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# The production of citric acid by using immobilized *Aspergillus niger* A-9 and investigation of its various effects

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**Abstract**

The production of citric acid was achieved by using *Aspergillus niger* conidiaspores, entrapped in Ca-alginate beads, and the factors that affect this production were investigated. The effects of starting sucrose concentration (100–180 g/l), nitrogen concentration (0–0.3 g/l), methanol concentration (0–6 ml) and finally ethanol concentration (0–5 ml) in 100 ml feeding medium on citric acid production were studied and optimum experimental conditions were determined. The starting nitrogen concentration (0.05 g/l) and the starting sucrose concentration (140 g/l) were optimized and maximum citric acid production observed under these given conditions. Maximum citric acid production was observed upon addition of 4.0 ml methanol and 3.0 ml ethanol.


*Keywords:* Immobilization; Alginate; Citric acid; Methanol; Ethanol; *Aspergillus niger*

## 1. Introduction

Citric acid, present in all citrus fruits, was first crystallized from lemon juice in the form of calcium citrate. Production of citric acid from sugar solutions by aerobic bioprocesses was first realized by using *Penicillium*. Due to low yields obtained from *Penicillium*, *Aspergillus niger* was utilized in subsequently developed processes (Shuler, 2002). Citric acid, with an estimated annual production of 500,000 tons, is produced almost exclusively by fermentation with *A. niger* and widely used in the food, chemical, pharmaceutical and other industries (Jianlong & Ping, 1996).

Cells have been commonly entrapped in a gel matrix through which substrates and products diffuse (in and out) easily. Agar, agarose, kappa-carrageenan, collagen, alginate, chitosan or cellulose could be used as a gel matrix. Some of these are expensive and also have weak mechanical strength. However, alginate is very mild and used for the entrapment of animal cells, mitochondria, chloroplasts, protoplasts and red blood cells (Park & Chang, 2000).

One of the earliest reports of immobilized enzymes was written by Nelson and Griffin (1916). They showed the adsorption of invertase on charcoal and alumina and demonstrated that these immobilized enzymes were retained. In addition, many techniques for immobilizaton have been developed in the past few decades. At present, enzymes as well as microbial cells, spores and organelles (such as chloroplasts and mitochondria) can be immobilized on suitable carriers while maintaining intact biochemical reactions (Tsay & To, 1987). Immobilized cells have been used for production of organic acids, amino acids, antibiotics, enzymes, alcohol and other compounds. Compared with free-cell systems, immobilized cell techniques have several advantages, such as higher production rate and easier product separation (Jianlong, 2000).

The aim of this work, the production of citric acid, was achieved by using *A. niger* conidiaspores, entrapped in Ca-alginate beads, and the effects of nitrogen, sucrose, methanol, ethanol concentration and re-use numbers on citric acid production were investigated.

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## 2. Materials and methods

### 2.1. Microorganism

Lyophilized cultures of *A. niger* A-9 were obtained from Ankara University Agriculture Faculty (Ankara/Turkey). A loopful of *A. niger* A-9 was spread on potato–dextrose–agar (PDA) and incubated for seven days at 30 °C. After seven days of incubation, 40 ml of sterilized cold water were added to each Petri dish and then the conidia suspension was prepared.

### 2.2. Fermentation medium

The fermentation medium for citric acid production had the following composition (g/l): sucrose, 140; $NH_4NO_3$, 0.05; $KH_2PO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, $0.6 \times 10^{-4}$; $CaCl_2$, 3.0; $ZnSO_4 \cdot 7H_2O$, $2.5 \times 10^{-4}$; $FeSO_4 \cdot 7H_2O$, $1.3 \times 10^{-3}$. The experiments were carried out in a constant-temperature shaker at 30 °C and 100 rpm.

### 2.3. Immobilization

*A. niger* was immobilized in Ca-alginate beads. About, 280 ml of conidiaspore suspension were added to 120 ml of Na-alginate solution (3% w/v) and then Na-alginate cell suspension solution was prepared. This solution was dropped into 0.27 M $CaCl_2$ solution by using 2 ml pipettes. Then, conidiaspores of *A. niger* were entrapped in Ca-alginate spherical beads. The immobilized conidiaspores were washed twice with distilled sterile water. Ca-alginate beads were stored in 0.027 M $CaCl_2$ solution at 4°C (Bayraktar & Mehmetoglu, 2000).

### 2.4. Preactivation of immobilized cells

Ten grammes of spherical immobilized cells were suspended in a 250 ml Erlenmeyer flask containing 100 ml of citric acid production medium. Immobilized cells were incubated in a constant-temperature shaker at 30 °C and 100 rpm during two days. The pH of the medium was 4.5 and airflow rate was 0.5 l/min.

### 2.5. Citric acid production

Ten grammes of the preactivated cells were washed thoroughly (three times) with distilled sterile water and then placed in a 250 ml Erlenmeyer flask containing 100 ml of substrate solution. The pH of the medium was 4.5 and the $O_2$ flow rate was 0.5 l/min. The experiments were carried out in a constant-temperature shaker at 30 °C and 100 rpm. Citric acid in the aqueous phase was analyzed by a spectrophotometric method (Marrier & Boulet, 1958).

