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Production of Citric Acid from *n*-Paraffins by Fluoroacetate Sensitive Mutant Strains of *Candida lipolytica*

Sir:

It is well known that many kinds of yeast produce citric acid, accompanied with a relatively large amount of (+)-isocitric acid, from various carbon sources.^{1~5)} However, from the viewpoint of economical production of citric acid from *n*-paraffins by *Candida lipolytica*, the accumulation of citric acid in a high yield without the formation of (+)-isocitric acid was desired.

Up to now, evidence has been presented that addition of fluoroacetate⁶⁾ or limited supply of ferrous ion⁵⁾ to the culture media gives a high yield of citric acid. On the other hand, it has been established that accumulation of citric acid from molasses by a strain of *Aspergillus niger* is caused by a extremely low aconitate hydratase activity in the mold cells during the production phase of the fermentation.⁷⁾

From these facts, it seemed to be indispensable to induce mutant strains with low aconitate hydratase activity in order to establish a suitable process for the production of citric acid. On the basis of the assumption that mutant strains incapable of assimilating citric acid or being sensitive to fluoroacetate would have a low aconitate hydratase activity, experiments were carried out in the attempt to obtain such mutant strains from *Candida lipolytica* IFO 1437 which produced citric and (+)-isocitric acid in the ratio of about 60:40 from *n*-paraffins.

Among the mutant strains obtained from the wild strain by N-methyl-N'-nitro-N-nitrosoguanidine treatment, a mutant strain, K-20, incapable of assimilating citric acid as the

sole carbon source, and a fluoroacetate sensitive mutant strain, S-22, were selected on the basis of high citric acid and low (+)-isocitric acid productivities. Figure 1(A) shows that growth of the two mutant strains in a medium containing sodium citrate as the sole

FIG. 1. Growth Response to Citrate(A) and to Fluoroacetate(B).

Medium: Sodium citrate 0.7% (A) or sodium acetate 0.7% plus sodium monofluoroacetate varied (B), NH_4Cl 0.1%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, NH_4NO_3 0.1% and thiamine hydrochloride 0.3 ppm. The cells were incubated at 28°C for 40 hr (A) or for 18 hr (B) in creased flasks on a rotary shaker of 200 rpm. The relative growth was calculated from O.D.₅₉₀ of the broth.

x---x wild strain; ●-----● mutant strain K-20; ○---○ mutant strain S-22.

TABLE I. COMPARISON OF ENZYME ACTIVITIES

The cells were grown at 28°C for 72 hr in a medium containing *n*-paraffins (a mixture of C₁₂-C₁₄) 8% (w/v), KH₂PO₄ 0.025%, MgSO₄·7H₂O 0.05%, (NH₄)₂SO₄ 0.4%, KCl 0.05%, NaCl 0.01%, CaCO₃ 0.1%, CuSO₄·5H₂O 0.1 ppm, ZnSO₄·7H₂O 1 ppm, FeSO₄·7H₂O 10 ppm, thiamine hydrochloride 0.05 ppm and bromocresol purple 10 ppm; and ammonia water was fed to the broth to keep the pH in the range of about 4 to 6 throughout the fermentation. The cells were harvested and washed with cold water and sonicated, and the sonicates were then centrifuged at 4000×*g* for 10 min. The supernatant was used as the crude enzyme preparation.

Enzyme	Wild strain	Mutant strain	
		K-20	S-22
Citrate synthase	100	92	72
Aconitate hydratase	100	11	2
Isocitrate dehydrogenase (NADP-linked)	100	135	123
Isocitrate lyase	100	97	124
Fumarate hydratase	100	103	106
Malate dehydrogenase	100	82	108

carbon source is extremely poor under aerobic conditions. The sensitivity of the two to monofluoroacetate was compared with that of the wild strain. As is obvious from Fig. 1(B), strain S-22 was shown to be extremely sensitive to the compound and strain K-20 to be moderately responsive. Next, enzyme activities related to the TCA cycle were compared. As shown in Table I, aconitate hydratase activity in the cells of K-20 and S-22 were approximately 1/10 and 1/100 that of the wild strain, respectively, and no remarkable differences in other enzyme activities were observed between these strains.

The citric and (+)-isocitric acid productivities of those strains were also compared. Each of these strains was inoculated into 25 ml aliquots of the medium, shown in Table I, in a 200 ml creased flask and incubated for 72 hr at 28°C on a rotary shaker of 200 rpm. As is clear from Fig. 2, the mutant strain, S-22, accumulated 110 mg/ml of citric acid and 4.5 mg/ml of (+)-isocitric acid in a ratio

FIG. 2. Comparison of Fermentation Time Course.

The fermentation was carried out under the conditions described in the Table I.

(A): wild strain; (B): mutant strain K-20; (C): mutant strain S-22.

○—○ citric acid; ●—● (+)-isocitric acid; ×---× aconitate hydratase activity.

of about 97:3.

The above results showed that the low aconitate hydratase activity in the cells caused sensitivity of the mutant strains to fluoroacetate and resulted in low productivity of (+)-isocitric acid. Moreover, it is believed that the effective technique developed by the present authors for the derivation of a mutant strain lacking aconitate hydratase activity will prove to be a very profitable tool for the elucidation of metabolism of the TCA cycle related metabolites.

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