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Production of Citric Acid from Starch by Submerged Culture of *Aspergillus niger*

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ABSTRACT

Citric acid can be produced by submerged culture of *Aspergillus niger* from starch at low pH. The optimum conditions for the production of the acid in a medium of 15% soluble starch (potato) were investigated by using a 10 ℓ jar fermentor with 8 ℓ working volume. The agitation rate was varied from 600 rpm to 800 rpm to keep aerobic condition while aeration rate was kept at 1 vvm throughout the experiment. The fermentation was carried out at 30°C. A higher yield was obtained by dynamic control of pH than that by constant value control. Cultivations with initial pH about 4 followed by controlling pH at 2.0 after 12 h by addition of 1% $CaCO_3$ or 6N NaOH resulted in almost the same yield of citric acid. The highest citric acid production was 91.4 g/ℓ after 5 days cultivation. Utilization of cassava as a raw material was also investigated using a 2.5 l jar fermentor. Cassava starch was liquefied by α-amylase before use. The working volume was 1.5 ℓ and dissolved oxygen was controlled to 10 - 30% sturation by varying agitation rate. The amount of citric acid produced from cassava starch was 109.3 g/ ℓ after 6 days cultivation.

The production of citric acid by tower fermentors was tested. A 6 ℓ glass column was used with a working volume of 5 ℓ. Air was supplied by a glass sparger at the bottom of the column and the fermentation was carried out for 7 - 9 days. Citric acid production was low due to inefficiency of oxygen supply.

INTRODUCTION

The production of citric acid by *Asperigillus niger* is one of the most commercially utilized examples of fungal overflow metabolism. Lockwood and Batti (1965) found that fermentation with *A. niger* can be successfully conducted even with crude carbon sources such as starch hydrolysates or incompletely refined sucrose. Various carbohydrate materials may be used in citric acid production such as cane or beet sugar, corn sugar molasses and crude unfiltered starch hydrolysates. Since early studies, it has been obvious that citric acid fermentation is extremely complex. A successful process depends both on an appropriate strain and on optimization of fermentation parameters. A number of reports have been published on the production of citric acid by submerged mold culture. It is well known that citric acid production by *A. niger* is subject to variation of various conditions and there have been various attempts to improve citric acid productivity of *A. niger* by heterokaryosis and polyploidy. For the development of submerged fermentation on a commerical scale it was of equal importance to elaborate conditions for the utilization of cheap raw materials of carbohydrate sources which apparently posed additional problems.

The purpose of this study was concerned about the optimum conditions for the submerged fermentation by *A. niger* from soluble starch (potato) and cassava starch.

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MATERIALS AND METHODS

Microorganisms *Asperigillus niger* obtained from the stock of International Center of Co-operative Research in Biotechnology, Osaka University, Japan, was used throughout the experiments. The culture was maintained on potato dextrose agar slants at 4°C and was subcultured at intervals of one month.

Starches Soluble starch (potato) from the Wako Pure Chemical Industried Ltd. and cassava starch from Thailand were used as raw materials in a production medium.

Medium The culture medium had the following compositions in 1 ℓ: soluble starch (potato) or cassava starch 150 g, $(NH_4)_2SO_4$ 3.5 g, KH_2PO_4 0.3 g, K_2HPO_4 0.3 g, $MgSO_4.7H_2O$ 0.3 g. The casava starch was liquefied by α-amylase before use. The medium pH was adjusted to 4.0 by hydrochloric acid before sterilization. This medium was used for inoculum and production medium.

Preparation of inoculum Potato dextrose agar slant was used for sporulation of *A. niger*. Spore suspension was prepared from one week culture and spore number was counted by Thomas hemacytometer to make a required inoculum. The spore suspension was transfered to the inoculation medium in order to allow the development of pellets in a 2 ℓ Sakaguchi flask which contained 300 - 400 ml of production medium for a 10 ℓ jar fermentor and 250 ml for a tower fermentor. The production medium contained 10^6 - 10^8 spores per 1 ℓ. The inoculum was incubated on a reciprocal shaker at 120 rpm for 2 days. 5% Inoculum was used for the citric acid fermentation.

Culture conditions The submerged fermentation was done both in 10 ℓ and 2.5 ℓ jar fermentors (with 6 ℓ, 8 ℓ or 1.5 ℓ working volume) and 6 ℓ tower fermentor (with 4 ℓ and 5 ℓ working volume). The agitation rate of a 10 ℓ fermentor was varied from 600 rpm to 800 rpm to keep aerobic condition while aeration rate was kept at 1 vvm and the temperature was controlled at 30°C throughout the experiment. Agitation rate of a 2.5 ℓ jar fermentor was automatically varied to keep dissolved oxygen tension between 10 - 30%. Foaming was controlled by the addition of 1 - 2 ml of antifoaming agent (Adecanol) during the first day of cultivation. The pH of production medium was varied and controlled by addition of 1% $CaCO_3$, 6 N NaOH or 3 N HCl during fermentation.

