Semicontinuous and Continuous Production of Citric Acid with Immobilized Cells of *Aspergillus niger*

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Aspergillus niger, Immobilized Cells, Citric Acid, Semicontinuous, Continuous

The citric acid excretion of Ca-alginate-immobilized cells of *Aspergillus niger* in batch culture decreased with a half-time of approximately 19 days. Reactivation of the biocatalysts by regeneration in growth medium was possible, but it was followed by a submerged sporulation of the fungus, and medium was highly contaminated with free cells. Citric acid production could better be prolonged by semicontinuous cultivation with medium exchange every 7 or 14 days, respectively. After 32 days the remaining activity in semicontinuous culture was 1.4-fold higher than in comparable batch experiments. Similar improvements were obtained with a continuous process at a dilution rate of 0.125 v/v·d, whereby medium efflux kept completely free of detaching mycelia.

Introduction

The successful technique of immobilized microorganisms as living biocatalysts, involving a more careful handling and showing often improved metabolic activities compared with free cells, was reported for the citric acid production of *Aspergillus niger* by several authors using different methods.

Adsorption of growing mycelia of *Aspergillus niger* was investigated using glass-carriers (raschig-rings) in a fixed bed reactor [1], or polypropylene discs in a rotating disc reactor [2]. Furthermore citric acid producing pellets were homogeneously immobilized by gel-entrapment in crosslinked collagen membranes [3], polyacrylamide cubes [4], agar- or k-carrageenan beads [5] and calcium alginate beads [6, 7].

But gel-entrapment of pregrown pellets was often effected by diffusional limitations of the matrices (especially for oxygen) and outgrowth of free mycelia, resulting in a diminished acid production and free-cell-contaminated media.

Therefore we employed the "immobilized growing cells technique" [8], starting with the Ca-alginate entrapment of spores of *Aspergillus niger* and their precultivation to achieve a growth-limited, citric acid producing, macroporous biocatalyst as it was previously reported [9–11]. In the present paper this work is continued towards the application of a semicontinuous and continuous culture.

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Material and Methods

Microorganism and media

Aspergillus niger-strain 180 (derived from ATCC 11414) was used in all experiments. For sporulation conditions and medium composition see Eikmeier and Rehm [10].

Immobilization

The spores of *Aspergillus niger* were immobilized in 3% Ca-alginate beads of 3 mm diameter, using a recently described immobilization equipment [11]. Na-alginate (Manugel DJX) was obtained from ALGINATE INDUSTRIES LTD. (Hamburg, FRG).

Cultivation conditions

A bubble column reactor according to Fig. 1 was filled with 150 ml of alginate beads and 250 ml medium. Sterile air was supplied from the bottom of the column at 3.0 v/v·m.

Analysis

Fermentation parameters were determined as previously reported [10].

