0273-2289/00/87/0117/$12.25

Production of Citric Acid Using Immobilized Conidia of *Aspergillus niger*

EMINE BAYRAKTAR AND ÜLKÜ MEHMETOGLU*

Chemical Engineering Department, Science Faculty, Ankara University, 06100 Tandogan, Ankara, Turkey, E-mail: mehmet@science.ankara.edu.tr

Received November 10, 1998; Revised October 14, 1999; Accepted November 4, 1999

Abstract

Conidia of *Aspergillus niger* were immobilized in calcium alginate gel for the production of citric acid. First, the type of the preactivation medium, together with the preactivation period, was investigated. It was found that *A. niger* requires a 2-d preactivation period at a 0.05 g/L NH_4NO_3 concentration. Second, preactivated cells were used to determine the effects of nitrogen concentration and the flow rate of oxygen and air on the production of citric acid. Maximum citric acid production was attained with medium containing 0.01 g/L of NH_4NO_3. The rate of citric acid production in the nitrogenous medium was 33% higher when oxygen was used instead of air during the production phase. This corresponds to an increase of 85% when compared to production when neither oxygen nor air was fed into the system. In the nonnitrogenous medium citric acid concentration remained similar regardless of the use of air or oxygen. However, in the nonnitrogenous production medium, citric acid production was not influenced considerably when oxygen was used instead of air. The advantage of using immobilized cells is that production is achieved easily in the continuous system. Therefore, citric acid production was also tested using a packed-bed bioreactor, and an increase in productivity by a factor of 22 was achieved compared to the batch system.

Index Entries: *Aspergillus niger*; citric acid; immobilization; Ca alginate particles; packed-bed bioreactor.

Introduction

Citric acid is a broadly used chemical with extensive industrial importance. However, it has limited production by *Aspergillus niger* owing to

*Author to whom all correspondence and reprint requests should be addressed.

severe technological difficulties. Citric acid has been produced by conventional submerged culture in which the biomass is suspended in the medium *(1)*. Therefore, its separation from the medium and the biomass is difficult. The fermentation method using the immobilized biomass, on the other hand, provides ease of separation for the product, and thus continuous production of citric acid can be readily achieved. By using immobilized cells, the process can be controlled more easily than with a batch system of free cells. In addition, immobilized cells are more stable than the free mass. Recently, various investigations concerning citric acid production with immobilized *A. niger* have been reported in the literature *(2–6)*. However, there is little information about the preactivation media and preactivation periods. The nitrogen concentration in the medium is essential for the growth of mycelium and production of citric acid. The growth of mycelium may be confined by limiting the nitrogen source.

In the present study, the effect of the nitrogen concentration of the preactivation medium and the effect of the preactivation period on the growth of the microorganisms as well as on the production of citric acid were investigated. In addition, the effect of nitrogen concentration of the production medium and the effects of air and oxygen flow rates on citric acid production were determined. Citric acid production was also carried out in a packed-bed bioreactor and the results were compared to those obtained from a batch system.

Materials and Methods

Microorganism and Immobilization

Lyophilized cultures of *A. niger* NRRL-2270 were obtained from the Northern Regional Research Laboratory (Peoria, IL). A loopful of *A. niger* conidia was inoculated in potato-dextrose agar slants and incubated at 30°C. After 7 d of incubation, 40 mL of sterilized cold water was added to each Petri dish. The conidia suspension was then collected. Thus, a 2.21×10^5 conidia/mL suspension was produced. By using this suspension as described above, 0.1 g of Na alginate/L of conidia solution was prepared and then dropped into 0.27 *M* $CaCl_2$ solution by using 2 mL-pipets in order to prepare the immobilized conidia. Then, conidia of *A. niger* were entrapped in Ca alginate pellets of approx 3 mm in diameter. The amount of conidia was 5.6×10^5 conidia/g of pellet. Ca alginate pellets were stored in 0.027 *M* $CaCl_2$ solution at 4°C.

