Bioprocess Engineering 15 (1996) 323–326

Citric acid production

Part 1: Strategies for reduction in cycle time for targeted yields

D. Mayilvahanan, G. Annadurai, V. Raju, M. Chellapandian, M.R.V. Krishnan, Kunthala Jayaraman

Abstract Techniques for possible higher and rapid production of citric acid from the well known industrial medium i.e. molasses has been reported using Aspergillus niger. This includes optimization of the total reducing sugar (TRS) and nutrients like nitrogen and phosphorous. The long and unproductive lag periods normally associated with this type of fermentation has been reduced. These strategies are discussed in detail.

1 Introduction

Commercially the most exploited biochemical product, namely, citric acid enjoys a good market demand which was about 300,000 tonnes per annum in the beginning of the 90's. With more applications coming to light, this tonnage is expected to register a rise with years. Citric acid is exclusively manufactured by fermentation. Literature is replete with data on several strains such as Aspergillus niger [1], Yarrowia lipolytica [2], candida lipolytica [3], Aspergillus foetidus [4], candida guilliermondi [5], acting on media ranging from glucose, cane/beet, molasses to paraffins. In citrate synthesis, biomass growth is the critical factor as uncontrolled growth results in poor yields. Investigations in this laboratory indicated that the pH for growth and citrate production were 6.5 and 3.0 respectively with 30 °C as the optimum operating temperature. Invariably the controlled parameters for maximum yields for a specified cycle time have been recognised as initial pH, concentration and type of carbon, nitrogen, phosphate and trace elements. These factors assume importance when one considers an industrial medium such as molasses since molasses is complex in nature and the use of sucrose is not economical.

Received: *27 February 1996*

D. Mayilvahanan, G. Annadurai, M. Chellapandian, M.R.V. Krishnan
Department of Chemical Engineering, Anna University, Madras-600 025, India

V. Raju, Kunthala Jayaraman
Centre for Biotechnology, Anna University, Madras-600 025, India

Correspondence to: M.R.V. Krishnan

Dr. M. Chellapandian is thankful to the Council of Scientific and Industrial Research, New Delhi, for the award of research associateship.

India is one of the largest sugar producers in the world with more than 430 sugar mills. The average capacity of each mill is about 2500 tonnes of cane crushed per day. Thus larger quantities of molasses result as by product (4–5% molasses based on sugar cane). On request from these industries, studies were conducted to effectively use this molasses for the production of citric acid. This investigation reports on production of citric acid using Aspergillus niger on molasses. A few preliminary studies were conducted to elucidate the variables that are likely to affect the progress of fermentation. Generally the yields were in the range of about 60 g/l (for the strain selected) over a span of 192 hours. Since this cycle time was too long for this average yield, a strategy was evolved to reduce the same. Thus the aim of this investigation is to promote strategies: 1. to increase citrate yield on the expense of growth by suitably designing the medium and 2. cut down on the unproductive lag period which tends to increase the cycle time.

2 Materials and methods

2.1 Microorganism

Aspergillus niger 684 was maintained on glycerol stock and preserved at −20 °C.

2.2 Media composition

Czapek-dox agar slants were made for working culture and they were maintained at 4 °C. This contained sucrose 30 g/l, $NaNO_3$ 2 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, agar 20 g/l (for slants) pH 5.0

2.3 Molasses medium

The molasses was pretreated before use. The following methods were tried with a view to testing its ability to support growth or citrate yield.

1. Sulphuric acid treatment (SA): The pH of the molasses (10% total reducing sugar) was adjusted to 3.0 by adding 0.1 N sulphuric acid. This was allowed to stand for 1.5 hours and then centrifuged at 3000 rpm for 15 minutes. The supernatant was collected and used.
2. Potassium ferrocyanide treatment (PFC): The molasses (10% TRS) was heated to 85 °C for 30 minutes and centrifuged at 3000 rpm for 15 minutes. 100 ml of the

supernatant was collected and 0.5 ml of $K_4Fe(CN)_6$ (10% solution) was added. The pH was adjusted to 6.5.