A tower fermentor modified from 6 ℓ glass column as shown in Fig. 1 was used in this study. Three types of aeration arrangement were used as follows: (1) with a single simple sparger, (2) with four small spargers and a draft tube, and (3) with a single sparger and rasching rings. They were run with a 5 ℓ of medium. Cultivations were carried out in a 30°C incubator room. The aeration was supplied by using a simple glass sparger (cylindrical shape, 20 × 30.5 mm) at the bottom of the column. Aeration rate was controlled at 1 vvm for 7 to 9 days.

Analytical methods Cell growth was measured in terms of mycelium dry weight of the culture broth. Twenty ml or ten ml or culture broth from fermentors were filtered through a pre-weighed Whatman glass microfiber filter paper (GF/B). The mycelium mass was washed twice with distilled water. When 1% $CaCO_3$ was added into the production medium, the mycelium mass was washed by 3 N HCl solution and followed subsequently with distilled water. The cell mass was dried together with the filter at 90°C for 24 h or until the dry weight was constant. The filtrate was used for analysis of total titratable acidity, citric acid, total sugar and glucose content. Total titratable acidity was estimated by titrating 5 ml aliquots with 0.1 N NaOH solution, using phenolphthalein as indicator and was expressed in terms of citric acid monohydrate. Citric acid was assayed by pentabromoacetone method of Stern J.R. (1957). Total sugar was determined by the phenol-sulfuric acid method of M. Dubois *et al.* 1956. Glucose was measured by a glucose oxidase method using a commercial assay kit (Glucostat, Toyobo Company, Japan) after appropriate dilution in distilled water.

RESULTS AND DISCUSSION

Effect of constant pH on citric acid production

Figure 2 shows the result of the submerged fermentation using soluble starch (potato) in which pH was controlled at 2 by 6 N NaOH throughout the cultivation. It shows that the citric acid production was highest on the 6th day of cultivation as 87.0 g/ℓ. Total sugar gradually decreased during the cultivation and glucose increased during the first 2 days and continued to decrease reaching almost zero at the 6th day. Production of citric acid started after 1 day cultivation and gradually increased to the maximum at the 6th day. After the 6th day the production gradually decreased. The highest yield was 82.4% The percentage yield was calculated by the ratio of citric acid produced (wt) to consumed sugar (wt) multiplied with 100.

Table 1 summarizes results of several batches of citric acid production from soluble starch at different values of constant pH in a 10 ℓ fermentor. When pH was controlled at 2 the highest citric acid production was obtained. It was observed that at higher pH, the production of citric acid and % yield were less than at lower pH but the pattern of sugar consumption was almost the same. It is possible that some by-products other than citric acid were formed at higher pH. From previous works the optimum pH condition were found to be different. From the work of Takahashi *et al.*, 1964, the pH of production medium was adjusted to initial pH 3.8 and showed the production at 1.32 g/100 ml during 4 - 6 days. The effect of various initial pH of citric acid production medium was studied by Ajibade, A. *et al.*, 1980. By variation of pH from 4.0 to 6.5, they found that the initial pH of 5.4 gave the highest citric acid production at 19.50% yield (w/v) using molasses as raw materials. Some papers reported that the optimum initial pH were 2.0, 3.0, 5.0 and 3.8.

For all conditions of constant pH, the citric acid production started after 1 day and gradually increased to the maximum at about the 5 - 6th day, except at pH 4 the maximum production was at the 4th day. It was observed that the culture produced materials of yellow color in the medium on the second day of fermentation. At lower pH, the color was produced earlier and it was more intense compared with that at higher pH. By using a 10 ℓ jar fermentor with 6 ℓ working volume and 600 - 700 rpm, the wall growth occured from the second day until the fermentation was finished. In some experiments, the wall growth was settled into the medium on the 5th day of fermentation resulting in a high unbelievable value of cell concentration. Due to the problem of wall growth, the fermentation at pH 4 and 5 were carried out with agitaion rate 600 rpm but the wall growth still occured during fermentation. It was found that controlling of pH 4 resulted in the highest cell growth rate during th e first day of fermentation.

Effect of dynamic pH control on citric acid production

Figures 3 and 4 show the effect of dynamic pH control on the production of citric acid. pH was initially controlled at 4 for 1 day (Fig. 3) or 2 days (Fig. 4). After that the pH was allowed to decrease, but kept at a pH not lower than 2.5 by using 6 N NaOH. The production of citric acid of the former case reached the highest value of 93 g/ℓ on the 7th day. But the production in the latter case was lower, 72.5 g/ℓ which was lower as compared to the production at constant pH 2 as shown in Table 2.

The citric acid production with pH control by addition of 1% $CaCO_3$ after 12 h fermentation was maximum after 5 days fermentation resulting in a high concentration, 91.4 g/ℓ as shown in Fig. 5. From the initial value of 3.47, pH gradually decreased and abruptly increased up to 6.4 after 1% $CaCO_3$ was added in the medium. After that the pH was quickly decreased to a low value about 2.4 during the second day of fermentation. From the experiment the agitation rate was kept at 600 rpm during 12 h and increased to 700 rpm after addition of 1% $CaCO_3$ until 24 h of fermentation, then followed by a high rate of 800 rpm throughout the experiment to keep arobic condition.