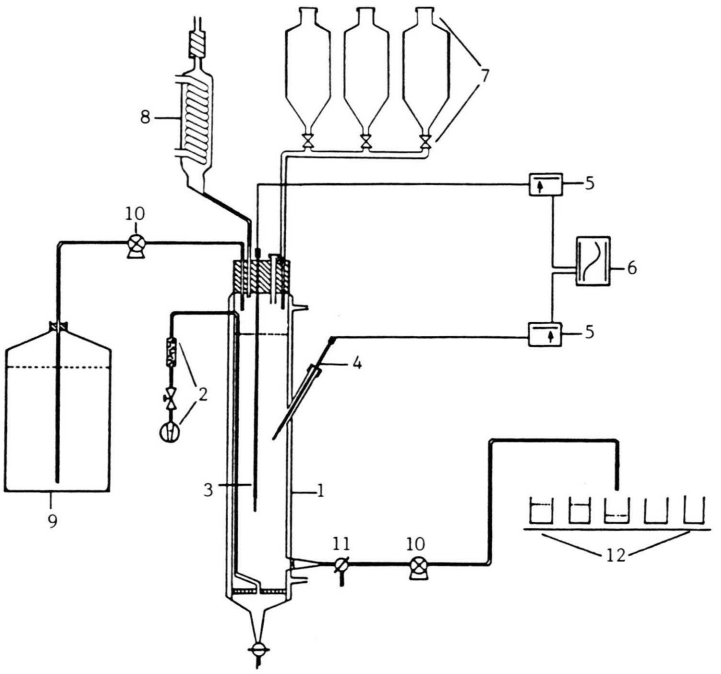

Fig. 1. Bubble column reactor for semicontinuous and continuous citric acid production. 1 = thermostated glass vessel, 2 = aeration unit, 3 = oxygen electrode, 4 = pH electrode, 5 = electrode amplifier, 6 = recorder, 7 = reservoirs for media and washing solution during semicontinuous cultivation, 8 = reflux cooling, 9 = production medium for continuous process, 10 = pump, 11 = samples, 12 = efflux.

Results

Citric acid production in batch culture

After 48 h of precultivation in growth medium the biocatalyst particles were washed with 0.9% NaCl-solution and supplied with the production medium. The development of citric acid excretion was observed for a 34 days period (Fig. 2), whereby halftime of the productivity could be estimated at 19 days as reported by Eikmeier and Rehm [10].

Citric acid production after regeneration of biocatalysts

At the end of the above described batch culture the immobilized cells of *Aspergillus niger* were regenerated by means of a secondary incubation in growth medium for 48 h, whereby the particle cell loading increased from 30 mg dry weight (dw)/ml gel to 72 mg dw/ml gel. This resulted in an improved citric acid production during the next batch culture,

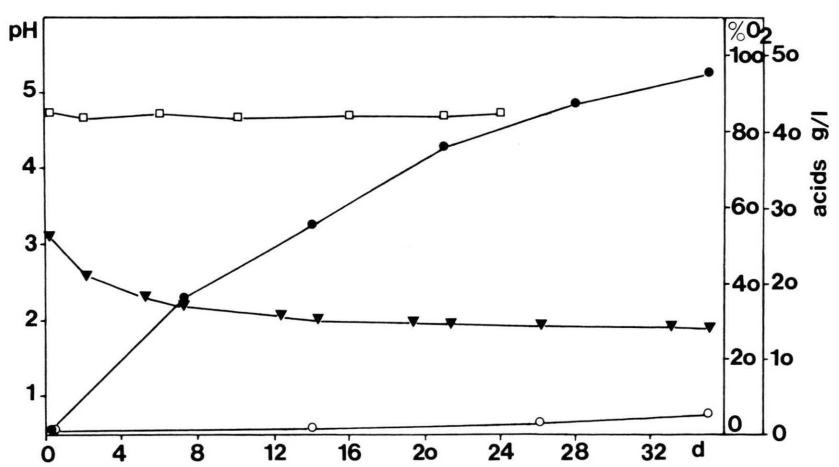

Fig. 2. Acid production, pH-development and oxygen saturation during citric acid production of immobilized cells of *Aspergillus niger* in batch culture. ●−−● = Citric acid, ○−−○ = gluconic acid, ▼−−▼ = pH-values, □−−□ = oxygen saturation.

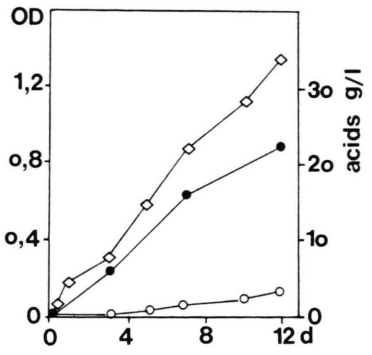

Fig. 3. Acid production and optical density in production medium of immobilized cells of *Aspergillus niger* after 48 h of regeneration in growth medium. ◇−−◇ = Optical density ($OD_{546\,nm}$), ●−−● = citric acid, ○−−○ = gluconic acid.

but parallely an intensive submerged sporulation of the fungus occurred, as it can be seen from the kinetics of optical density in Fig. 3. During further cultivation after 12 days germination and growth of free cells blocked the sintered glass at the bottom of the column and aeration and circulation broke down.

