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SEMI-CONTINUOUS FERMENTATION OF CITRIC ACID BY SUBMERGED FERMENTATION

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Key words: citric acid fermentation, *Aspergillus niger*, semi-continuous fermentation.

The semi-continuous fermentation of the citric acid was carried out at a temperature of 30°C on a synthetic medium (sucrose+mineral salts) using the *Aspergillus niger* B-64-5 strain. The acid-forming activity of the mould and its morphological properties were found to change gradually during the cultivation period of 16-32 days. Compared with batch fermentation, a 20-50% increase in the productivity of the citric acid was obtained, depending on the dilution rate, the yield having been maintained on the same level.

A decided majority of studies in the range of mastering continuous microorganism cultivation methods deal with bacteria and yeasts; quite a few works have been devoted, so far, to the continuous cultures of moulds. This results not only from the specific difficulties encountered in this methods but also, and first of all from the lack of adequate data from the sphere of the mould growth kinetics in continuous cultivation [3]. Another difficulty is the fact that there is a phase difference in the moulds between the growth of vegetative mycelium and the reproduction. Under these conditions it is possible to maintain the flow-type culture for some period of time in the case of the development of the thread-like mycelium being torn by the movement of the stirrer blades, or the mycelium which undergoes spontaneous fragmentation of the fimbrias.

In the multi-stage cultures a great technical difficulty is the flow of the medium with the mycelium between the fermentors, especially in the investigations carried out on a smaller scale. This is caused by the inclination towards the clogging of pipes and valves and by the development of surface mycelium on the vessel walls [2].

Investigations in the range of the semi-continuous and continuous cultivation of the moulds mainly embrace the following problems: development and selection of the necessary equipment and investigations into the kinetics of growth and biosynthesis and the effect of continuous cultivation on the morphological and physiological changes in the mould [6]. The majority of these studies deal, however, with obtaining antibiotics and enzymes [1, 5, 9, 13, 15].

The possibilities of using semi-continuous and continuous fermentation in the sphere of citric acid biosynthesis should be considered against the background of the methods of cultivation of the *Aspergillus niger* mould as used here, i.e. the surface- and submerged-fermentation methods. Leaving aside the advantages and drawbacks of these two methods it should be said that from the technical point of view in both cases it is possible to carry out both the semi-continuous and continuous fermentation [11, 15, 16, 17].

Compared with the batch process in the continuous and semi-continuous methods, not only increased yield is obtained, but also, and first of all, the productivity of the citric acid fermentation is increased [18, 19].

The purpose of the present study was to determine certain technological variables of the citric acid submerged fermentation semi-continuous process, and to find out what is the effect of the *Aspergillus niger* mould cultivation period on the course and yield of this process.

MATERIALS AND METHODS

Tests were carried out in two stages: in stage I in flat-bottomed 750 cm^3 flasks containing 125 cm^3 of the medium [7] on a shaking — shaker — sieve put into reciprocating motion of a stroke of 6 cm at 200 rpm, at a temperature of 26 ± 1°C; in stage II in stainless steel fermenter of a capacity of 30 dm^3, at a temperature of 30 ± 1°C. The fermenter was provided with facilities for automatic control of temperature, foaming and dissolved oxygen concentration, made within the framework of the authors' study [8, 9].

During fermentation total acidity and the content in the solution of sugar, citric acid and mycelium dry matter were determined. Total acidity was determined by titration of 2 cm^3 samples with 0.1 n NaOH in the presence of phenolpthalein. The content of reducing sugars was determined by the Lane-Eynon method as modified by Soczyński [14]. Sucrose inversion was carried out with 2 n of hydrochloric acid at a temperature of 70-75°C during 10 minutes. The citric acid content was determined colorimetrically by the Saffran and Denstedt method [12] in Ilczuk modification [4]. The dry matter of the mycelium was determined by weight after it has been separated and dried at a temperature of 100-105°C, to constant weight.

RESULTS AND DISCUSSION

In the first series of the experiments semi-continuous fermentation was carried out using four fermentation media:

— synthetic (sucrose + mineral salts),

— synthetic with an addition of the *Aspergillus niger* conidia in an amount of $10^5/cm^3$,

— synthetic with a 24-hour mycelium of the *Aspergillus niger*,

— synthetic with a 48-hour mycelium of the *Aspergillus niger*.