Due to high agitation rates and high working volume, the wall growth was suppressed during the fermentation. The maximum yield was 72.1% (Table 2). The citric acid production with the stepwise addition of 0.5% $CaCO_3$ after 12 and 24 h, shown in Fig. 6, was still almost high as same as with 1% $CaCO_3$ addition after 12 h fermentation. It can be concluded that dynamic control of pH with higher pH on the first day followed by lower pH until the final day of fermentation is better for the production of citric acid compared with constant pH. Dynamic pH control by addition of 1% $CaCO_3$ is the best way for citric acid production in this study.

Effect of Ca^{++} ion on citric acid production

The effect of Ca^{++} ion on citric acid production was studied by the addition of 1% $CaCl_2$ in the production medium and the pH of medium was controlled at 2 throughout the experiment. The result is shown in Fig. 7 and Table 2. The highest concentration obtained was 80.0 g/ℓ with 59.5% yield. The productivity from this experiment was still lower than by using 1% $CaCO_3$. It is considered that Ca^{++} ion in the form of $CaCl_2$ had no effect on improvement of citric acid production at low pH.

Citric acid production from cassava starch

The citric acid production from cassava starch was studied by using a 2.5 ℓ jar fermentor with 1.5 ℓ working volume. Two batch runs were made by controlling pH at 2 and 1% $CaCO_3$ addition after 12 h fermentation. As shown in Figs. 8 and 9, the amount of citric acid produced at controlled pH 2 and 1% $CaCO_3$ addition were at 86.1 g/ℓ and 109.3 g/ℓ respectively. Both conditions gave better mycelial growth from the first day of fermentation as compared to that using soluble starch. Available sugar had been reduced to about 10 g/ℓ due to the good growth in cassava medium with high number of spores used in inoculum medium. In both conditions, the production medium contained 6.5×10^8 spores per liter. The good growth in cassava starch medium may be due to the presence of more readily consumable components in the starch caused by liquefaction by α-amylase. It was also observed that the culture grown in the inoculum appeared in mycelial form. Consequently it could effectively use sugar in the medium.

Tower fermentor

Tower fermentors of three different aeration arrangements as shown in Fig. 1 were utilized for citric acid fermentation. The variation of cultivation conditions using soluble starch (potato) and cassava starch as raw materials were studied. In Table 3, it is shown that the citric acid production from soluble starch (potato) with addition of 1% $CaCO_3$ was higher than other experiments resulting in high accumulation of citric acid, at 36.6 g/ℓ after 9 days fermentation. The starch was consumed very slowly and remained at a high concentration during fermentation as shown in Fig. 10. By dynamic pH control using NaOH, controlling pH 4 during the first day and allowed to decrease not lower than 2, the citric acid production was still very low. The lower productivity of citric acid in cases of tower fermentor can be ascribed to inefficiency of oxygen transfer rate. The results of experiments of measuring $k_L a$ value in 15% soluble starch medium using a 10 ℓ jar fermentor with 8 ℓ working volume comparing with 6 ℓ tower fermentors with various types of air supply are summarized in Table 4. Aeration rate was 1 vvm in all cases. It is shown that the $k_L a$ values of tower fermentors were lower than those of a jar fermentor. The $k_L a$ value of tower fermentor using a single sparger gave the lowest value at 16.5 h^{-1}. There was improvement of oxygen transfer when four single spargers were used with a draft tube. The $k_L a$ was highest when rasching rings were used but $k_L a$ value was still low as compared to those obtained by using an agitated and aerated jar fermentor. It can be clearly stated that a good O_2 transfer rate is necessary for an efficient citric acid production by submerged fermentation.

CONCLUSION

The Citric acid can be produced by submerged culture of *Aspergillus niger* from soluble starch (potato) and cassava starch as raw materials. By dynamic pH control, it has more effect on citric acid production than by constant pH control. The fermentation of citric acid by aerated and agitated fermentor using jar fermentor is more efficient than by a tower fermentor. From this study the maximum production of citric acid from soluble starch (potato) is 91.4 g/l with 72.1% yield after 5 days fermentation using 10 ℓ jar fermentor and controlling pH by addition of 1% $CaCO_3$ and from cassava starch is 109.3 g/ℓ. after 6 days fermentation using 2.5 ℓ. jar fermentor. The citric acid production from a tower fermentor was low due to inefficiency of oxygen supply.

Table 1. The citric acid production at different conditions of constant pH.

pH	Citric acid (monohydrate) (g/ℓ)	Yield (%)	Time for maximum production (day)
2	87.0	82.4	6
2.5	82.5	67.6	5
3	62.5	59.0	5
3.5	53.0	46.6	5
4	48.7	51.2	4
5	50.0	36.9	6

*Yield (%) = $\frac{\text{citric acid produced (wt)}}{\text{consumed sugar (wt)}} \times 100$

Table 2. The citric acid production with various pH control.

Fermentation with various pH control	Citric acid (monohydrate) (g/l)	Yield (%)	Time for maximum production (day)
Constant at pH_2	87.0	82.4	6
1% $CaCO_3$ was added after 12 hr fermentation	91.4	72.1	5
0.5% $CaCO_3$ was added after 12 and 24 hr fermentation	90.6	71.9	5
pH 4 for 1 day and pH 2.5 thereafter	93.0	63.0	7
pH 4 for 2 days and pH 2.5 thereafter	72.5	79.2	7
Constant pH at 2 and addition of 1% $CaCl_2$	80.0	59.5	6

Table 3. The citric acid production in a tower fermentor.

