

The effect of *Aspergillus niger* mutagenization on citric acid biosynthesis

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The industrial *A. niger* strain producing citric acid was mutagenized with the use of new chemical mutagens: free nitroxyl radicals. Strains of higher citric acid production yield were obtained. Citric acid was produced in a shorter time compared to the initial strain. During 6-12 months of storage most of the strains preserved their positive features which proves that mutants with profitable biotechnological properties were obtained. These mutants are used in industrial process.

INTRODUCTION

In citric acid production the most important factor is the use of high yield fungus strain characterized by high homogeneity of citric acid biosynthesis. It is widely known that depending on raw material and applied technology proper *A. niger* strain or its mutant suitable for production conditions should be used. Microbiological laboratories are careful to produce strains characterized by high yield citric acid biosynthesis. These effects can be obtained by constant clone selection and by mutagenization of collected strains in order to improve them.

The most frequently used mutagenic factors are UV rays, Gamma rays and among chemical factors nitrosoguanidine. The investigations were never carried at to find new, more effective mutagens.

For the last years chemistry of free nitroxyl radicals has been developing (Rozancew, Szolte, 1985). Depending on the concentration free nitroxyl radicals have either inhibitory or toxic effects on live cells (Smith, Schriener, Marsh, 1975). Nitroxide spin-labels are stable synthetic organic free radicals that can interact either covalently or noncovalently with biological macromolecules (Dugas, 1977). These radicals have active group = N - O' with one unpaired

electron which when introduced into the cell induces the formation of new radical products resulting in cell structure disorder (Brustad et al., 1972; Pryor, 1973). This disorder concerns also cell DNA which can lead to stable mutant appearance characterized by useful biotechnological properties.

In the studies on gibberellins production with use of *Gibberella fujikuroi* fungus, free nitroxyl radicals were used with good results to obtain more effective mutants (Walisch, 1989). In our experiments we checked mutagenic influence of some nitroxyl radicals on industrial *A. niger* strains producing citric acid.

MATERIAL AND METHODS

Microorganisms. In our experiments we used 4-D *Aspergillus niger* strain from the collection of Research Laboratory of Citric Acid Factory in Zgierz. The strain was previously adapted for submerged biosynthesis of citric acid in synthetic medium with sucrose as substrate (Nowakowska-Waszczyk, Sokółowski, 1987). The strain was stored as lyophilizate or on potato slants in temperature +5°C (Kędziora, Juda, Walisch, 1982).

Media. Medium 1 (for screening): malt wort (3° Blg) 100 cm³, sucrose 8 g, pepton 0.005 g, NH₄NO₃ 0.01 g, KH₂PO₄ 0.01 g, K₂HPO₄ 0.01 g, MgSO₄ · 7H₂O 0.01 g, bromothymol blue 0.002 g, agar 2 g, pH = 6.5. Medium 2 (for fermentation test): sucrose 10 g, NH₄NO₃ 0.2 g, MgSO₄ · 7H₂O 0.015 g, KH₂PO₄ 0.015 g, tap water to 100 cm³, pH = 2.3. The media were sterilized at 120°C for 30 min.

Mutagens. We used classic mutagen 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) produced by Sigma USA and free nitroxyl radicals: di-tert-butyl-nitroxyl (DTBN), 4-iodoacetoxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (IAMP), and 4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (HAMP) synthesized in the Institute of Organic Chemistry, University of Łódź. Besides chemical mutagens we used UV rays sent by the lamp UV type EMITA VP-60, 185 W, produced by Famed (Poland).

Cultivation technique. *A. niger* strains were cultivated on potato slants at 30°C for 7 days. Screening test (medium 1) was carried at 30°C for 48 h. The most active clones which produced dark pink zones around colonies were collected for further experiments. Fermentation tests were carried out by cultivation of selected clones (expected mutants) in liquid medium (medium 2) on the shaker 225 rpm and amplitude 3 cm at temp. 30°C, for 6 days. The cultivation was carried out in 500 cm³ flasks in 75 cm³ of medium inoculated with *A. niger* conidia in the amount of 10⁶.

Mutagenization technique. The swelling conidia suspension in the amount of 10⁷/cm³ were mutagenized in phosphate buffer, pH = 5.8, at 30°C for 30-60 min. Mutagen doses were from 0.05 to 100 mg/cm³. In some cases conidia suspension was additionally irradiated by UV rays for 30 min., in 15 cm away from

the radiator. Under these conditions the survival rate of conidia depending on the kind of mutagen and exposition time, ranged from 0.002 to 60.3 %.

