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Protoplast Fusion in *Aspergillus niger* Strains Accumulating Citric Acid

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ABSTRACT. Auxotrophic strains of *Aspergillus niger* were obtained from citric-acid-producing strains of the fungus after irradiation with UV light. Protoplasts were isolated from young hyphae of the auxotrophic strains after treatment with snail enzyme and than treated with polyethylene glycol (30 %, *W/V*), in a Ca^{2+} (10 mmol/L) solution. The pH value of the suspension was adjusted to 9.0. The frequency of the heterokaryons (related to the number of protoplasts reverting after PEG treatment) was 0.67 %. Prototrophic heterozygous spores were isolated from a heterokaryon with the frequency of 1.2×10^{-6}. Citric acid production in the best heterozygous strains was about 15 % higher than that of the high-production parent strain.

Mutagenesis is the oldest and most common method for the improvement of citric-acid-accumulating *Aspergillus niger* strains. Parasexual hybridization of these strains has also been described later (Ikeda 1961; Seichertová and Leopold 1969; Ilczuk 1971; Das and Roy 1978; Das 1980; Bonatelli *et al.* 1983). The disadvantages of these methods (decreasing variability of the strains after repeated mutagen treatment in the case of mutagenesis and low frequency of hybrid formation and incompatibility of the strains in the case of parasexual hybridization) could be avoided when using protoplast fusion. First studies on protoplast fusion in citric-acid-producing *A. niger* strains were published by Kirimura *et al.* (1986, 1988*b*). In that work diploid strains were obtained whose productivity was better than that of both parental strains in the solid culture. Further improvement of citric acid production in both solid and submerged culture was achieved by haploidization of *A. niger* diploid strains (Kirimura *et al.* 1988*a*). In the present study, protoplasts of two *A. niger* strains with a different citric acid production and of different origin were fused and the obtained hybrid strains were tested in submerged culture in a beet molasses medium.

MATERIALS AND METHODS

Auxotrophic mutants *A. niger* 97A/61 (requiring arginine for growth) and *A. niger* 137 (requiring nicotinamide for growth) were isolated after UV irradiation of a spore suspension of the prototrophic strain 97 and of the prototrophic strain 84/151, respectively (*unpublished results*).

The protoplasts were isolated according to Musílková and Fencl (1968). The method of protoplast fusion is described in Fig. 1: Suspension containing an approximately equal number of protoplasts of both the parental strains was centrifuged (500 ***g***, 10 min) and resuspended in 30 % polyethylene glycol (molar mass 4–6 kg/mol). The pH value of the suspension was adjusted to 9.0 with NaOH

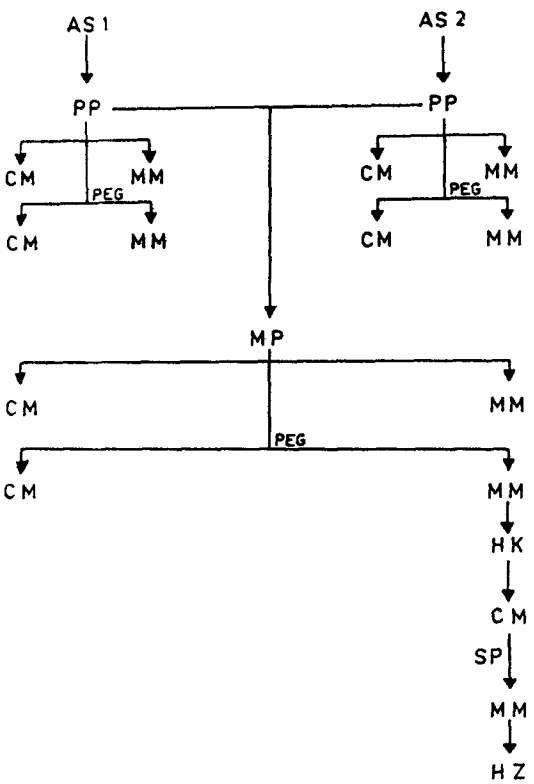

Fig. 1. Protoplast fusion of two complementary auxotrophic *A. niger* mutants; AS – auxotrophic strain, CM – complete medium, HK – heterokaryons, HZ – heterozygotes, MM – minimal medium, MP – mixture of protoplasts of both parent strains (cell concentration 1–10/nL), PP – protoplasts, SP – spores.

and concentration of Ca^{2+} to 10 mmol/L with $CaCl_2$. After a 15-min incubation at 30 °C the suspension was centrifuged (500 ***g***, 10 min), the protoplasts were washed twice with NaCl (0.7 mol/L) and resuspended in the same stabilizer. The final suspension of protoplasts was spread on a minimal (Czapek-Dox) and complete medium. The suspensions of protoplasts of individual strains before and after PEG

treatment and the mixture of protoplasts of both strains before PEG treatment were spread on the same media (see Fig. 1). The conidial diameter was measured with a micrometer eyepiece.