These media were placed in the flasks at daily intervals in the amounts of 50, 40, 30, 20 and 10 cm^3 — previously the same amounts of the fermenting medium together with the mycelium were removed therefrom. The total volume of medium in the flask being 125 cm^3, a daily dilution rate was: 0.40, 0.32, 0.24, 0.16 and 0.08, respectively. The proportioning of the media was started on the fifth day of fermentation and it was continued for 22 days, i.e. till the 27th day of fermentation.

The results shown in the Fig. 1 indicate that in the course of fermentation total acidity and, consequently, the acid formation rate decrease gradually, the drop being the fastest for high dilution rate (0.40 and 0.32). Beginning from the 22nd day of fermentation the acidity was held approximately at the same level in all the flasks, which is characteristic of a steady state in a continuous process.

From the data presented here it also results that the kind of medium introduced is of a great significance for the course of the fermentation process. Best results have been obtained adding the medium with the 2-day mycelium followed by 1-day mycelium, because the medium with the *Aspergillus niger* conidia gave nearly identical results as those obtained on a synthetic medium. These data find a full confirmation in the shape of the average citric acid formation rates depending on the kind of medium being added and dilution rate — Fig. 2.

The results obtained indicate that the cause of the rapid decrease in total acidity at higher dilutions rates partly lies in the restricted growth of the mycelium and, partly, in the reduction of its acid-forming activity. The weight of the mycelium dry matter varied within 7-8 g/dm^3 at a 0.40 dilution rate to some 9-10 g/dm^3 at a 0.08 dilution rate. The morphology of the mycelium cultivated on individual media also showed great differences. The mycelium on a synthetic medium after several days began to develop in the form of big, compact pellets of a diameter of 2-4 mm, whereas in the tests with the successively introduced fresh mycelium it developed in the form of loose fragments or small pellets up to 1 mm in diameter, which is characteristic of batch fermentation. The higher activity of such a mycelium is indicated also by the fact that after the medium proportioning was finished the fermentation went on in the correct way unlike the fermentation with the mycelium having