Analytical methods. In fermentation tests, acid concentration was determined by titration of the samples with 0.1 N NaOH. Homogeneity of citric acid biosynthesis i.e. the presence of other acids (oxalic, malic, succinic, gluconic and lactic) was checked by chromatographic analysis, according to Hulme (1961) using Whatman paper 1, and butanol, formic acid, water (4:1:5 v/v) as a solvent and bromocresole blue as an indicator. After drying chromatograms at room temperature, yellow spots of acids were visible on the blue background.

RESULTS AND DISCUSSION

Using nitrosoguanidine as a mutagen, over 150 clones of expected mutants were obtained. In screening tests they showed positive features, but in fermentation tests no isolated clone exceeded the citric acid yield of parent strain. Therefore, in further work we used mutagens of younger generation i.e. free nitroxyl radicals.

Using DTBN as a mutagen, from over 50 clones preliminary isolated, we obtained 3 strains which in fermentation test exceeded the citric acid yield of the control strain. In particular B₉ strain produced about 10 % more of citric acid as compared to the control strain, but the acceleration of acid biosynthesis was not observed in these experiments (Fig. 1A).

In further work, using IAMP as a mutagen and additionally UV rays, several dozens of clones were isolated and 5 of them gave very interesting results in fermentation tests. They produced citric acid faster, as compared to the control. This was already noticeable on the 4th day of fermentation (Fig. 1C). Citric acid biosynthesis was finished on the 5th day. Strains J₇, Ju₁₆, Ju₂₆ and Ju₂₈ showed higher citric acid yield compared to the parent strain (Fig. 1C). Strain J₇ was lyophilized and its activity was checked after 6 months of storage of the lyophilizate. After this time its acid-forming activity did not change but the activity decreased during 12 months of storage on potato slant (Fig. 2). Strain Ju₁₆ changed its activity neither during storage as a lyophilizate nor during 12 months of storage on potato slants. The citric acid yield of the latter was even higher on the 4th day of fermentation compared to the lyophilizate (Fig. 2). In case of strain Ju₂₆ the acid-forming activity was kept on the same level in the stored lyophilizate as well as on potato slants. The high yield was already observed on the 4th day of fermentation (Fig. 6).

Using HAMP as a mutagen, and additionally UV rays, 3 clones were isolated. They exceeded the citric acid production yield (about 14-16 %) on the 4th day of fermentation, of the parent strain (Fig. 1B). One of these strains (OH₁) was lyophilized and stored for 12 months. After this time its acid-forming activity even exceeded that of the control i.e. the sample right after lyophilization (Fig. 2).

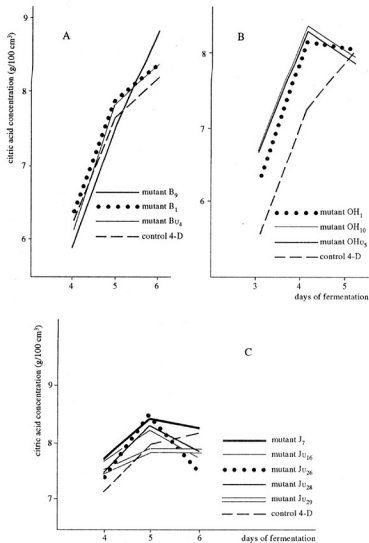


Fig. 1. Activity of *A. niger* mutants after: A - DTBN; B - HAMP and HAMP + UV treatment, C - IAMP and IAMP + UV

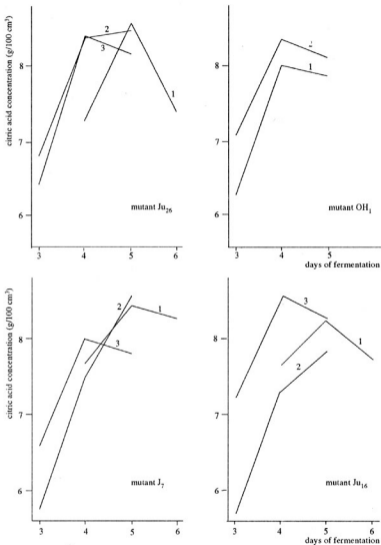


Fig. 2. Activity of mutants after lyophilization:

1 - after lyophilization, 2 - after 6 months of storage, 3 - after 12 months of storage

In all fermentation samples chromatographic analysis was carried out on the last day to check the presence of organic acids. Except for citric acid no other organic acids were found. In some fermentation samples the content of citric acid decreased at the end of fermentation, which may be due to the consumption of the acid by *A. niger* when other carbon sources were lacking. On the basis of the results we can conclude that it is possible to obtain mutants with stable features using new chemical mutagens – free nitroxyl radicals which outgrow the initial strains as far as positive biotechnological properties are concerned. Among the mentioned mutants the following are used in industrial process: Ju₁₆, Ju₂₆, OH₁.

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