## 3. Results and discussion

### 3.1. Effect of fermentation period on the citric acid production

Citric acid production with immobilized *A. niger* cells at pH 4.5 is showed in Fig. 1. Maximum citric acid production was observed at four days and citric acid production was decreased after four days because of the feed-back inhibition. By the end of this period, a great amount of citric acid was produced, and it could not penetrate to the outside of the cell. The accumulated citric acid in the cell inhibited citrate synthase (Cevrimli, 2000; Jianlong, 2000).

### 3.2. Effect of initial nitrogen concentration on citric acid production

The effect of the initial nitrogen concentration on citric acid production, by using immobilized *A. niger* cell during the four day fermentation period, was studied at a constant temperature of 30 °C.

To investigate the effect of initial nitrogen concentration on citric acid production, a low concentration of nitrogen was used. As shown in Fig. 2, the maximum citric acid production was obtained at 0.05 g/l of nitro-

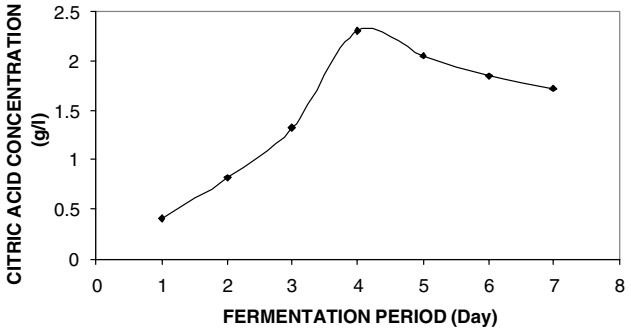


Fig. 1. Effect of fermentation period on citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l.

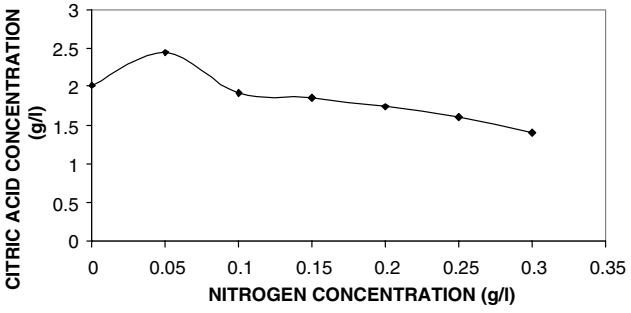


Fig. 2. Effect of initial nitrogen concentration on citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $t = 4$ days.

gen. At higher nitrogen concentration citric acid production decreased. Citric acid production significantly decreased at 0.3 g/l of nitrogen.

The influence of nitrogen on the production of citric acid can be explained by the observations of Kristiansen and Sinclair (1979). The cytoplasm in the hyphae flows toward the tip where the new cells are formed. Meanwhile, aged cells suffer from nitrogen limitation, become carbon stores, and will produce citric acid. The number of cells produced will increase with the nitrogen concentration, and a similar increase will be observed in the flow of cytoplasm toward new cells. If the nitrogen concentration were increased, the rate of formation of storage cells would increase, resulting in higher yields of citric acid. It is known that citric acid is produced in the mitochondria. If the flow is significant, streaming of cytoplasm is transported to nonproducing tip of hyphae, which is not suffering nitrogen limitation and citric acid production. According to these facts, citric acid concentration would decrease at both lower and higher nitrogen levels. For this reason, the optimum nitrogen concentration must be used.

### 3.3. Effect of initial concentration of sucrose on citric acid production

The effect of initial sucrose concentration on citric acid production, by using immobilized *A. niger* cell for a four day fermentation period, was studied under optimum conditions (pH 4.5, nitrogen concentration 0.05 g/l) at a constant temperature of 30 °C.

As shown in Fig. 3, maximum citric acid production was obtained at 140 g/l of sucrose. Tsay and To (1987) reported that maximum citric acid production was obtained at 140 g/l of sucrose. When the sucrose concentration was higher than 140 g/l, citric acid production decreased, due to polyalcohol formation (Gutierrez-Rozas, Cordova, Auria, Revah, & Favela-Torres, 1995). Citric acid production decreased at lower sucrose concentration because of oxalic acid formation (Honecker, Bisping, Yang, & Rehm, 1989).

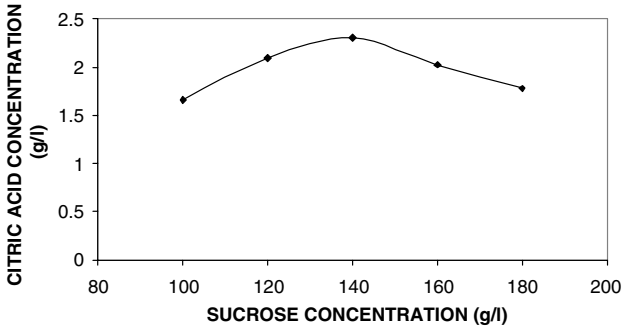


Fig. 3. Effect of initial sucrose concentration of citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_N = 0.05$ g/l, $t = 4$ days.