3. Tricalcium phosphate treatment (TCP): The pH of the molasses (10% TRS) was adjusted to 7.0 by the addition of 0.1N NaOH and treated with 2% (W/V) tricalcium phosphate followed by heating at 105°C for 5 minutes. The mixture was cooled and centrifuged at 3000 rpm for 15 minutes. The supernatant was used.
4. Tricalcium phosphate with hydrochloric acid treatment (TCPH): The TCP treated liquor was adjusted to pH 2.0 by the addition of 0.1N HCl followed by vigorous shaking. The mixture was allowed to stand for 6 hours. The supernatant was used after centrifugation at 3000 rpm for 20 minutes.
5. Bentonite treatment (BT): The pH of molasses (10% TRS) was adjusted to 7.0. 2% (W/V) of bentonite was added and kept in a boiling water bath for 30 minutes. The solution was then centrifuged at 3000 rpm for 15 minutes. The supernatant was collected and used.

2.4 Inoculum

Fresh spore slants, 3 to 5 days old, were used for preparing spore suspension. The suspension was made by adding 1% sodium chloride solution to the slant and gently scraping the surface. The size of the inoculum was approximately 2×10^7 colony forming units. For fermentation studies, spore suspension was inoculated into 100 ml sulphuric acid treated molasses at a pH of 6.5. After 24 to 30 hours, the inoculum was transferred to a fermenter (Marubishi MS 500, aeration 1.7 VVM, agitation 400 rpm, temperature 30°C) at 10% concentration based on fermentation medium.

2.5 Analytical methods

Biomass concentration was estimated by dry weight method. 50 ml of the sample was filtered through a preweighed and dried filter paper. The filter paper was dried in an oven at 80°C to get two consecutive constant weights. Citric acid was estimated by the method of Leopold and Valtr [6].

The variables investigated included:

1. Pretreatment of molasses – SA, PFC, TCP, TCPH and BT
2. Effect of various concentrations of TRS – 0 to 15%
3. Effect of ammonium nitrate – 0, 1.5 and 3.0 g/l
4. Effect of phosphate – 0, 0.2 and 0.4 g/l
5. Effect of inoculum concentration – 2, 4 and 10%
6. Batch fermentation with 10% TRS and 10% inoculum
7. 2 stage batch fermentation
8. Fed batch fermentation (Batch with intermittent feeding)

3 Results and discussion

The citric acid and biomass profiles using Aspergillus niger with pretreated molasses is shown in Fig. 1. The trace metal ions which have major influence are Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}. The Aspergillus niger grown on tricalcium phosphate molasses (TCP) gave the maximum citric acid yield; whereas for high biomass production, the sulphuric acid-treated molasses (SA) was found to be good. The higher yields of citric acid in the TCP and bentonite-treated molasses (BT) could be due to the partial removal of heavy metals. Further experiments on citrate yields were carried out using TCP treated molasses as substrate.