Fermentation condition	Citric acid (monohydrate) (g/ℓ)	Yield (%)	Time course (day)
No pH control (potato)[a]	0.3	3.6	8
	(0.4)[c]	(2.8)[c]	(9)[c]
1% $CaCO_3$ (potato)[a]	30.8	62.8	8
	(36.6)[c]	(76.4)[c]	(9)[c]
pH 4 (1 day) → pH 2.0 (cassava)[a]	4.0	10.0	8
pH 3.4 → 2.0 (cassava)[b]	14.5	31.7	6

[a] Using a single glass sparger.
[b] Using a single glass sparger with rasching rings.
[c] Figures in ( ) are the data based or the a day fermentation.

Table 4. The k_L a value of a jar fermentor (10 ℓ) and a tower fermentor (6) with soluble starch.

Fermentor type	Agitation rate (rpm) and modification on tower fermentor	k_La value (h^{-1})
Jar fermentor	600	87.3
	700	107.4
	800	152.1
Tower fermentor	simple	16.5
	+ Draft tube	36.9
	+ Raschig ring	48.9

Aeration rate was 1 vvm.

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LITERATURE CITED

Ajibade A. *et al.:* Enzyme Microb. Technol., *2,* 61 (1980).

Al Obaidi, Z. S., Berry, D.R.: Biotechnol. Lett., *1,* 221 (1979).

Al Obaidi, Z. S., Berry, D.R.: Biotechnology Letters, *2,* 6 (1980).

Chaudhary, K. *et al.:* J. Ferment. Technol., *56,* 554 (1978).

Dubois M., *et al.:* Anal. Chem., *28,* 350 (1956).

Kubicek, C.P., Röhr, M.: Eur. J. Appl. Microbiol., *4,* 167 (1977).

Lockwood, L. B., Batti, M. A.: US Patent 3 189 527 (1965).

Rahmatullah, M. *et al.:* J. Ferment. Technol., *57,* 379 (1979).

Rehm, H. T., Reed, G.: Biotechnology, vol. 3, p. 436, Weinheim; Deerfield Beach, Florida (1983).

Stern, J. R.: Methods in Enzymology (S.P. Colowick, N. C. Kaplan), vol. III, 426, Academic Press, New York (1957).

Takahashi, J. *et al.:* Agri. Biol. Chem., *29,* 331 (1965).