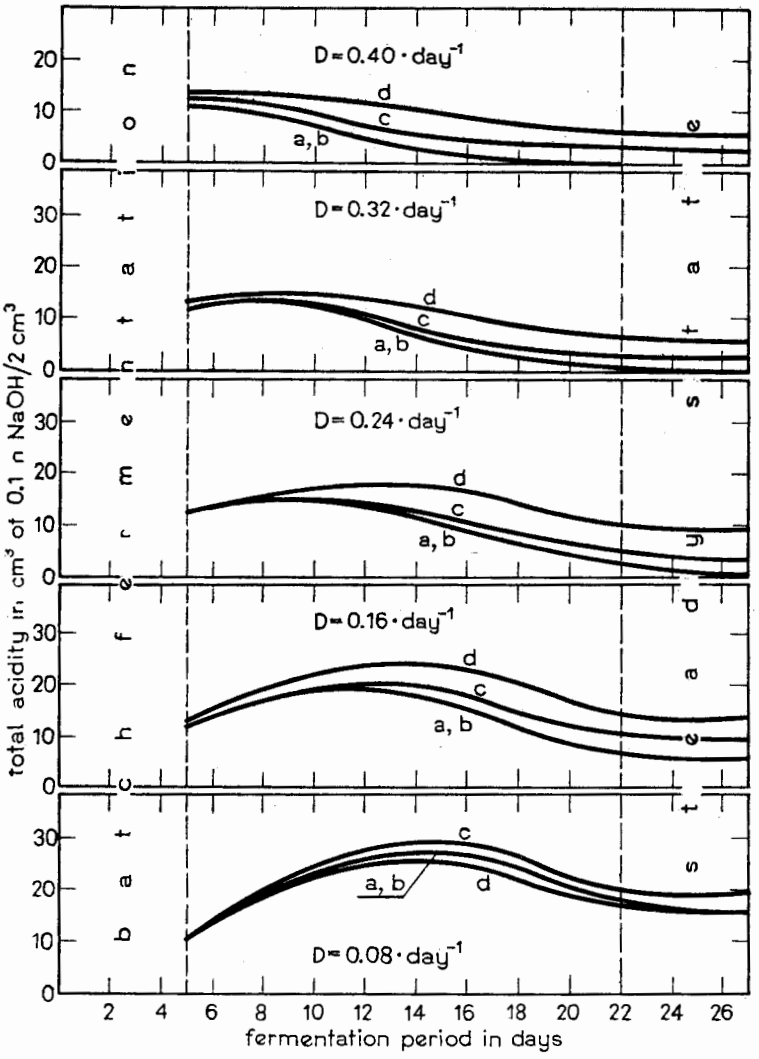

Fig. 1. The shape of total acidity in the citric acid semi-continuous fermentation process using various fermentation media and different dilution rates; a — synthetic medium (sucrose+mineral salts), b — synthetic medium with *Aspergillus niger* conidia, c — synthetic medium with 1-day *Asp. niger* mycelium; d — synthetic medium with 2-day *Asp. niger* mycelium

the form of pellets, which was inhibited. Where the dilution rate was low, the mycelium had a similar form and activity in all the samples irrespective of the kind of medium being dosed. In connection with this, in the next series of experiments synthetic medium was proportioned in the course of fermentation so as to keep the sugar and citric acid concentration variations at an as low level as possible. Medium proportioning was started on the successive days of fermentation beginning

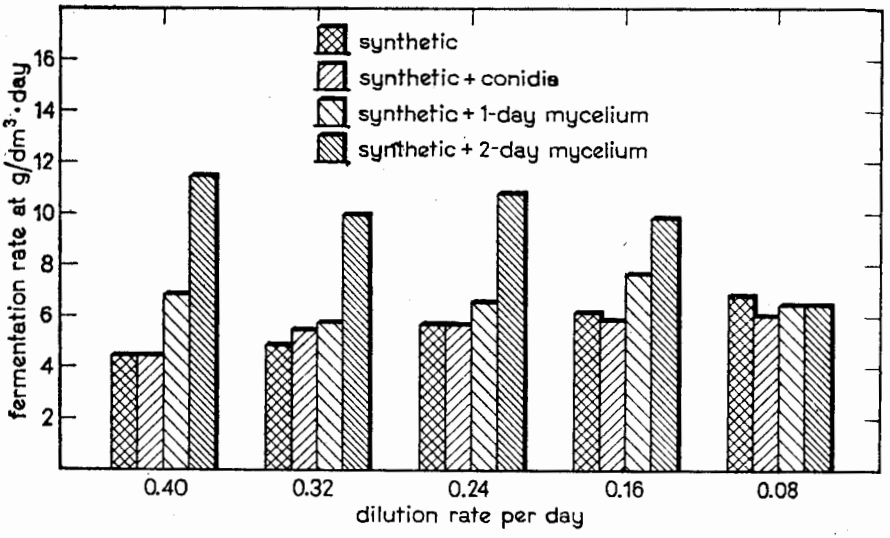

Fig. 2. Citric acid formation rate depending on dilution rate and the kind of medium used

from the 4th day for fermentation No. 1 (initial total acidity = 9.0) to the 10th day for fermentation No. 6 (initial total acidity = 30.0). Depending on total acidity, the fermenting medium was being drawn off from the flasks, at daily intervals, in the amounts from 50 to 20 cm³ adding instead the same amount of the fresh medium. If the acidity was found to gave grown, the amount of medium replaced by the fresh one was being increased; if the acidity dropped, less fresh medium was added instead of the unchanged amount of the used medium removed. The exemplary results shown in Fig. 3. for the low, average and high content of the acid indicate that the fermentation rate slows down also where this method of medium exchange is used. The average rate of production

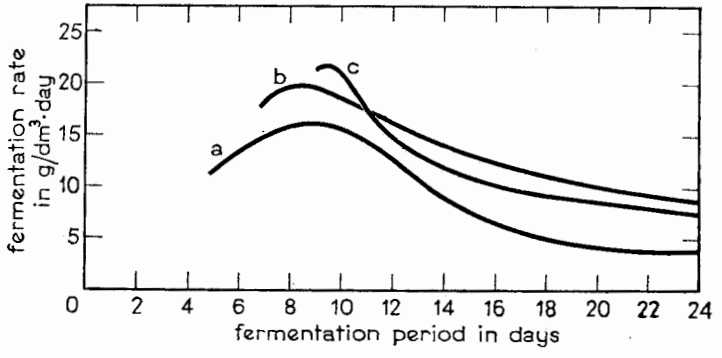

Fig. 3. Citric acid formation rate in the semi-continuous fermentation process at low (a — 31 g/dm³), average (b — 57 g/dm³) and high (c — 92 g/dm³) acid concentration in the medium