Production of both heterokaryotic and heterozygous isolates was tested by a submerged two-stage laboratory cultivation in a synthetic medium with 14 % sucrose according to Sanchez-Marroquin *et al.* (1970) (inoculation stage) and in a beet molasses medium with 15 % sugar (0.0065 % H_3PO_4, 0.03 % $K_4Fe(CN)_6$, pH 6.4) (production stage) in 300-mL Erlenmeyer flasks containing 50 mL of the media on a rotary shaker (3.7 Hz, radius 25 mm) at 30 °C. Factors required for growth were added to a concentration of 1 g/L. The inoculation and production stage took 2 and 6 d, respectively. Citric acid was determined by isotachophoresis (leading electrolyte: HCl (10 mmol/L) + β–alanine, pH 4.0; terminating electrolyte: glutamic acid (10 mmol/L)).

RESULTS AND DISCUSSION

The reversion of the protoplasts of individual strains and of a mixture of both strains before and after PEG treatment is shown in Table I. After the PEG treatment

TABLE I. Reversion (%) of protoplasts of *A. niger* 97A/61 and 137 before and after PEG treatment

Strain	Minimal medium		Complete medium	
	before	after	before	after
97A/61	0	0	47.1	6.5
137	0	0	67.9	7.3
97A/61 + 137	0	0.02	45.3	6.0

the frequency of protoplast reversion in a complete medium strongly decreased. A similar conclusion was reached by Homolka (1988). This phenomenon could be caused by lysis of the protoplasts or by the formation of protoplast clusters. Protoplast formation and PEG treatment did not induce reverse mutations of the parental auxotrophic strains. The fact that the mixture of the protoplasts of both strains did not form prototrophic colonies before PEG treatment shows that protoplast fusion was not induced by centrifugation.

Protoplasts capable of reversion in the minimal medium were only detected in the protoplast mixture after PEG treatment. The fusion frequency was estimated as the ratio of the protoplast reversion in the minimal and complete medium and was 6.7×10^{-3} per protoplast pair.

TABLE II. Changes in the accumulation of citric acid in strains *A. niger* 971/61 and 137 after protoplast fusion (number of isolates in individual production lasses, *n*)

ercentage (%) of productivity of the parent strain 971/61							
%	101–110	111–120	121–130	131–140	141–150	151–160	161–170
n	1	1	2	9	4	2	1
Percentage (%) of productivity of the parent strain 137							
%	71–80	81–90	91–100	101–110			
n	2	9	6	3			

The production of most of the heterokaryons was between those of the parental auxotrophic strains. Only three isolates were better than either parent strain (see Table II). The maximum production increase was 4.6 % over the high-production strain 137.

From the spores of the best heterokaryon 97/137/III 25 prototrophic colonies were obtained. The frequency of prototrophic spores was 1.2×10^{-6}. Most prototrophic isolates produced roughly the same amount of organic acids as the original heterokaryon, only in four cases was the production activity increased by more than 10 %. The isolation of the best hybrids is illustrated in Fig. 2. These results provide

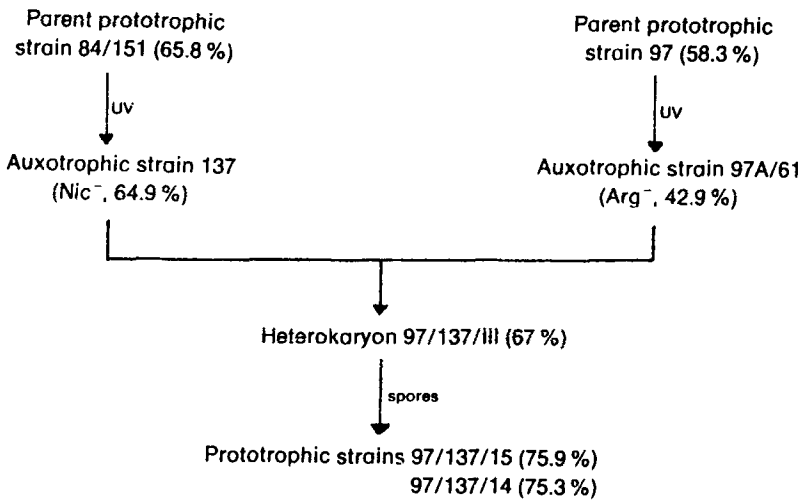

Fig. 2. Isolation of the best hybrids obtained by protoplast fusion of the auxotrophic strains *A. niger* 97A/61 and 137. The data in *parantheses* correspond to citric acid production per sugar supplied in the beet molasses medium with 15 % sugar.

further evidence that the strains with improved citric acid production can be obtained by hybridization. Similar conclusions were reached by Ikeda (1961), Seichertová and Leopold (1969), Das and Roy (1978) and Kirimura *et al.* (1986, 1988*a*, *b*). The maximum production increase observed here was about 15 % in comparison with the high-production prototrophic parent strain, *i.e.* a little lower than that observed by Kirimura *et al.* (1988*a*).

The diameter of conidia of the parent auxotrophic strains was 3.3–5.0 μm, that in the best prototrophic isolates 5.0–6.6 μm (strain 97/137/14) and 5.0–5.8 μm (strain 97/137/15). These data indicate that the strains are diploid.

The results suggest that diploidization by protoplast fusion may be useful for improving *A. niger* strains producing citric acid in submerged culture.

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