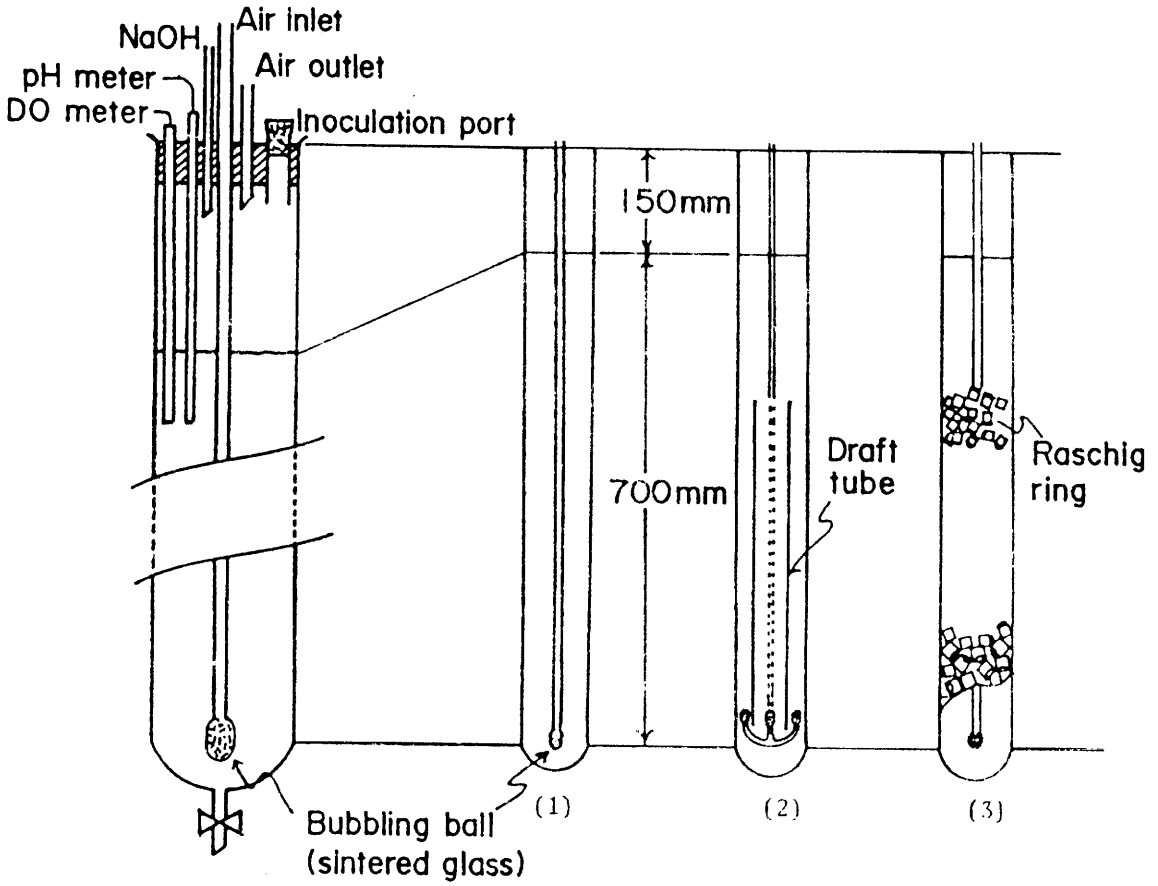

Fig. 1. Schematic diagram of tower fermentor.
(1) with a single simple sparger
(2) with four small spargers and a draft tube
(3) with a single sparger and raschig rings

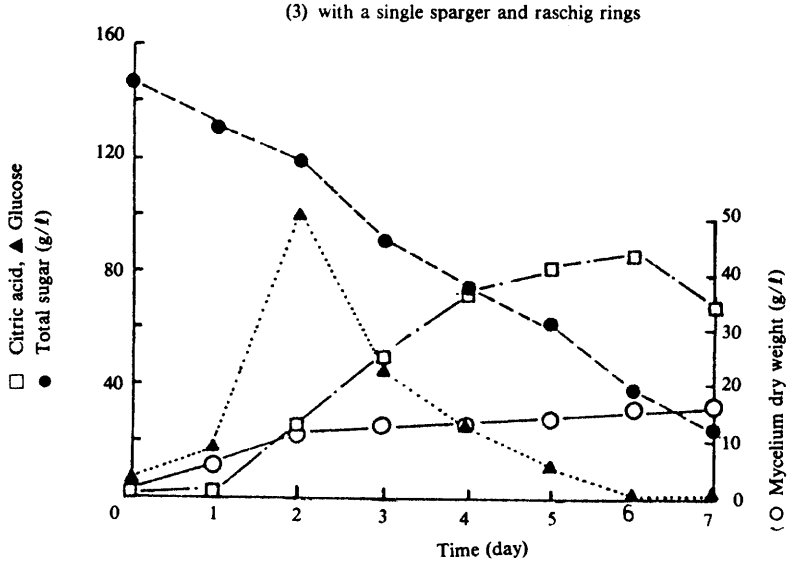

Fig. 2. Citric acid production form soluble starch (potato) at controlled pH 2.0
(Using a 10 *l* jar fermentor with 6*l* working volume, agitation rate 700 rmp.)

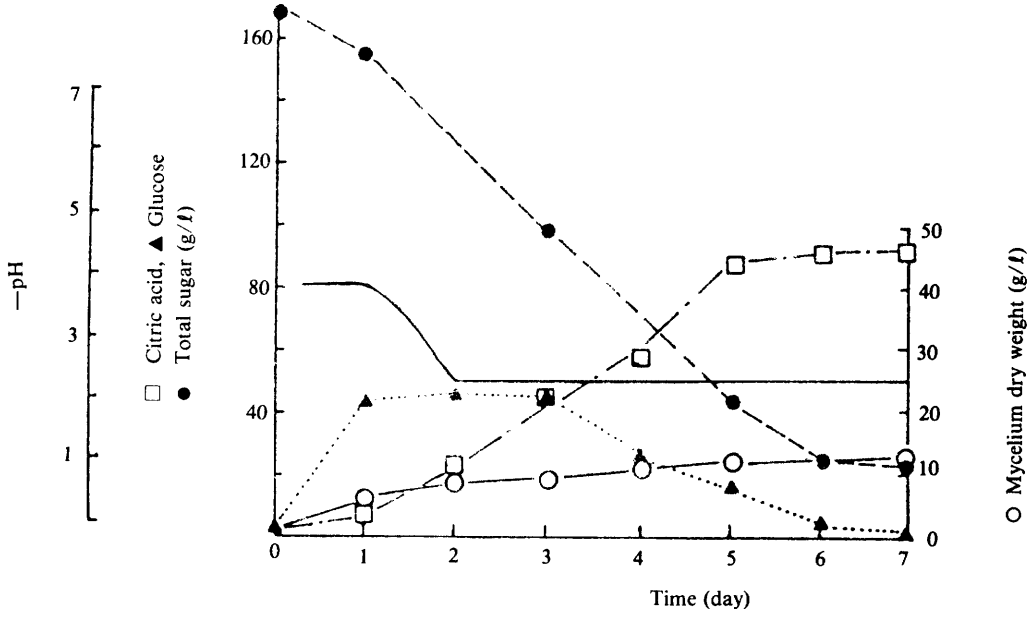

Fig. 3. Citric acid production from soluble starch (potato). pH was controlled at 4 for 1 day after that pH was contolled at 2.5. (using a 10 *l* jar fermentor with 8 *l* working volume, agitation rate 700 rpm.)

