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SELECTION OF 5-FLUOROCYTOSINE-RESISTANT MUTANTS FROM AN *ASPERGILLUS NIGER* CITRIC ACID-PRODUCING STRAIN

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SHORT COMMUNICATION

ABSTRACT

Mutants of *Aspergillus niger* N402, induced by UV mutagenesis, were selected and tested for resistance or sensitivity to 5-fluorocytosine. Some mutants showed increased citric acid production, which did not correlate with the intracellular amount of protein or ammonium ion. The resistance to 5-fluorocytosine proved to be a rational approach for isolation of new mutants with improved production of citric acid. The best mutant (FR13) accumulated double the amount of citric acid produced by the parental strain.

Key words: *Aspergillus niger,* citric acid, 5-fluorocytosine

Because of its high solubility, palatability and low toxicity, citric acid is one of the most commonly used acids in the food and pharmaceutical industry. There are many microorganisms, including fungi, yeasts and bacteria, that can produce citric acid by fermentation. Since *Aspergillus niger* is one of the best known citric acid producers, it is widely used for industrial-scale production (14).

In spite of the large number of producer strains available, it is still important to generate strains with some advantageous characteristics, such as enhanced citric acid production and increased rate of fermentation. To improve citric acid producing strains, selection after random mutation has been generally carried out (10,13).

Since *A. niger* shows impaired protein synthesis during citric acid fermentation, the disturbance of protein synthesis has been claimed to be an important phenomenon (5,12). The idea is that mutants having impaired protein synthesis should accumulate large amounts of citric acid and this property has thus been proposed as a new tool for mutant screening (15). Antibiotics and metabolic antagonists are the first choice for sensitive or resistant mutant isolation with impaired protein synthesis. The nucleotide analogue 5-fluorocytosine (5-FC) is a metabolic antagonist which promotes impaired protein, RNA and DNA synthesis (3,4), and has been used to test the hypothesis that other mechanisms besides disturbance in protein synthesis might be involved in citric acid production by *A. niger*.

The *A. niger* strain N402, a *csp*A1 (conferring short conidiophores) derivative of ATCC 9029 (1) is not a hyper-accumulating strain used by the citric acid industry. This strain was used in the present work as a model strain for the selection of mutants. Strain N402 was kept on yeast extract-glucose agar slants and subcultured each month. For citric acid production, and for selection of mutants, the medium and conditions described by Rugsaseel *et al*. (15), was used, except for the isolation medium, which was 0.1 ug 5-FC/mL. All cultivations were carried out at 30ºC.

After UV mutagenesis (8), a conidial suspension was transferred to the isolation medium. For the isolation of resistant mutants, after 24, 48 and 72 h, the broth was filtered through a glass wool filter to remove the germinated conidia, which were immediately plated onto medium containing 5-FC. To confirm resistance, the colonies were replica plated onto

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the same medium and cultured for seven days. The sensitive mutants were collected as conidia in the filtrate, which was centrifuged, concentrated and plated. To confirm sensitivity, the mutants were replica plated onto media with and without 5-FC and cultivated for 72 h. Twenty-two mutants were isolated, 20 of them resistant and 2 sensitive, and inoculated in the presence of 5-FC to test growth impairment. Most of the mutants showed almost the same radial growth as the parental strain after 72 hours of incubation (data not shown). Citric acid was determined in the broth of cultures inoculated with 10^6 conidia/mL after nine days of cultivation. The amount of acid in broth was measured by titration with 0.1 M NaOH against phenolphthalein. After thrichloroacetic acid (TCA)-insoluble protein removal, glucose in the broth was measured enzymatically using the glucose GO-test kit (Sigma, St Louis, MO). The resultant mycelium was treated as described before (15) and intracellular protein was measured in the pellet kept at -20°C (11). Growth was estimated by measurement of mycelial dry weight.

Mycelium (20 mg) plus 200 mg of washed sand, 200 mg of glass beads and 400 uL of 0.1 M NaOH was placed in a microcentrifuge tube and submitted to maximum speed vortexing for 10 min. The homogenate was then centrifuged at 14,000 g for 5 min and the supernatant (250 uL) was placed in a tube with 250 uL of 10% (W/V) TCA. The mixture was homogenized and centrifuged at 14,000 g for 5 min. The free protein supernatant was used to measure the ammonium ion (NH_4^+) with the Nessler reagent (19). Nucleic acids were extracted from mycelium (2) and the extracted amount was determined with a Gene Quant spectrophotometer (Pharmacia Biotech).

Citric-acid-accumulation tests were applied to the parental strain and also to the selected mutants. The sensitive mutants were extremely difficult to obtain and always showed a smaller production of citric acid than the parental N402. Five mutants selected for 5-FC resistance showed a small increase in the rate of citric acid accumulation as shown in Table 1. *A. niger* mutants resistant to the metabolic antagonist 2 deoxy-D-glucose (8) also showed a small increase in citric acid accumulation, although in this case some mutants showed a reduction in the fermentation time to five days, while another mutant, a cycloheximide-sensitive mutant of the hyper-accumulating citric acid strain WU-2223L, showed a four-fold increase in citric acid production after nine days of fermentation (15).

5-FC is taken up by fungal cells through a cytosine permease, deaminated to 5-fluorouracil, converted into the nucleoside triphosphate and incorporated into RNA, where it causes miscoding. In addition, 5-fluorouracil is converted into deoxynucleoside, which inhibits thymidylate synthase and thereby DNA biosynthesis (4).