Preactivation of Immobilized Cells

The immobilized cells were washed thoroughly three times with distilled sterile water, and 20 g of spherical immobilized cells were suspended in a 250-mL Erlenmeyer flask containing 100 mL of substrate solution. The growth medium contained 140 g/L of sucrose, 0.05–0.4 g/L of NH_4NO_3, 1.0 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4{\cdot}7H_2O$, 3.0 g/L of $CaCl_2$, 1.3 mg/L of

$CuSO_4{\cdot}5H_2O$, 0.06 mg/L of $ZnSO_4{\cdot}7H_2O$, and 0.25 mg/L of $FeSO_4{\cdot}7H_2O$. The pH of the medium was 4.5 and the O_2 flow rate was 0.075 L/min. Microorganisms were incubated in a constant-temperature shaker at 30°C and 150 rpm.

Citric Acid Production with Preactivated Immobilized Cells

Batch Operation

Twenty grams of the preactivated cells was washed thoroughly three times with distilled sterile water and then added into a 250-mL Erlenmeyer flask containing 100 mL of substrate solution. The production medium contained 40–180 g/L of sucrose, 0–0.4 g/L of NH_4NO_3, 1.0 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4{\cdot}7H_2O$, 3.0 g/L of $CaCl_2$, 1.3 mg/L of $CuSO_4{\cdot}5H_2O$, 0.06 mg/L of $ZnSO_4{\cdot}7H_2O$, and 0.25 mg/L of $FeSO_4{\cdot}7H_2O$. The pH of the medium was 4.5 and the O_2 flow rate was 0.075 L/min. The experiments were carried out in a constant-temperature shaker at 30°C and 150 rpm.

Continuous Operation

Continuous operations were carried out in a temperature-controlled packed-bed bioreactor with a height and an inner diameter of 0.35 and 0.028 m, respectively. Preactivated cells (244 g) were prepared as described in Materials and Methods, and the immobilized cells were supported on a sintered glass distributor plate with a 90- to 150-μm pore size. The production medium contained 40–80 g/L of sucrose, 1.0 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4{\cdot}7H_2O$, 3.0 g/L of $CaCl_2$, 1.3 mg/L of $CuSO_4{\cdot}5H_2O$, 0.06 mg/L of $ZnSO_4{\cdot}7H_2O$, and 0.25 mg/L of $FeSO_4{\cdot}7H_2O$. The pH of the medium was 3.5 and the O_2 flow rate was 0.075 L/min.

Analyses

Immobilized cells were washed thoroughly three times with distilled sterile water and dried at ambient temperature for 24 h. Dried particles were weighted and counted. The number of conidia was determined by using the Thoma Lam under a microscope *(7)*. Citric acid was analyzed according to the method of Marier and Boulet *(8)*. Sucrose concentration was determined by a Shimadzu Model HPLC using an Aminex CR column. H_2SO_4 (pH 2.0) was used as the eluent, and the flow rate was kept at 0.6 mL/min. The temperature of the column was maintained at 65°C.

Results and Discussion

Citric acid fermentation was generally considered type II fermentation *(1)*. This type is characterized by two phases: growth and product formation. The first phase can also be described as the growth phase and the second one as the production phase. In this study, the first phase was achieved during the preactivation period. It is known that nitrogen affects both mycelium formation and citric acid production. Therefore, the effects of nitrogen concentration on both phases were determined.