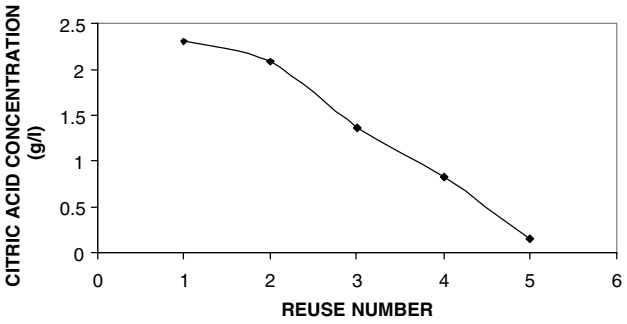


Fig. 4. Reuse number of immobilized *A. niger* on citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l, $t = 4$ days.

### 3.4. Reuse number of immobilized A. niger on citric acid production

One of the most important benefits of immobilized cells is their re-use. In other words, they can be used with media replacements. Reuse numbers of immobilized *A. niger* was investigated in citric acid production. Immobilized *A. niger* is taken from fermentation media after four days and put into new sterile fermentation media. This process was repeated five times and the obtained experimental results are shown in Fig. 4.

Citric acid production decreased while re-use number increased. This behaviour could be attributed to clogging of the pores of the *A. niger* immobilized with Ca-alginate gel. Thus, citric acid produced inside the gel accumulated and could not penetrate to the outside of the gel. As a result, the accumulation of the citric acid inside the cell inhibited the citrate synthase found in the citric acid cycle (Lehninger, Nelson, & Cox, 1993) and citric acid production decreased (Ates, Dingil, Bayraktar, & Mehmetoglu, 2002).

### 3.5. Effect of methanol and ethanol concentration on citric acid production

The effect of the amounts of both methanol and ethanol were investigated to obtain optimum citric acid production. The experimental results are shown in Tables 1 and 2.

Table 1
Effect of methanol concentration on citric acid production

| Methanol concentration (%) (v/v) | Citric acid concentration (g/l) |
|---|---|
| 0 | 2.31 |
| 3 | 2.34 |
| 4 | 3.49 |
| 5 | 2.03 |
| 6 | 1.15 |

$T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l, $t = 4$ days.

Table 2
Effect of ethanol concentration on citric acid production

| Ethanol concentration (%) (v/v) | Citric acid concentration (g/l) |
|---|---|
| 0 | 2.31 |
| 2 | 2.60 |
| 3 | 3.42 |
| 4 | 1.71 |
| 5 | 1.19 |

$T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l, $t = 4$ days.

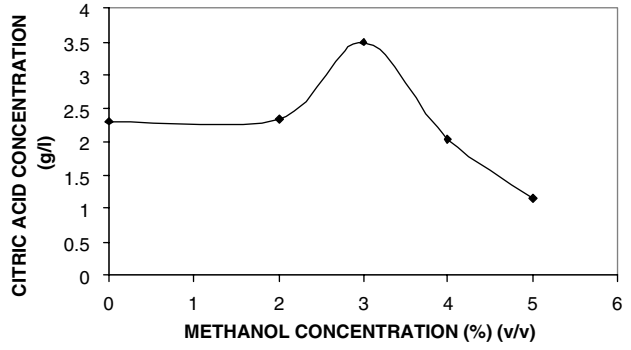


Fig. 5. The effect of methanol concentration on citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$ l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l, $t = 4$ days.

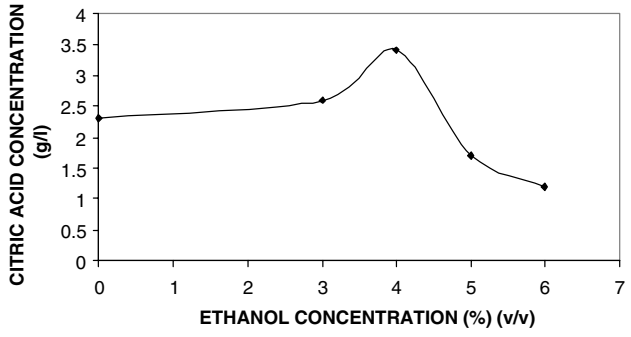


Fig. 6. The effect of ethanol concentration on citric acid production. $T = 30$ °C, $N = 100$ rpm, pH 4.5, $Q_v = 0.5$l/min of air, 0.1 g of pellet/ml of fermentation media, $C_{Su} = 140$ g/l, $C_N = 0.05$ g/l, $t = 4$ days.

Citric acid production continuously increased by adding up to 4 ml methanol and 3 ml ethanol per 100 ml fermentation media (Figs. 5 and 6). Maximum citric acid production was observed at these methanol and ethanol concentrations. The observed increments in citric acid concentration showed that methanol and ethanol had a profound effect on the metabolism of sugars by *A. niger*. The mechanism by which methanol and ethanol stimulate citric acid production from sugars is not clear (Maddox, Hossain, & Brooks, 1986) and the effects of methanol and ethanol are at the cell permeability level, allowing metabolites to be excreted from the cell (Haq, Ali, Qadeer, & Iqbal, 2003; Rouskas, 2000).

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