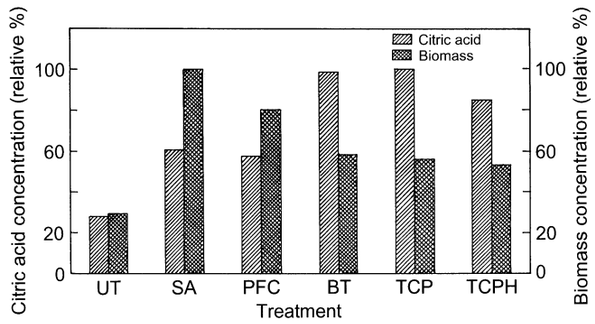

Fig. 1. Pretreatment of molasses

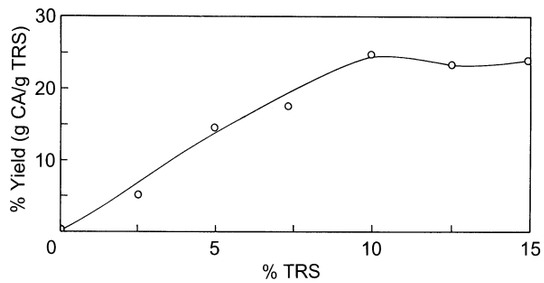

Fig. 2. Effect of TRS concentration

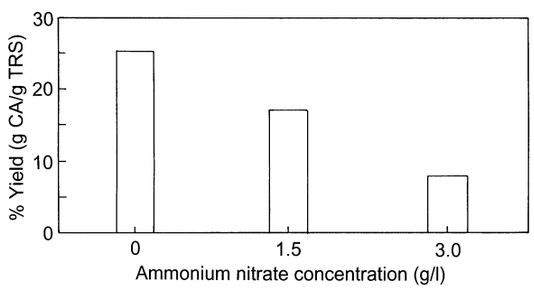

Fig. 3. Effect of ammonium nitrate

The production of citric acid depends on the initial concentration of total reducing sugar (Fig. 2). Maximum citric acid yield was obtained at a concentration of 10% total reducing sugar. This may favour the acidogenesis at high concentration of sugar [7]; a TRS below 2.5% leads to a lower yield of citric acid and the accumulation of oxalic acid [8]. Citrate production was influenced by the intracellular ammonium ion concentration. In an earlier report, the maximum amount of citric acid was produced at an ammonium ion concentration of 2 to 3 μ.mol/g. cell [9]. In this study three different concentrations of ammonium nitrate 0, 1.5 and 3 g/l were tried. Results showed that medium without the nitrogen source gave higher yields of citric acid (Fig. 3). A decrease in the yield leads to increased specific growth rate of Aspergillus niger, due to a delay in the biosynthesis of citric

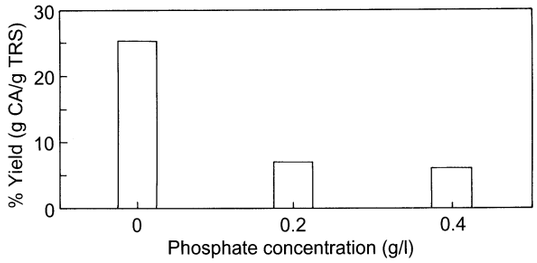

Fig. 4. Effect of phosphate

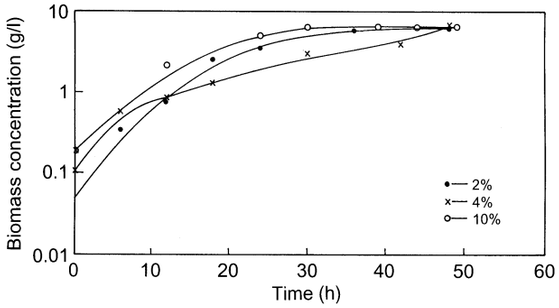

Fig. 5. Effect of inoculum concentration on biomass production

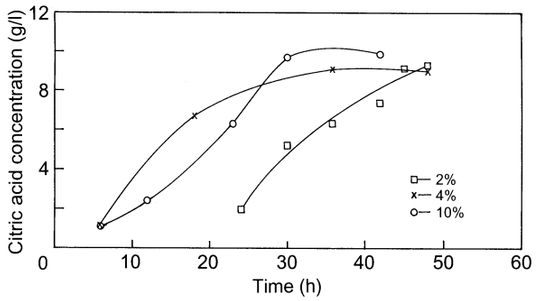

Fig. 6. Effect of inoculum concentration on citric acid production

acid at higher concentration of nitrogen [10]. The higher yield of citric acid without the addition of ammonium nitrate may be due to the presence of nitrogen in the molasses which is sufficient for the biosynthesis of citric acid. Similar results were obtained when dihydrogen potassium orthophosphate was tried at a concentration of 0, 0.2 and 0.4 g/l. The amount of phosphate present in molasses is enough for the production of citric acid (Fig. 4). The biomass concentration at pH 6.5 (19 g/l) was higher compared to that at 3.0 (8.2 g/l). But the citric acid yield of 17.8 and 8 g of citric acid per gram of total reducing sugar at a pH 3.0 and 6.5 respectively.