Citric acid production in semicontinuous culture

As the regeneration procedure was accompanied by the undesired contamination of the medium with free cells, the prolongation of the citric acid producing activity of the immobilized biocatalysts was aspired by semicontinuous cultivation.

Therefore the production medium was changed every 7 days or 14 days, respectively, involving a preceeding washing step of the gel beads with physiological NaCl-solution (Fig. 4A and 4B). Within both experiments the residual productivity after 32 days remained 1.4-fold higher in semicontinuous culture than in a comparable batch experiment, which was prepared from the same inoculum. Moreover the fermentation media kept absolutely free from contamination by outgrowing mycelia.

Citric acid production in continuous culture

According to Fig. 1 the bubble column reactor was continuously fed with production medium at a flow rate of 0.125 v/v·d. An equilibrium between citric acid production of the immobilized cells and its dilution by the continuous influx/efflux was reached after 12 days with a maximum concentration of 14 g/l (Fig. 5). During further fermentation the citric acid content slightly decreased, which was due to the above mentioned loss of activity.

The sucrose content of the production medium was initially inverted to glucose and fructose (see also [10]), but within proceeding cultivation time secondly little amounts of sucrose were observed (Fig. 5).

A comparative figuration between citric acid production in continuous and batch cultivation of immobilized cells of *Aspergillus niger* can be based upon the absolutely excreted amounts (g citric acid), which can be estimated from formula 1.

Calculation of absolute citric acid production per day

$$\text{g citric acid}/d_x = \frac{C_e \cdot V_e}{d_x} + \frac{(C_c - C_{c\text{-}1}) \cdot V_c}{d_x}. \quad (1)$$

Legends: d_x, day of measurement; C_e, citric acid concentration in efflux (g/l); V_e, volume of efflux (l); C_c, citric acid concentration in the column (g/l); $C_{c\text{-}1}$, C_c the day before d_x; V_c, working volume of the column.

For simplification set $C_e = C_c$.

Running addition of each amount per day (g/d_x) leads to the kinetics given in Fig. 6, which shows a similar improvement of citric acid production for the continuous process as it was observed with the semicontinuous cultivation (Fig. 4A and 4B).

Discussion

The citric acid production of Ca-alginate entrapped *Aspergillus niger* gradually decreases in the production medium, although the sugar concentration (initial: 20% sucrose) remains high enough for a prolonged acid excretion, provided metabolism has once turned to citric acid overproduction [12, 13]. This loss of activity might be due to the growth limited character of the producing, resting cells of *Aspergillus niger*, which failed to regenerate themselves in case of senescence.

Therefore necessary regeneration was carried out by interval cultivation in nutrient medium [14], leading to a secondary mycelium growth, which is responsible for an improved acid production. But on the other hand the new mycelium growth will be concentrated on the outer layer of the gel beads, thereby enhancing the risk of cell-detachment as it was previously reported [9, 10] and was also observed with regenerated polyacrylamide immobilized