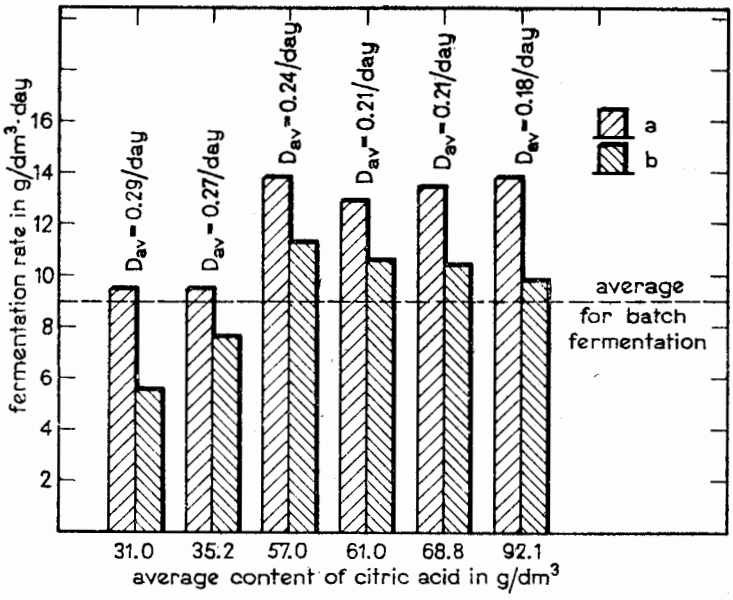

Fig. 4. Average citric acid formation rate for the whole period (a) and for the last 10 days (b) of semi-continuous fermentation, at various acid concentrations

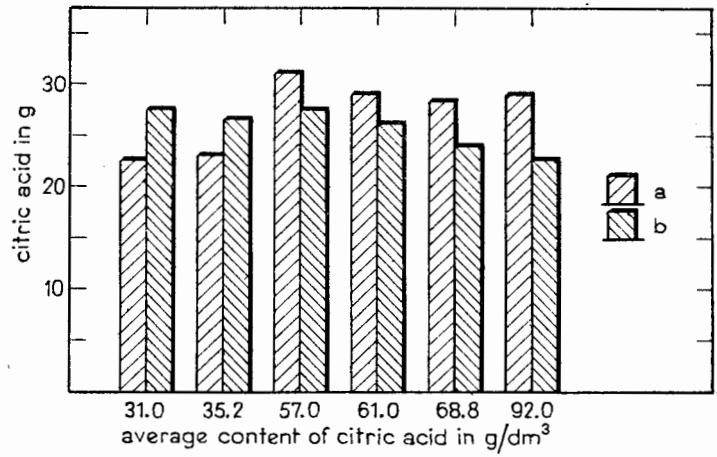

Fig. 5. Amount of citric acid formed in a 125 cm³ flask (a) and per 1 g of mycelium (b) in the semi-continuous fermentation process carried out at various acid concentrations

of the citric acid, calculated for the fermentation period as a whole, varies from 0.4 g/dm³ per day for fermentation No. 1, to 13.8 g/dm³ per day for fermentation No. 6 (Fig. 4). For the last 10 days of fermentation this rate, for all the acid concentrations, has been calculated to be much lower and to amount from 5.4 g/dm³ per day for fermentation No. 1,

to 9.8 g/dm³ for fermentation No. 6 (Fig. 4). This is a proof of the gradual decrease of the acid-forming activity of the mycelium cultivated by this method.

To determine the productivity in individual samples, the total amount of the citric acid produced in them has been calculated. These amounts varied from approximately 22.5 g for fermentation No. 1 and No. 2 to some 30 g for the remaining samples (Fig. 5). The smaller amount of acid obtained in the first two samples is connected with the slower growth of the mycelium, because its acid-forming activity was even higher than in the remaining samples (Fig. 5). In these fermentations the form of the mycelium was better than in the former series of experiments, changing gradually from loose thread-like fragments into small pellets 0.6 to 2.0 mm in diameter depending on the day of fermentation and the dilution rate.