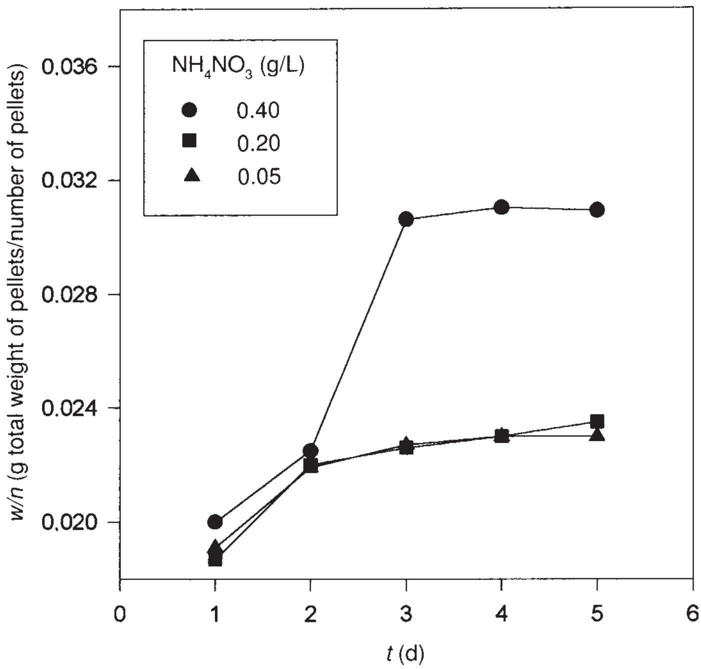

Fig. 1. Effect of nitrogen concentration and time on mycelium formation. $T = 30°C$; $N = 100$ rev/min; $C_{S0} = 140$ g/L; pH = 4.5, $Q_v = 0.1$ L/min air; $d_p = 3$ mm; 0.2 g of pellet/mL of fermentation media.

Determination of Optimum Nitrogen Concentration and Preactivation Period

To determine the effect of nitrogen on the preactivation of immobilized microorganisms, experiments were conducted at concentrations of 0.40, 0.20, and 0.05 g/L of NH_4NO_3. The results indicate that the weight per pellet (grams of total weight of pellets/number of pellets) is maximum for the 0.4 g/L NH_4NO_3 medium (Fig. 1). The amount of microorganism increased with increasing nitrogen concentration. However, with media containing 0.05 and 0.20 g/L of NH_4NO_3, no change in weight was observed after 2 d. To examine the effect of preactivation at various NH_4NO_3 concentrations on citric acid production, the preactivated pellets were transferred into the medium containing 0.05 g/L of NH_4NO_3. Contrary to our expectations, we found that the pellets preactivated under lower nitrogen concentration resulted in higher citric acid yield (Fig. 2). This may be explained by the extensive mycelium formation on the surface of the pellets when preactivated at higher NH_4NO_3 concentrations. Excessive mycelium might clog the pores and decrease the rate of citric acid production. Accordingly, the nitrogen concentration during the preactivation period should not exceed 0.05 g/L of NH_4NO_3, and the preactivation period of 2 d was found to be satisfactory for the experiments. Eikmeier and Rehm *(9)* suggested the preactivation period as 2 d, whereas Gupta and Sharma *(10)* reported a period of 6 d.

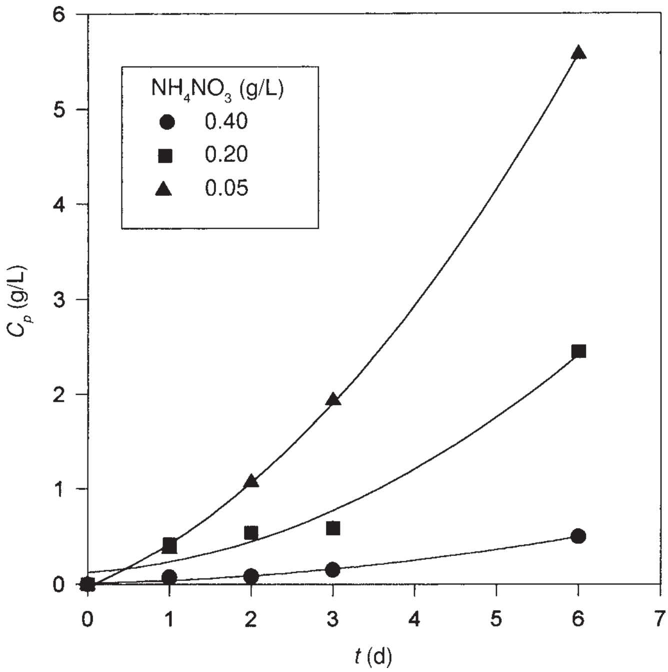

Fig. 2. Effect of preactivation in various nitrogen concentrations on citric acid production. $T = 30°C$; $N = 100$ rev/min; $C_{S0} = 140$ g/L; pH = 4.5; $Q_v = 0.1$ L/min air; $d_p = 3$ mm; 0.2 g of pellet/mL of liquid.