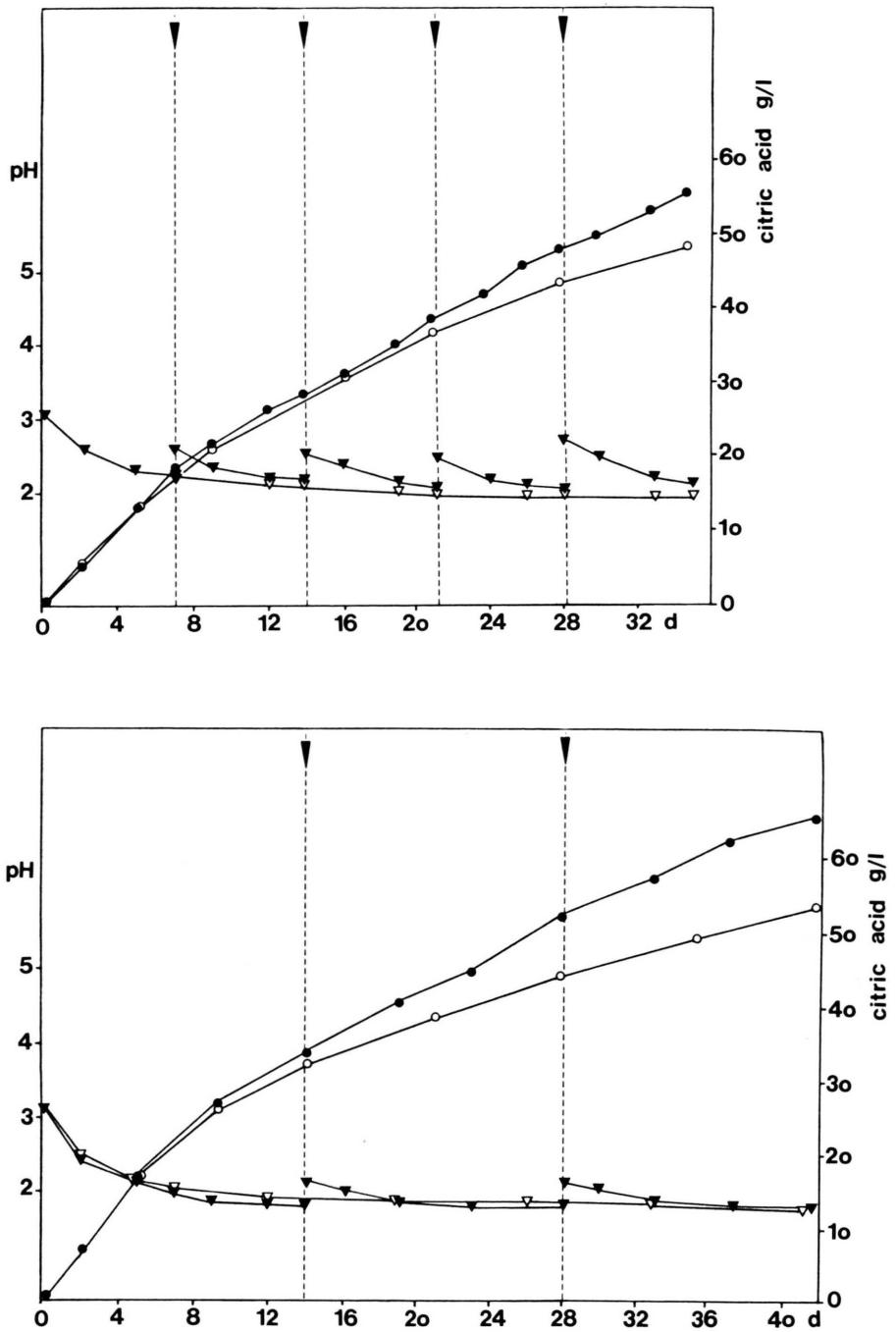

Fig. 4. Citric acid production with immobilized mycelia of *Aspergillus niger* in batch and semicontinuous culture. For semicontinuous cultivation production media were changed every 7 days (A) or 14 days (B) as marked by arrows/broken lines. The successive acid productions were added to single kinetics. Batch culture: ○−−○ = citric acid, ▽−−▽ = pH-values; semicontinuous culture: ●−−● = citric acid, ▼−−▼ = pH-values.

