a	86.1 ± 1.5a	1.00 ± 0.01a	3.3 ± 0.2a

Each value is an average of three replicates. ± shows standard deviation among the replicates. The values followed by different letters vary significanty at $P \geq 0.05$.

Table 3. Kinetic parameters for production of citric acid from sugars in molasses following growth of *A. niger* and its mutant derivatives.

Kinetic parameter	Parental strain GCB 75	Mutant GCM 45	Mutant GCM 7
Substrate consumption parameters			
μ (h^{-1})	0.20 ± 0.02a	0.23 ± 0.02a	0.26 ± 0.02a
$Y_{x/s}$ (g cells/g)	0.39 ± 0.03c	0.44 ± 0.04b	0.49 ± 0.04a
Q_s (g/l/h)	0.38 ± 0.02c	0.47 ± 0.03b	0.93 ± 0.04a
q_s (g/g cells/h)	0.51 ± 0.03a	0.52 ± 0.03a	0.53 ± 0.04a
Q_x (g cells/l/h)	0.33 ± 0.03c	0.39 ± 0.03b	0.74 ± 0.04a
Citric acid formation parameters			
Q_p (g/l/h)	0.38 ± 0.03b	0.39 ± 0.03b	0.74 ± 0.04a
$Y_{p/s}$ (g/g)	0.45 ± 0.04c	0.65 ± 0.04b	1.00 ± 0.11a
$Y_{p/x}$ (g/g cells)	1.90 ± 0.1c	2.60 ± 0.20b	3.20 ± 0.20a
q_p (g/g cells/h)	0.38 ± 0.02c	0.60 ± 0.04b	0.83 ± 0.05a

Each value is an average of three replicates. ± indicates standard deviation among replicates. $Y_{x/s}$ = g cells/g substrate utilized, Q_s = g substrate consumed/l/h, q_s = g substrate consumed/g cells/h, Q_x = g cells formed/l/h, Q_p = g citric acid produced/l/h, $Y_{p/s}$ = g citric acid produced/g substrate consumed, $Y_{p/x}$ = g citric acid produced/g cells, q_p = g citric acid produced/g cells/h.

Maximum growth in terms of specific growth rate (μ) was only marginally different during growth of the wild parent and its two mutant derivatives on 15% carbohydrates in molasses. However, when the cultures were monitored for $Y_{x/s}$, Q_s and q_s, there was significant enhancement ($P < 0.05$) in these variables in mutant cultures over those obtained for wild-type cultures of *A. niger* (Table 3). This indicated that the mutant derivatives were faster growing organisms.

Specific yields of citric acid, $Y_{p/x}$ (g citric acid /g cells), product yield coefficient, $Y_{p/s}$ (g citric acid produced/g substrate utilized), Q_p (volumetric productivity) or q_p (specific productivities) are presented in Table 3. *Aspergillus niger* mutants exhibited improved production kinetic parameters over the parental strain. Maximum $Y_{p/s}$, $Y_{p/x}$, Q_p, and q_p (Table 3) were several-fold improved over those from some other *A. niger* cultures or mutants (Roukas & Harvey 1991; Kirimura *et al.* 1992; Gupta & Sharma 1994; Sanjay & Sharma 1994; Shoukat *et al.* 1997). Succinic acid, malic acid and fumaric acids were also produced but their highest volumetric productivities, in mutant GCM 7, were only 0.14, 0.13 and 0.1 g/l/h.

This mutational work has given a potent organism for production of citric acid from molasses. Further work is needed to improve the substrate consumption rate by isolating mutants which are resistant to higher concentrations of deoxy-D-glucose and are aspartate-requiring. Such mutants have been shown to exhibit high substrate consumption and product formation rates (Kirimura *et al.* 1992).

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