Effect of Nitrogen Concentration on Citric Acid Production

As previously mentioned, nitrogen affects both the growth of microorganism and the production of citric acid. Therefore, the effect on production was examined with media containing 0.4, 0.05, 0.01, and 0.0 g/L of NH_4NO_3 (Fig. 3). The maximum amount of citric acid was produced with 0.01 g/L of NH_4NO_3. However, the variation in citric acid production was not significant under NH_4NO_3 concentrations of 0.05 and 0.01 g/L. On the other hand, citric acid production decreased remarkably under a nitrogen concentration of 0.4 g/L. The influence of nitrogen on the production of citric acid can be explained by the observations of Kristiansen and Sinclair *(11)*. Accordingly, the cytoplasm in the hyphae flows toward the tip where the new cells were 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 apparently significant, streaming of cytoplasm is transported to nonproducing tip of hyphae, which is not suffering nitrogen limitation and the citric acid production. According to these facts, citric acid concentration would decrease

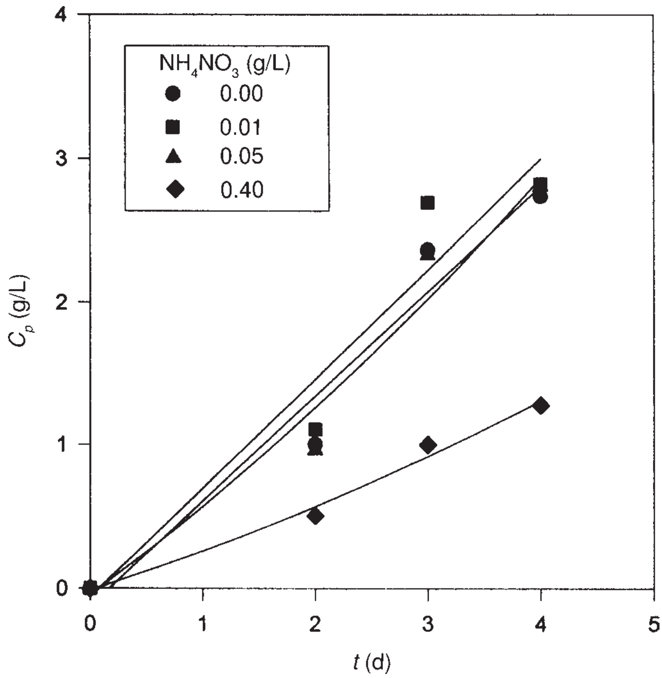

Fig. 3. Effect of nitrogen concentration on citric acid production medium. $T = 30°C$; $N = 100$ rev/min; $C_{S0} = 140$ g/L; pH = 4.5; $Q_v = 0.1$ L/min air; $d_p = 3$ mm; 0.2 g of pellet/mL of fermentation media.

at both lower and higher nitrogen levels. For this reason, the optimum nitrogen concentration must be used.

Effect of Air and Oxygen on Citric Acid Production by Immobilized Microorganisms

To determine the effects of air and oxygen on citric acid production, experiments were conducted with and without feeding oxygen or air. The media were used with and without NH_4NO_3 in these experiments. The flow rates of oxygen and air were 0.075 and 0.1 L/min, respectively.

Figure 4 illustrates the effect of air and oxygen in the nitrogenous medium. Citric acid concentration increased with O_2 feeding. The production rate was 33% higher when oxygen was used instead of air, which also corresponds to an increase of 85% compared to the production rate when neither oxygen nor air was fed into the system.

On the other hand, for the nonnitrogenous system, Fig. 5 shows a change in citric acid concentration with O_2 feeding. Citric acid concentration in the presence of O_2 increased by 42% compared to without feeding O_2 or air. Also, citric acid concentration did not change much when O_2 was fed instead of air in the nonnitrogenous medium. Because the existence of nitrogen provokes cell production, the conclusion may be reached that oxygen is necessary for more cell production.

