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博 士 論 文 概 要

論 文 題 目

Improvement of Citric Acid-Producing
Fungi by Alteration of Metabolic Regulation

代謝制御機構の改変によるクエン酸
生産糸状菌の改良

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1994 年 6 月

More than 350,000 tons per year of citric acid are produced on a worldwide scale to be used in the food, pharmaceutical and detergent industries. Most industrial citric acid fermentation processes use genetically improved strains of *Aspergillus niger*. Since this filamentous fungus lacks a sexual cycle, parasexual recombination, diploidization, and heterokaryon formation have been explored as possible means of strain improvement, but most industrial strain development methods have probably relied on mutagenesis and screening for the derivation of efficient producers of citric acid. Various culture methods, *i.e.* semi-solid, surface and submerged cultures, have been used in the industrial production of citric acid, however, among them the submerged culture is the method that gives the best performance: it is more easily controlled at various stages, uses raw material more efficiently and yields more product.

On the other hand, South East Asian countries are known to be those where the agricultural products and wastes are more abundant and used as suitable substrates for citric acid production. At present, however, in this area only a semi-solid culture is successfully used for citric acid production. The submerged culture method is considered by the author to be the most efficient for the increase of citric acid production. Although submerged culture has been suggested to be the best performance method for citric acid production, it does have negative sides: it is strongly affected by the composition of the culture media and some other culture conditions that cause an impairment of the production. Therefore, to increase citric acid production in submerged culture so that the agricultural products could be employed more effectively, the generation of strains that could overcome the above effects or an improvement of culture conditions would be necessary.

This thesis was put forward in an attempt to enhance citric acid productivity of *A. niger* in submerged culture and especially under unfavorable condition for acidogenesis. Therefore, the induction of mutants having some characteristics important to the overproduction of citric acid in *A. niger*, *e.g.* an impaired protein synthesis or an increased glycolytic flux at the expense of the pentose phosphate pathway etc., was performed. In addition, some effects of culture conditions on citric acid production and the accumulation of related metabolite, extracellular polysaccharide in shake culture were also investigated.

This thesis contains 8 chapters and was organized in the following way:

Chapter 1 introduces fundamental concepts about citric acid, the filamentous fungus *A. niger* in relation to its ability to produce citric acid, and states the purpose of this thesis.

Chapter 2 describes the materials and the experimental methods used for this thesis.

Chapter 3 describes the enhancement of citric acid production by mutants of *A. niger* from soluble starch in shake culture. The aimed mutants were induced from *A. niger* WU-2223L, a hyper-producer of citric acid in shake culture, through selection of enhanced amylolytic activity and acid productivity on modified starch-methyl red agar plates. All the selected mutants exhibited a considerable increase in glucoamylase activity and citric acid productivity in shake culture containing soluble starch as a sole carbon source. A representative mutant, 2M-43, produced 48.0 mg citric acid/ml from 120 mg soluble starch/ml after 9 d of cultivation in shake culture, whereas WU-2223L produced 35.1 mg/ml. Maximum glucoamylase activities in the culture filtrates of 2M-43 and WU-2223L were detected after 3 d of cultivation (3.62 U/ml and 2.11 U/ml, respectively).

Chapter 4 is devoted to the enhancement of citric acid productivity of *A. niger* in shake culture under conditions unfavorable for acidogenesis. On the basis of evidence to the effect that impaired protein synthesis through addition of methanol contributed to an enhancement of *A. niger* citric acid productivity, mutants having an impairment of protein synthesis, *i.e.* cycloheximide-sensitive mutants, were induced from *A. niger* WU-2223L. The obtained mutants exhibited a drastically increased citric acid productivity in a production medium containing a high amount of Mn^{2+} ions (92.8 μM) without the addition of methanol. Under these conditions, the best mutant: CHM 100-13, produced 69.4 mg citric acid/ml, whereas WU-2223L produced 19.9 mg/ml from 120 mg glucose/ml after 9 d of cultivation. In contrast to the results obtained from WU-2223L, the addition of 2% (v/v) MeOH or 0.02 mg cycloheximide/ml to the production medium resulted in a remarkably decreased citric acid productivity of the mutants. In comparison with WU-2223L, CHM 100-13 exhibited a significantly decreased level of extra- and intracellular protein accumulation but remarkably increased level of intracellular $NH4^{+}$ accumulation in shake culture without the addition of methanol.

Chapter 5 is devoted to citric acid accumulation by mutants of *A. niger* with reduced effects of extracellular citric acid in shake culture. The effects of product inhibition and the ability to reassimilate the excreted citric acid contributed to the tremendous problems encountered in determining the accumulation of high amounts of citric acid by *A. niger*. Therefore, to obtain over-accumulation of citric acid, mutants able to overcome the above problems were induced from WU-

2223L. Five mutants showed a considerable increase of citric acid accumulation in shake culture, namely about 1.2-1.4 times that of WU-2223L. In comparison with WU-2223L, M-125 revealed almost no capacity to assimilate exogenous citric acid, while in contrast M-26 and M-156 still maintained and remarkably increased their abilities. Nevertheless, the effect of product inhibition upon these three mutants was clearly reduced. Consequently, the 3 mutants exhibited an extremely high citric acid accumulation in shake culture without the addition of 2% (v/v) methanol, about 2.7-3.6 times that by WU-2223L, indicating that their acidogenesis might be far less sensitive to the inhibition by citric acid than WU-2223L.

Chapter 6 deals with stimulation of citric acid production in *A. niger* by addition of viscous substances in shake culture. *A. niger* Yang no.2, a hyper-producer of citric acid in semi-solid culture, was strongly influenced by the culture conditions. In shake culture, citric acid productivity of Yang no. 2 was low but increased about 2.5-3.6 times through the addition of some viscous substances, such as agar, gelatin, and polyethylene glycol 6000 *etc.*, to the culture medium. However, no influence of viscous substances was observed in semi-solid and surface cultures, *i.e.* under static cultivation conditions. Since Yang no.2 did not utilize the viscous substances used, these results suggested that they functioned as protectants of the mycelium from physiological stresses due to shaking, and this resulted in a remarkable increased citric acid productivity in shake culture.

Chapter 7 deals with effects of culture conditions on extracellular polysaccharide accumulation by citric acid-producing strain of *A. niger*. For *A. niger* Yang no.2, culture conditions such as viscosity of medium, volume of medium and shaking speed influence citric acid and extracellular polysaccharide production. The increase of shaking speed caused a remarkable increase in the extracellular polysaccharide production, and consequently a great decrease of citric acid production. The addition of the excreted extracellular polysaccharide to the culture medium resulted in a considerable decrease of extracellular polysaccharide production but an equally considerable increase of citric acid production. Therefore these results support the idea that *A. niger* Yang no.2 excretes the extracellular polysaccharide to protect itself from physiological stresses in shake culture.

Chapter 8 concludes the thesis with a few final remarks.