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Effect of the Copper Concentration on Citric Acid Productivity by an *Aspergillus niger* Strain

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INTRODUCTION

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. Although citric acid can be obtained by chemical synthesis, its cost is much higher than fermentation (*1*). There are many microorganisms, including fungi, yeasts, and bacteria, that can produce citric acid by fermentation. *Aspergillus niger* is one of the most well-known citric acid producers (2). This mold is commonly used as a "pellet" in the submerged processes of citric acid production. The structure of these pellets has a great influence on productivity. In citric acid production, small pellets with fluffy centers and lax surfaces are specially advised (3). The structural properties and mold physiology in pellet growth are strongly dependent on medium composition and operating conditions. These factors are decisive to pellet productivity and stability. The influence of ion concentration in the production stage has been studied by several workers (4,5). From the results of preliminary assays, we concluded that copper

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concentration at the stage of pellet formation can be very important. In order to confirm this assumption, suitable studies were carried out. Pellet formation was performed in the presence of different Cu(II) concentrations. The pellets obtained were used as the inoculum for the production stage. The corresponding citric acid accumulation is being reported; the results obtained are discussed in this paper.

MATERIALS AND METHODS

Strains isolated from the original *A. niger* NRRL 1419 (Northern Regional Research Laboratory, US Department of Agriculture) were selected by methods developed in our laboratory (*6*). Mold culture was carried out on agar medium.

Culture Media

Media formulation is based on Shu and Johnson's proposed (*7*) sporulation. Medium 1, (pH 5.5–6.0): 7.5 g/L glycerine; 5 g/L yeast extract; 10 g/l NaCl; 0.005 g/L $CuSO_4$; 0.006 g/L KH_2PO_4; 0.0016 g/L $(NH_4)_2(FeSO_4)_2$; 0.0001 g/L $CuSO_4 \cdot 5H_2O$; 0.25 g/L $CaSO_4$; 25 g/L agar-agar. Medium 2, pellet formation (pH 3.0): 150 g/L sucrose; 2 g/L yeast extract; 0.1 g/L KH_2PO_4; 2 g/L NH_4NO_3; 0.2 g/L $MgSO_4$; 0.00016 g/L $FeCl_3$; 0.078 g/L $CuSO_4 \cdot 5H_2O$. Medium 3, production (pH 3.0): 150 g/L sucrose; 2 g/L yeast extract; 0.1 g/L KH_2PO_4; 0.2 g/L $MgSO_4$; 0.0016 g/L $FeCl_9$; $CuSO_45H_2O$ it changed in each case. (HCl 1,5 N). All chemicals are in analytical grade.

Culture

Static culture on solid media were incubated at 28–30°C for an appropriate time. The submerged cultures were carried out in 1-L Erlenmeyer flasks with stainless steel beffles (UNSL, Argentine). Media and equipment were sterilized at 120°C for 15 min. The medium volume used in all cases was one-fourth of the flask volume. A spore suspension from selected cultures was prepared. This was used as inoculum so that the end concentration was 500–700 spores/mL of medium 2. The flasks were incubated at 28–30°C on a rotating shaker (2.5 eccentricity) at 140 rpm operating speed. All the pellets obtained were placed in medium 3 at different Cu concentrations and incubated as described.

Analytical Techniques

Dry Weight

The sample taken from the flask was filtered on no. 2 filter paper. The filter cake was washed twice with 50-mL aliquots of distilled water, dried at 85°C for 12 h, and cooled in a desiccator.

Citric Acid

The modified spectrophotometric method by Hartford (*8*) was used to determine the amount of citric acid in the filtrate.

RESULTS AND DISCUSSION

The germination of *A. niger* spores in a standard medium for pellet formation (no. 2, proposed by Shu and Johnson [7]) was observed microscopically. At 8 h of culture, the appearance and elongation of the germinal tube was detected. Proliferation and swelling of hyphae took between 25 and 30 h. After 36 h of cultivation, the microorganism was present in the form of pellets having thick and bulbous hyphae; after which the diameter of the pellets slightly increased.

The following experiments were made to confirm if either the development or the final result of these stages was altered by changes in Cu(II) concentration. The *A. niger* culture was carried out in medium 2 under the conditions described in Materials and Methods by changing the Cu(II) concentration present in the medium as shown in Table 1, from 0, 78, 100, 120 to 156 mg of $CuSO_4 \cdot 5H_2O$/L. The pellets obtained are the inoculum for the production stage. Medium 3 was used for citric acid production (Materials and Methods). The final citric acid concentrations are shown in Table 1. The experiments were carried out fourfold. It was observed that, in medium 2 without Cu(II), the formation stages described are not fulfilled. The pellets formed are less (20 pellet/mL) irregular in form (0.3 at 0.9 mm diameter) with abundant filamentous mycelium growth increasing in time. This inoculum yielded a very low citric acid content (4 g/L) in the production medium.

As desired, fluffy loose and 0.5 mm diameter pellets were formed by adding 78 mg/L of $CuSO_4 \cdot 5H_2O$. The acid concentration reached 60.2 g/L, which was very interesting for experiments in Erlenmeyer flasks (Table 1). The dry weight was slightly higher in this case.