The positive results obtained during testing on a shaker have induced the authors of the study to carry out the semi-continuous fermentation of the citric acid in a 30 dm³ fermenter. The mixing and aerating conditions were selected so as to keep the oxygen dissolved concentration in

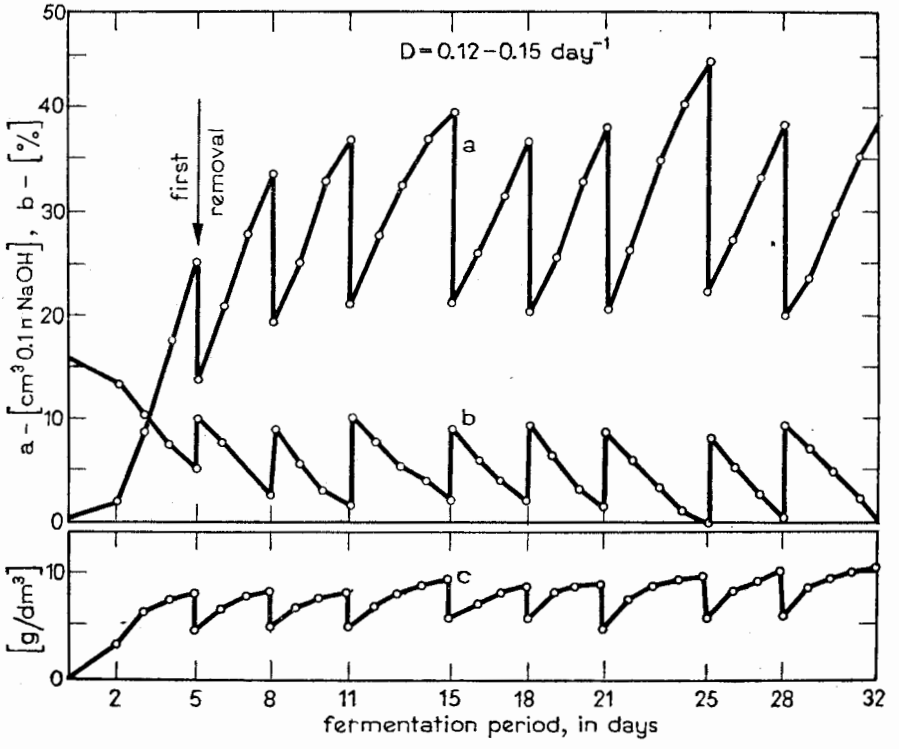

Fig. 6. Curves of total acidity (a), sugar content (b) and dry mycelium mass (c) in the citric acid semi-continuous fermentation process 10 dm³ removals every 3 or 4 days

the solution on a level of approximately 30% saturation. Fermentation was continued in such a way that beginning from the 5th day, 10 cm^3 of the fermentation medium were being drawn off from the fermenter, together with the mycelium (40% of the volume of medium), to be replaced by the same amount of sterile fresh medium. The course of the process and the results obtained are shown in Fig. 6.

Total acidity at individual stages varied within approximately 20-40 cm^3 0.1 n NaOH/2 cm^3 of the medium; the content of sugar decreased from 10% to 2%, respectively. Only during the last three withdrawals, when efforts were being made to bring the fermentation to an end, sucrose concentration decreased below 0.5%. On the other hand, the content of the dry matter of the mycelium increased from approximately 5 g/dm^3 after adding fresh medium to some 10 g/dm^3 towards the end of the given stage of fermentation.

The microscopic observation of the mycelium indicates that with the progress of the process of fermentation its structure changes gradually, from loose fragments at the beginning of the culture (photo 1) through jagged pellets — photo 2 to the tightly compacted forms of elongated

Photo 1. After 6 days (80×)

Photo 2. After 12 days The changes of the mycelium during fermentation (Photo 1-4). (80×)

Photo 3. After 25 days (40×)

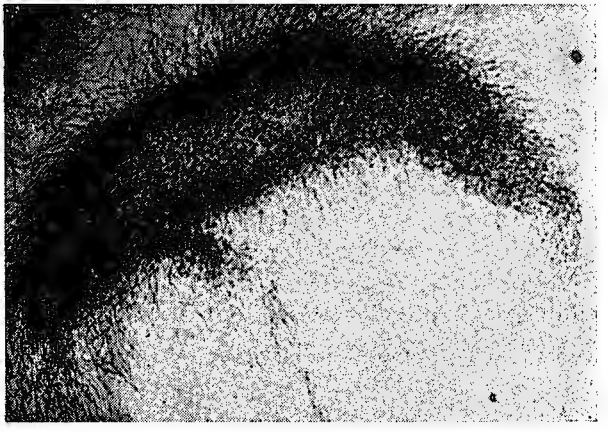

Photo 4. After 30 days (40×)

shape, their length being up to 3 mm and diameter up to 1 mm (photos 3-4), in the final fermentation stages. The development of such forms of the mycelium is not desirable, because a specific number of the cells inside the compact pellet become restricted or even excluded from the process of biosynthesis due to the lack of the inflow of the nutritive substances, especially oxygen.