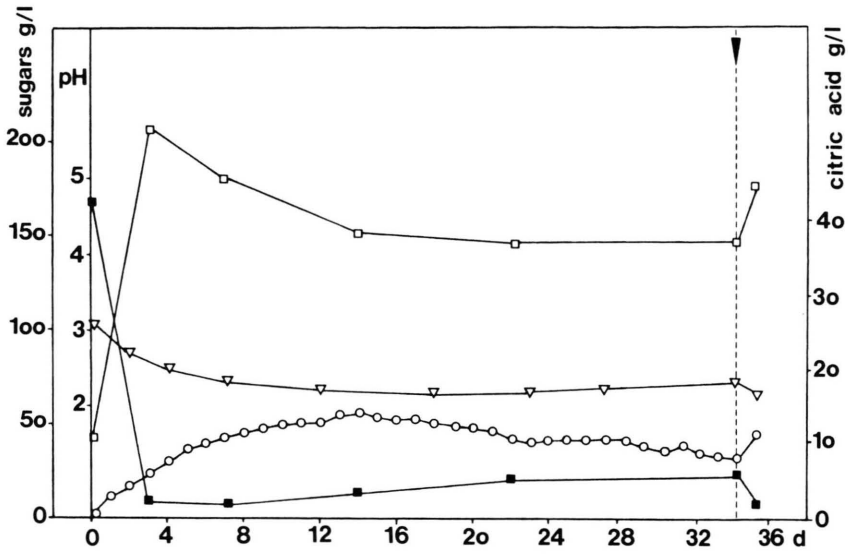

Fig. 5. Continuous citric acid production with immobilized cells of *Aspergillus niger* at 0.125 v/v·d. Residual activity was shown in a one-day batch-phase at the end of the fermentation (marked by arrow/broken line). ○−−○ = Citric acid, ▽−−▽ = pH-values, ■−−■ = sucrose, □−−□ = inverted sugar.

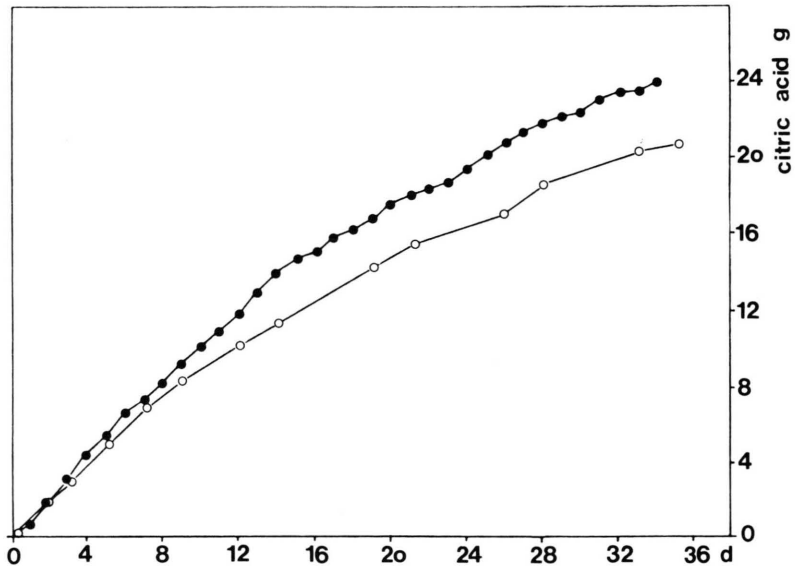

Fig. 6. Absolute citric acid production (g) of immobilized cells of *Aspergillus niger* in batch (○−−○) and continuous culture (●−−●).

Aspergillus niger [4, 15, 16]. Moreover the secondary cell propagation is accompanied by an increasing sporulation of the fungus, leading to a highly spore- and free cell-contaminated medium. Galleraith and Smith [17] reported a submerged sporulation of free cells of *Aspergillus niger* after addition of little NH_4NO_3-concentrations. Therefore the NH_4NO_3-content of the regeneration medium (150 mg/l) could be responsible for the described sporulation after regeneration of the immobilized cells.

The citric acid producing activity of the immobilized biocatalysts can better be prolonged by a semicontinuous or continuous cultivation, whereby the efflux medium remained free of detached mycelia. Similar observations with gel-immobilized fungi are reported for the alkaloid production of *Claviceps purpurea* [18], the penicillin production of *Penicillium chrysogenum* [19, 20] and the itaconic acid and glucoamylase production of *Aspergillus niger* [15, 21, 22].

In case of citric acid production with immobilized cells of *Aspergillus niger*, a reason for the improved excretion during semicontinuous and continuous cultivation might be derived from the reduced actual citric acid concentration of the media compared with a one-step batch culture, because recent results indicate an inhibition of glucose uptake by free cells of *Aspergillus niger* at increasing citric acid concentrations [23].

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