The presence of $CuSO_4 \cdot 5H_2O$ concentration higher than 100 mg/L did not have any [major] effect on the structure and external aspect of the pellets, but [it] affected acid production which remarkably decreased (35.5 g/L; 32.0 g/L and 18.66 g/L).

The results presented show that the great number of pellets with a suitable structure improves citric acid production. According to Kristiansen and Sinclair (*9*), cells capable to accumulating and excreting citric acid would be the "oldest" cells. These are behind the free extremes of the hyphas. Therefore, the more free extremes, the more "oldest" cells of adequate production age. In order to get a large number of these free extremes, it is necessary to get more pellets to equal biomass. Table 1 shows that the Cu concentration is very important in getting fluffy and smaller pellets. The highest pellet number was found at 78 mg/L of $CuSO_4 \cdot 5H_2O$.

Table 1
Pellet Structure and Final Citric Acid Concentration by *A. niger* with Different Cu(II) Concentrations in Medium 2

$CuSO_4 \cdot 5H_2O$ in medium 2, mg/L	Pellet structure, 36 h	Citric acid, 120 h, g/L
0	Irregulars in form Less (20 pellet/mL) Lax and fluffy Filamentous growth Aleatory	4
78	Regulars in form Smalls (0.5 mm) Laxs and fluffy Abundants (200/mL) Repeater	60.5
100	Regulars in form	35.5
120	Smalls (0.7–1 mm)	32.0
156	Laxs and fluffy Abundants (150/mL) Repeater	18.6

This effect is related to an influence on the mechanism for pellet formation. In the literature review, the general influence of metallic ions on pellet structure and morphology was reported (*3*). (In analyzing citric acid data, it was also observed that there is an influence of Cu(II) concentration higher than that of structure change.) Some authors believe that Cu may stimulate citric acid production by inhibiting aconitase (EC 4.2.1.3) (*10*); others reject this possibility, finding no alterations in the citrate-isocitrate ratio when adding Cu under the conditions of citric acid production. However, they observed favorable changes in citric acid yield as well (*11*). In our experiments, Cu concentration changes were carried out at the "pellet formation stage," during the mold growth. Thus, the existence of an inhibition on the part of Cu during the aconitase synthesis can be assumed. Since new biomass formation is avoided later at the "production stage," this inhibition would be irreversible. A possible form of inhibition would be competing with Fe (II), which is a structural component of the aconitase molecule. The gradual decrease in the number of pellets of Cu concentration higher than 78 g/L of $CuSO_4 \cdot 5H_2O$ becomes stronger and more evident from 156 g/L owing to the toxic effect that this cation exerts on the growth (*12*). The experiments carried out by adding different Cu(II) quantities in the production stage (medium 3) (once the pellets are formed) only produce a slight increase in productivity, and without a remarkable influence (Table 2), as reported by other authors (*11*).

Table 2
Citric Acid Production by *A. niger*
with Different Cu(II) Concentrations in Medium 3

$CuSO_4 \cdot 5H_2O$ in medium 2, mg/L	$CuSO_4 \cdot 5H_2O$ in medium 3, mg/L	Citric acid, 120 h, g/L
78	0	55.0
78	78	60.0
78	100	57.0
78	120	51.0

From the present work, we conclude that the presence of different Cu(II) concentrations in the pellet formation medium is very important in order to enhance a suitable pellet strucutre (fluffy center and lax surface) related to major cellular physiology to citric acid production. The optimum initial $CuSO_4 \cdot 5H_2O$ concentration in our experiment was 78 mg/L. Citric acid was the only organic acid produced.

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REFERENCES

1. Kapoor, K., Chaudhrary, K., and Tauro, P. (1981), in *Prescot & Dunn's Industrial Microbiology*, Reed, G., eds., McGraw-Hill, New York, pp. 709–715.
2. Arnaud, A. and Guiraud, J. (1985), in *Biotecnologia*, Scriban, R., ed., El Manual Moderno, Mexico, pp. 84–96.
3. Metz, B. and Kossen, N. (1977), *Biotech. Bioeng.* **19,** 781.
4. Kubicek, P. and Rohr, M. (1977), *Eur. J. Appl. Microbiol. Biotechnol.* **4,** 167.
5. Sanchez-Marroquin, A., Carreño, R., and Ledezma, M. (1970), *Appl. Microbiol.* **20,** 888.
6. Benuzzi, D. and Segovia, R. (1989), *Acta Cientifica Venezolana* **40,** 195.
7. Shu, P. and Johnson, M. (1947), *J. Bacteriol.* **54,** 161.
8. Hartford, C. (1962), *Analytical Chemistry* **34,** 426.
9. Kristiansen, B. and Sinclair, C. (1979), *Biotech. Bioeng.* **21,** 297.
10. Bruckhman, E. (1961), *Biochem. Z.* **335,** 199.
11. Kubisec, C. and Rohr, M. (1985), *Appl. Environ. Microbiol.* **50,** 1336.
12. Dunn, G. M. (1985), in *Comprehensive Biotechnology*, Murray Moo-Yang, ed., Pergamon, Oxford, pp. 120.