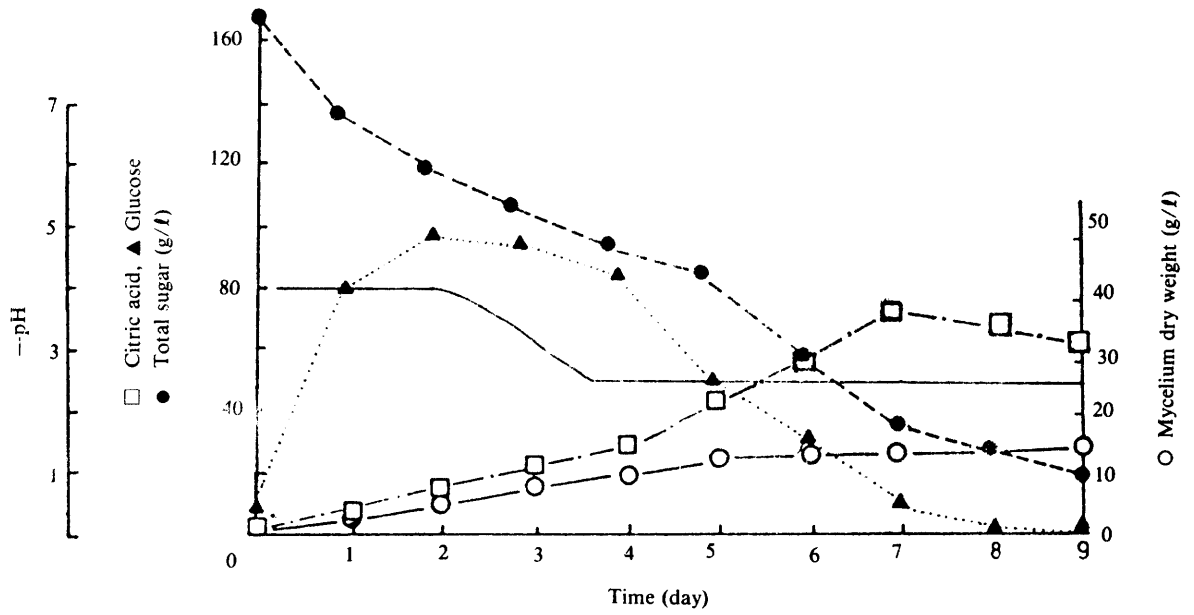

Fig. 4. Citric acid production from soluble starch (poatato). pH controlled at 4 for 2 days, after that pH was controlled at 2.5, (using a 10 *l* jar fermentor with 8 *l* working volume, agitation rate 700 rpm.)

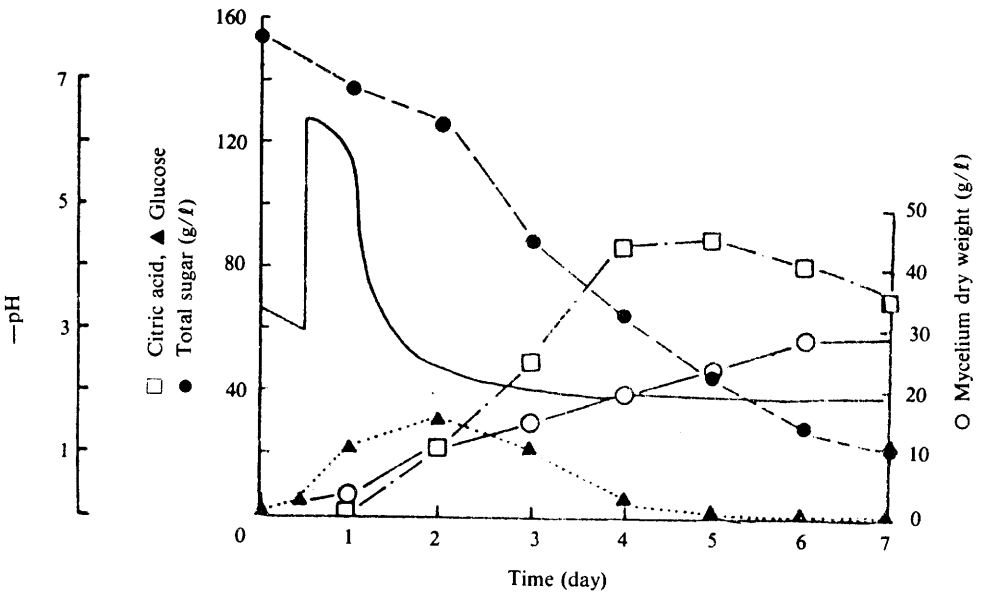

Fig. 5. Citric acid production from soluble starch (potato) with 1% $CaCO_3$ addition after 12 h fermentation. (Using a 10 ℓ jar fermentor with 8 ℓ working volume, agitation rate 600 rpm (0 - 12 th h), 700 rpm (12th - 24th h) and 800 rpm (24th h up to the final fermentation)