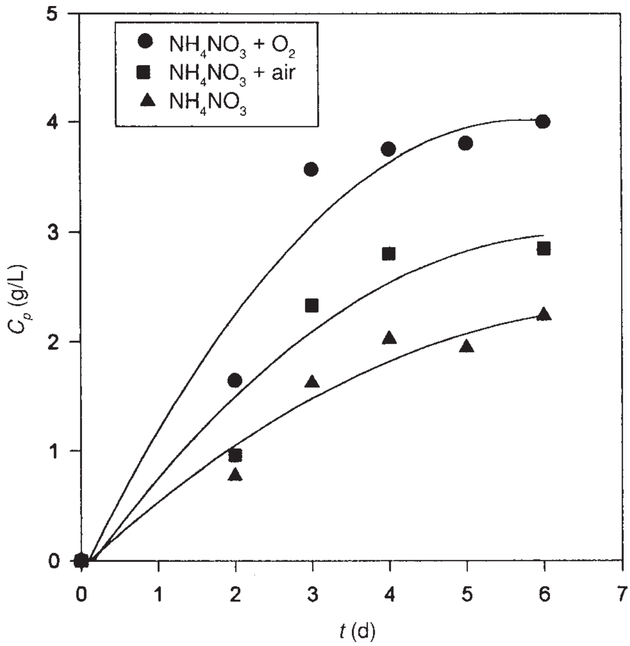

Fig. 4. Effects of oxygen and air on citric acid production in nitrogenous medium. $T = 30°C$; $N = 100$ rev/min; $C_{S0} = 140$ g/L; pH = 4.5; $d_p = 3$ mm; 0.2 g of pellet/mL of fermentation media.

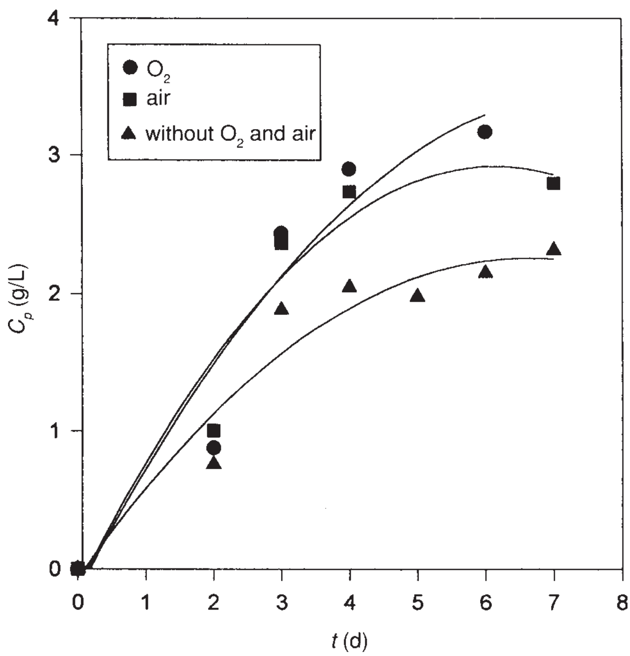

Fig. 5. Effects of oxygen and air on citric acid production in nonnitrogenous medium. $T = 30°C$; $N = 100$ rev/min; $C_{S0} = 140$ g/L; pH = 4.5; $d_p = 3$ mm; 0.2 g of pellet/mL of fermentation media.

Table 1
Changes of Citric Acid Productivity and Sucrose Concentration with Dilution Rate in Packed Bioreactor

	Y_{CA} (g/[L·h])		C_S (g/L)	
C_{S0} (g/L)	D_1 = 0.12 (1/h)	D_1 = 0.23 (1/h)	D_1 = 0.12 (1/h)	D_1 = 0.23 (1/h)
40	0.165	0.291	22.5	27.6
60	—	0.311	—	39.0
80	0.189	0.314	29.0	48.4
100	—	0.344	—	58.7

The effect of O_2 or air on citric acid production under different nitrogen concentrations has not been reported in the literature. However, it has been indicated that an increase in O_2 concentration leads to an increase in citric acid concentration in nitrogenous medium *(1,11,12)*.