The next fermentation was carried out in the fermentor under the conditions determined on the shaker as the optimum ones (dilution rate D = 0.24/day; total acidity approximately 20.0; withdrawal of the medium at daily intervals). The basic parameters of the process are shown in Fig. 7.

Total acidity, after the establishment of the steady state varied from some 20 to 27 cm^3 0.1 NaOH; the content of sugar was from about 5% after adding the fresh medium to some 2% towards the end of the given stage of the fermentation process; the mycelium dry matter content varied from 6 to 9 g/dm^3.

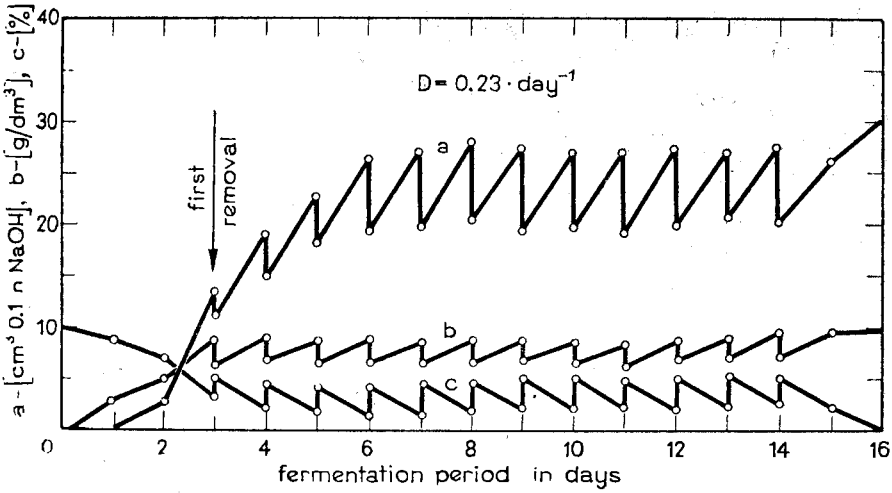

Fig. 7. Curves of total acidity (a), dry mycelium mass (b) and sugar content (c) in the citric acid semi-continuous fermentation process 5 dm³ removals every 24 hours

The average citric acid production rate for the individual stages — i.e. the measure of the mycelium acid-forming activity (Fig. 8) — in both fermentations shows a distinct downward tendency. In the case of fermentation with medium added in an amount of 10 dm³, it decrease from 21.7 to 16 g/dm³ per day; for the 5 dm³ additions of the medium this decrease is from approximately 27 to 20 g/dm³ per day. The average acid production rate for the whole period of the semi-continuous fermentation was 18.0 and 22.3 g/dm³ per day, respectively, the average yield being 77% and 80%.

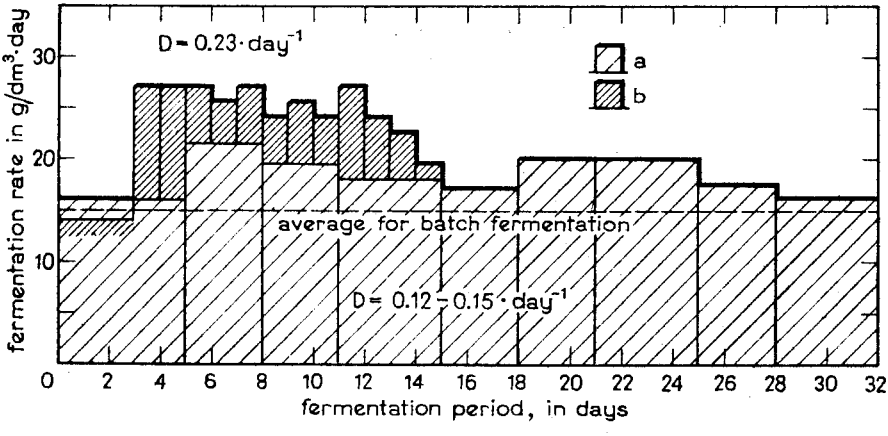

Fig. 8. Average citric fermentation rate in the semi-continuous fermentation process carried out in a 30 dm³ fermentor; a — removals every 3-4 days, b — removals every 24 h