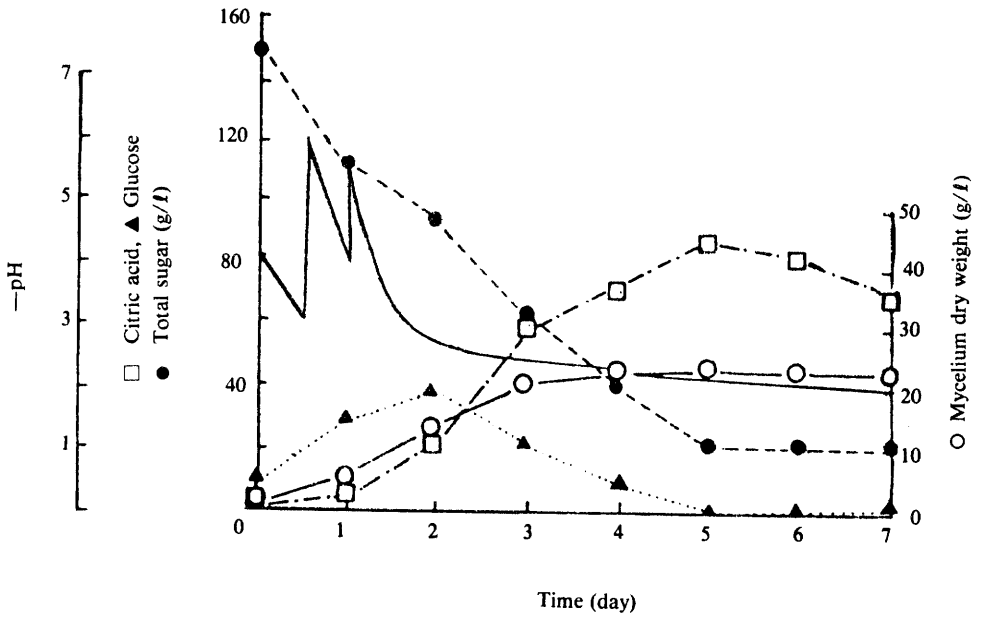

Fig. 6. Citric acid production from soluble starch (potato) with 0.5% $CaCO_3$ addition after 12 and 24 h fermentation. (Using a 10 ℓ jar fermentor with 8 ℓ working volume, agitation rate 600 rpm (0-12th h), 700 rpm (12th-24th h), and 800 rpm (24th up to the final fermentation).

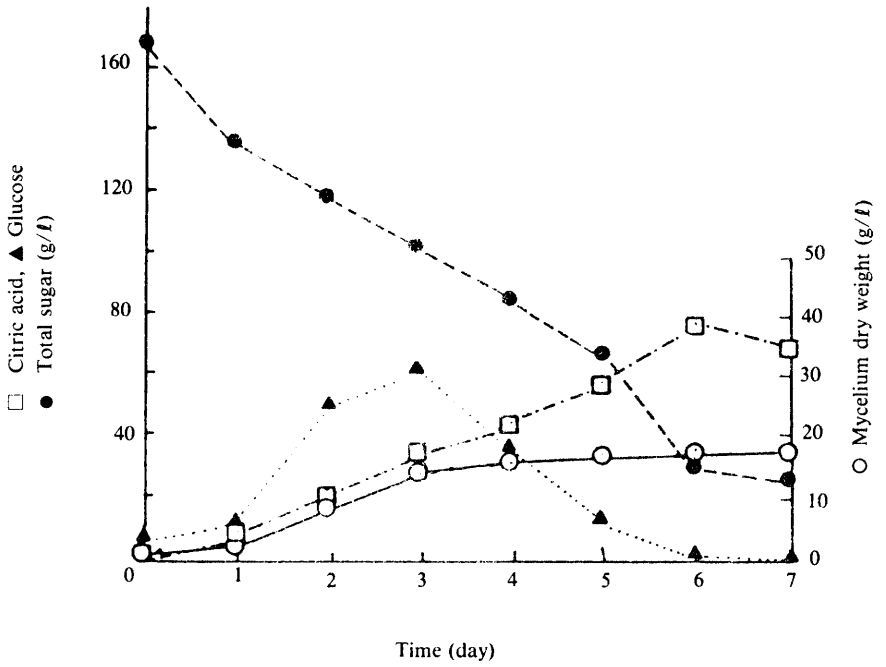

Fig. 7. Citric acid production from soluble starch (potato) with 1% $CaCl_2$ addition at controlled pH 2.0. (Using a 10 ℓ jar fermentor with 8 ℓ working volume, agitation rate 600 rpm (0-24th h), 800 rpm (24th up to the final fermentation).

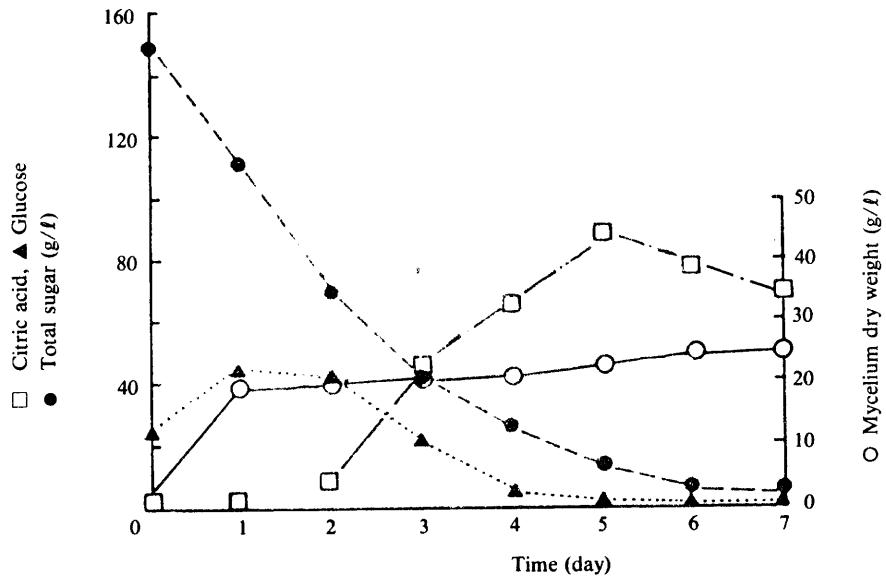

Fig. 8. Citric acid production from cassava starch. pH was controlled at 2.0 (using 2.5 ℓ jar fermentor with 1.5 ℓ working volume).