It was previously shown that protein content decreases in *A. niger* under conditions of citric acid accumulation (6,9) while the content of intracellular NH_4^+ increases due to protein degradation (12). This was also observed by Rugsaseel *et al* (15). However, as shown in Table 2, the 5-FC-resistant mutants presented a higher level of intracellular protein than the parental strain N402, while the ammonium ion concentration was always lower than in the parental one. Also, the content of nucleic acids in these mutants was two to three times higher relative to the parental. This may represent an advantage by permiting a more extended period of citric acid fermentation, an interesting economic characteristic.

It is important to consider that the generally observed decrease in the total amount of intracellular protein, as well as the consequent increase in the amount of NH_4^+ ions could not be sufficient to explain the whole mechanism of citric acid accumulation, which remains controversial for different authors. It is widely accepted that there is an increase in the carbon flux through the glycolytic pathway under the conditions of citric acid fermentation, and many authors have attempted to explain this increase in glycolysis as being caused by phosphofructokinase stimulation by NH_4^+ ions (18). Such a stimulus would then result in enhanced production of pyruvate, from which both the precursors of citric acid are produced: acetyl-CoA and oxaloacetic acid, whose condensation in the citric acid cycle is the major route of citrate synthesis.

Table 1. Citric acid accumulation by the 5-fluorocytosine-resistant mutants of *Aspergillus niger* in shake culture.

Strains	Dry weight (mg/mL)	Citric acid productivity* (mg/mg DW)
N402	13.5	1.6
FR5	16.9	1.9
FR7	17.7	2.0
FR10	20.8	2.0
FR12	23.1	1.7
FR13	9.2	3.0

* Citric acid productivity was described as the ratio of amount of citric acid to mycelial dry weight. DW, dry mycelium weight. Cultivations were done for 9 days.

Table 2. Total amount of proteins, ammonium ions and nucleic acids in the mycelia of the *Aspergillus niger* parental strain and mutants.

Strain	Protein (mg/ g-DW*)	NH_4^+ (mg/ g-DW)	Nucleic acids (ug / uL)
N402	115	1.5	1.8
FR5	378	0.9	2.8
FR7	223	0.6	6.7
FR10	266	1.2	2.8
FR12	199	0.5	4.0
FR13	177	1.3	3.2

* DW, dry mycelium weight. Cultivations were done for 9 days.

However, several reports have shown that the single stimulation of phosphofructokinase is not the unique aspect leading to citric acid production. In fact, it was shown that overexpression of the glycolitic enzymes phosphofructokinase and/or pyruvate kinase in *A. niger* did not increase citric acid production by the fungus significantly (16). It ultimately means that the increase in the activity of one or two enzymes would not explain the strong accumulation of citric acid because other components of the pathway could then turn into a blockage to the metabolic flux. The same authors concluded that phosphofructokinase and pyruvate kinase seem not to contribute in a major way to flux control of the metabolism involved in the conversion of glucose to citric acid.

Furthermore, other authors verified that addition of 5-fluorouracil to the fermentation media resulted in citric acid production even when Mn^{2+} ions are present (12). It should be noticed that Mn^{2+} absence or limitation is considered to be responsible for protein degradation and NH_4^+ accumulation during citric acid fermentation. Thus, it is possible to conclude that 5-FC and ultimately 5-fluorouracil might have other metabolic effects leading to citric acid accumulation which are not dependent on protein decrease. Besides, Mn^{2+} deficiency might have another role, e.g. in decreasing the formation of the by-product oxalic acid, since a recent report showed that oxalate production is mediated by the Mn^{2+} dependent enzyme oxaloacetate acetylhydrolase in an *A. niger* citric acid producing strain (17).

Several mechanisms can result in resistance to 5-FC, as verified in the model organism *Saccharomyces cerevisiae* (7). Hyper production of pyrimidines is one of these mechanisms that might be occurring in the mutants isolated in the present work and that helps to explain the increase in the total amount of nucleic acid and protein in the mutants studied. This could mean that such mutants have a more efficient metabolism, and therefore grow better than the parental strain under the limiting conditions of citric acid fermentation, what in fact is shown by their increase in biomass production. Thus, it is possible that the metabolic advantage of the 5-FC-resistant mutants result in a better turnover and reposition of anabolic components (enzymes, cofactors) ensuring the maintenance of an adequate flux through the pathways involved in citric acid accumulation.

Taken together, the results indicate that 5-FC resistance might be a useful criterium for the rational screening of *A. niger* mutants in order to increase citric acid accumulation.

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RESUMO

Seleção de mutantes resistentes à 5-fluorocitosina isolados de uma linhagem de *Aspergillus niger* produtora de ácido cítrico

Através de mutagênese com UV, foram isolados mutantes de *Aspergillus niger,* verificando-se sua resistência ou sensibilidade à 5-fluorocitosina. Alguns mutantes apresentaram um aumento na produção de ácido cítrico sem um aumento correspondente da quantidade de proteína intracelular ou de ions amônio. A resistência à 5-fluorocitosina mostrou ser uma abordagem racional para o isolamento de mutantes com maior capacidade de produção de ácido cítrico. O melhor mutante selecionado, FR13, produziu o dobro da quantidade de ácido cítrico em relação à linhagem parental.

Palavras-chave: *Aspergillus niger*, ácido cítrico, 5-fluorocitosina.

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