Compared with the batch process carried out in a fermenter (at productivity of 15 g/dm³ per day and an yield of 80%), first of all an increase of the order of 20-50% in the citric acid productivity was obtained, at unchanged yield. This can easily be understood, because due to the removal of some part of the medium together with the mycelium some part of the sugar had to be used to build the cellular mass of the mycelium, as in the case of the batch process.

Higher productivity and smaller morphological changes of the mycelium observed in the process where 5 dm³ doses of the medium were being added, permit one to state that for the semi-continuous fermentation of the citric acid it is better to add the medium in smaller doses but at shorter time intervals. This offers a ground for a conclusion that in a fully continuous process even better fermentation parameters can be obtained.

CONCLUSIONS

1. To maintain the acid-forming activity of the mycelium in semi-continuous fermentation, the 1- or 2-day mycelium should be added with the *Aspergillus niger* medium.

2. When introducing a synthetic medium with no mycelium, good fermentation parameters can be obtained by selecting the optimum dilution rate.

3. During the semi-continuous fermentation of the citric acid the *Aspergillus niger* mycelium gradually changes its structure from loose microscopic fimbries in the first days to the strongly compacted forms of elongated shape and size up to 3 mm in the final cultivation stage. This is reflected in the decreasing productivity and yield of the fermentation process.

4. Under the conditions applied in the present study the citric acid productivity in the semi-continuous fermentation process was 20-50% higher than in the batch process, the yield being at the same level (approximately 75-80%).

5. It has been found that in the citric acid semi-continuous fermentation process it is better to add smaller doses of fresh medium but at shorter time intervals; this gives a higher productivity of the fermentation process.

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FERMENTACJA PÓŁCIĄGŁA KWASU CYTRYNOWEGO METODĄ WGŁĘBNĄ

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Streszczenie

Fermentację prowadzono dwuetapowo, początkowo na trząsawce w kolbach płaskodennych o poj. 750 cm^3 wypełnionych 125 cm^3 pożywki, a następnie w fermentorze o pojemności całkowitej 30 dm^3 i roboczej 22 dm^3.

Badano przebieg fermentacji półciągłej przy różnych stopniach jej rozcieńczenia [D] oraz przy dozowaniu zmiennych podłoży (rys. 1). Stosowano podłoże syntetyczne (sacharoza+sole mineralne), podłoże syntetyczne z konidiami oraz z jedno- i dwudobową grzybnią *Aspergillus niger*.

Stwierdzono, że przy niskich D rodzaj wprowadzonego podłoża nie wpływa na przebieg fermentacji, natomiast przy wyższych D najlepsze wyniki uzyskuje się wpro-

wadzając podłoże z dwudobową grzybnią *Aspergillus niger* (rys. 2).

Dozując podłoże syntetyczne najwyższe szybkości fermentacji uzyskuje się prowadząc proces przy stężeniach kwasu cytrynowego 50-70 g/dm^3 i przy D = 0,17-0,20 (rys. 3 i 4). W tych warunkach najwyższa jest również ilość kwasu cytrynowego wytworzonego w jednostce objętości (rys. 5).

Stwierdzono również, że w procesie fermentacji półciągłej korzystniej jest dozować mniejsze objętości pożywki w krótszym czasie (rys. 7) aniżeli większe objętości pożywki ale w dłuższym czasie (rys. 6), gdyż w pierwszym przypadku uzyskuje się wyższe szybkości fermentacji (rys. 8).

Zaobserwowano także, iż w czasie hodowli *Aspergillus niger* trwającej 16-32 dni zmienia się stopniowo aktywność kwasotwórcza grzybni i jej cechy morfologiczne (fot. 1-4). W porównaniu z procesem okresowym w fermentacji półciągłej uzyskano wzrost produktywności kwasu cytrynowego o 20-50% przy utrzymaniu wydajności na tym samym poziomie ok. 80% w stosunku do cukru.