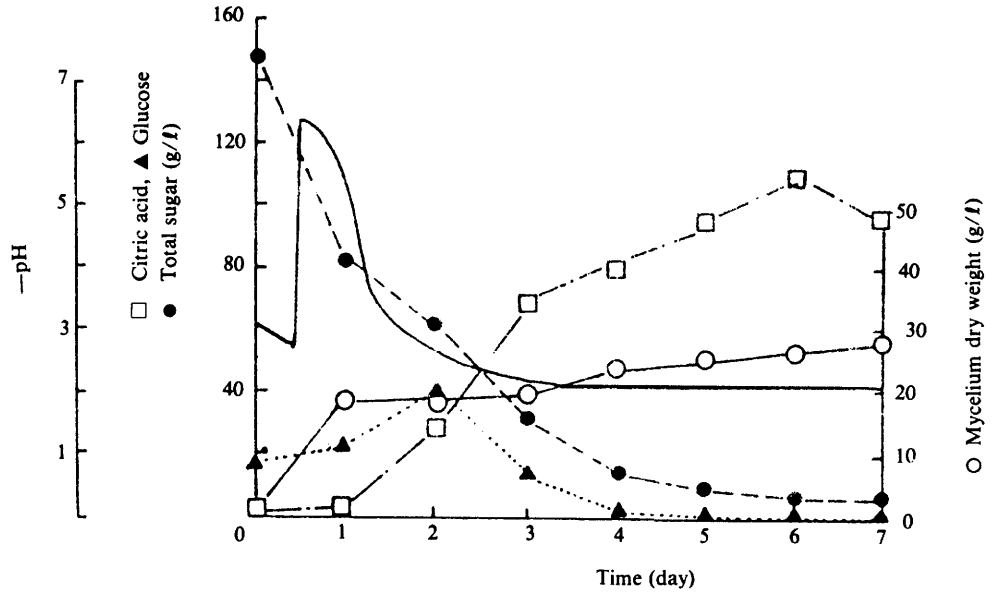

Fig. 9. Citric acid production from cassava starch with 1% $CaCO_3$ addition after 12 h fermentation. (Using a 2.5 *l* jar fermentor with 1.5 *l* working volume).

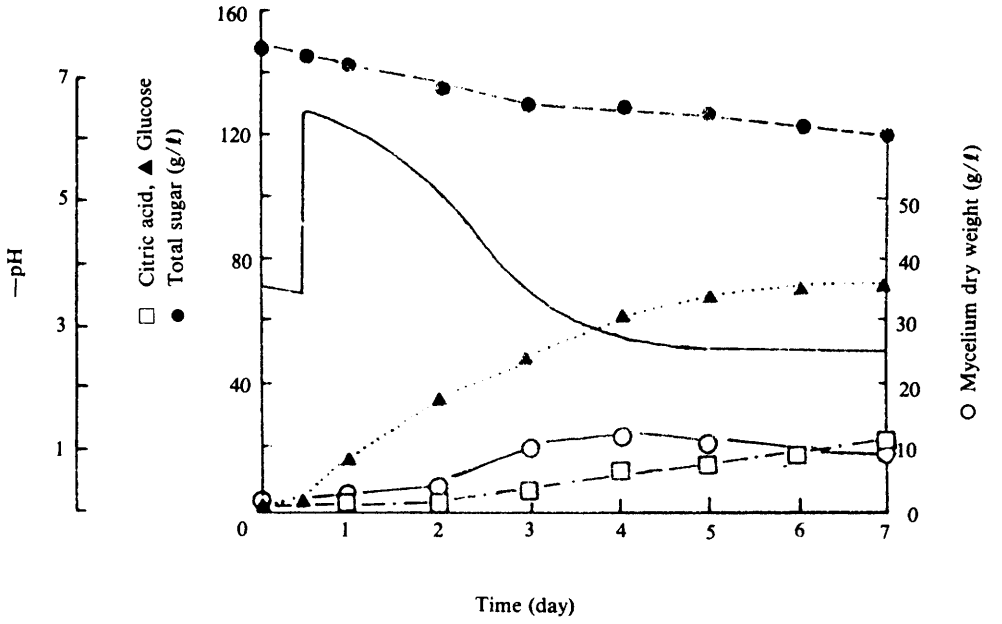

Fig. 10. Citric acid production from soluble starch in a tower fermentor 1% $CaCO_3$ was added after 12 h fermentation. (The fermentor has single sparger without draft tube and raschig rings.)