Citric Acid Production in Packed-Bed Bioreactor

Citric acid production was also carried out in a packed-bed bioreactor using pellets preactivated in a batch bioreactor. Experiments were performed with 0.12 and 0.23 h^{-1} dilution rates; 0.15 L/min O_2 flow rate; and concentrations of 40, 60, and 80 g/L of sucrose. Citric acid production in the packed-bed bioreactor was compared to that of the batch reactor (*see* Table 1). Table 1 also shows the change of sucrose concentration with dilution rates. Citric acid production increased and sucrose concentration decreased with increasing dilution rates (from 0.12 to 0.23 h^{-1}) for all initial sucrose concentrations. Citric acid production in the packed-bed bioreactor increased by about 12–22 times with respect to the productivity in the batch bioreactor, depending on the dilution rate. The major difficulty encountered with the system used in our study was the decrease in citric acid production observed after about 7 d of continuous operation. This finding was also reported previously for a similar system *(3)*.

Conclusion

The results have shown some important aspects of citric acid production by immobilized *A. niger* using sucrose solutions. The presence of nitrogen in either the preactivation medium or the production medium was essential to achieve maximum citric acid production. Citric acid production increased when pure O_2 instead of air was fed into the nitrogenous medium. However, using either O_2 or air in the nonnitrogenous medium did not affect production. We studied citric acid production by immobilized *A. niger* in a continuous system as well as a batch system. The maximum productivity was about 22 times higher in the continuous reactor compared to the batch one. Unfortunately, in the continuous system, the loss of activity and reactor blockage was encountered during prolonged reaction times. Therefore, further studies are necessary in order to overcome these problems.

Acknowledgments

We gratefully acknowledge the funding of this work by Ankara University Research Fund (project no. 98-05-04-07) and Statement Planning Organization, DPT (project no. 96K120410).

Nomenclature

C_p = citric acid concentration (g/L)
C_S = substrate concentration (g/L)
C_{So} = initial substrate concentration (g/L)
D_1 = dilution rate (1/h)
d_p = pellet diameter (mm)
N = stirring rate (rpm)
Q_v = aeration rate (vvm)
T = temperature (°C)
t = time (d)
Y_{CA} = productivity of citric acid (g/[L·h])
wn = g total weight of pellets/number of pellets

References

1. Röhr, M., Kubicek, C. P., and Kominek, J. (1983), *Biotechnology*, vol. 3, Rehm, H. J. and Reed, G., eds., Verlag Chemie, Weinheim, Germany, pp. 420–465.
2. Eikmeier, H. and Rehm, H. J. (1987), *Appl. Microbiol. Biotechnol.* **26,** 105–111.
3. Horitsu, H., Adachi, S., Takahashi, Y., Kawai, K., and Kawano, Y. (1985), *Appl. Microbiol. Biotechnol.* **22,** 8–12.
4. Honecker, S., Bisping, B., Yang, Z., and Rehm, H. J. (1989), *Appl. Microbiol. Biotechnol.* **31,** 17–24.
5. Tsay, S. S. and To, K. Y. (1987), *Biotechnol. Bioengin.* **29,** 297–304.
6. Hamamci, H. and Hang, Y. D. (1989), *Biotechnol. Techniques* **3,** 51–54.
7. Bayraktar, E. (1998), PhD thesis, Ankara University, Ankara, Turkey.
8. Marier, J. R. and Boulet, M. (1958), *J. Dairy Sci.* **4,** 1683–1692.
9. Eikmeier, H. and Rehm, H. J. (1984), *Appl. Microbiol. Biotechnol.* **20,** 365–370.
10. Gupta, S. and Sharma, C. B. (1994), *Biotechnol. Lett.* **16(6),** 599–604.
11. Kristiansen, B. and Sinclair, C. G. (1979), *Biotechnol. Bioeng.* **21,** 297–315.
12. Dawson, M. W., Maddox, I. S., and Brooks, J. D. (1989), *Biotechnol. Bioeng.* **33,** 1500–1504.