In order to step up the citric acid and biomass yields, different concentrations of inoculm such as 2, 4 and 10 percent were tried in a 10 l fermenter. There was a time lag between the three samples to reach the stationary phase (Figs. 5 and 6); experiments showed that 10% inoculum concentration gave the highest yield with the shortest possible fermentation time. Based on shake flask studies, the molasses medium (10% TRS)

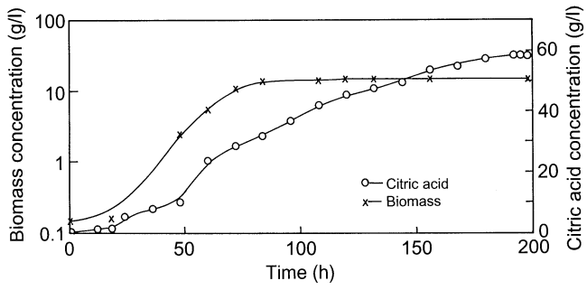

Fig. 7. Batch fermentation in stirred tank fermenter (with 10% TRS & 10% Inoculum)

was used and at regular intervals the biomass and citric acid concentration were analyzed (Fig. 7). The production of citric acid depends on the concentration of sugar available, so at higher concentration more citric acid will be produced. The maximum amount of citric acid (58.73 g/l) was produced at a fermentation period of 192 hours. The longer duration was mainly due to long initial lag period because of high sugar concentration [7].

In order to obtain more citric acid in a shorter period 2 strategies were evolved. (i) A two stage batch operation of the fermenter and (ii) a batch with intermittent feeding using high concentration of TRS. In two stage batch operation, the biomass concentration was first built up at low concentration of sugar (5% TRS). During the exponential phase, high concentration of molasses (high concentration of TRS) was added completely at once, so that the final concentration would be 10% TRS. 58.04 g/l of citric acid production was obtained over a period of 182 hours fermentation. During batch with intermittent feeding, the inoculation was made at a low concentration of sugar (5% TRS). In the exponential phase, high concentration of sugar (16% TRS) was added at a rate of 0.5 ml/min. Within 165 hours of fermentation, 60.1 g/l citric acid was produced and also the lag period in both the batch and 2 stage batch process were eliminated.

4 Conclusion

The production of citric acid using molasses medium has been studied with Aspergillus niger by submerged fermentation. Among the various pretreatment methods, tricalcium phosphate treatment was found to give maximum citric acid yield. Sulphuric acid treated molasses was used to prepare the required amounts of biomass. Since this resulted in higher concentration of biomass. The effect of initial pH shows that pH 3.0 and 6.5 are favourable for the production of maximum citric acid and biomass concentration respectively. For the preparation of inoculum, the initial pH was maintained at 6.5. However, the nitrogen and phosphate present in the molasses is sufficient and further addition decreased the yield of citric acid. 10% inoculum was found to give maximum yield of citric acid in a short duration. Intermittent addition of high concentration of sugar improved the yield of citric acid production in a short duration when compared to conventional batch and two stage batch fermentation process.

References

1. **Kapoor, K.K.; Chaudhary, K.; Tauro, P.:** Citric acid. In prescott and Dunn's Industrial Microbiology. AVI publishing Co., Westport, CT, (1982) 709–747
2. **Moresi, M.:** Effect of glucose concentration on citric acid production by Yarrowia lipolytica. J. Chem. Technol. Biotechnol. 60 (1994) 387–395
3. **Moresi, M.; Cimarelli, D.; Gasparrini, G.; Liuzzo, G.; Marinelli, R.:** Kinetics of citric acid fermentation from n-paraffins by yeasts. J. Chem. Technol. Biotechnol. 30 (1980) 266–277.
4. **Chen, H.C.:** Response surface methodology for optimizing citric acid fermentation by Aspergillus foetidus. Process Biochem. 29 (1994) 399–405.
5. **Ward, O.P.:** Industrial Chemicals. In Fermentation biotechnology, Open University Press, Milton Keynes, (1989) 135
6. **Leopold, H.; Valtr, Z.:** Ability of an aliphatic carboxylic acid and of sugars to form soluble copper complexes. Naturwissenschaften. 44 (1957) 558
7. **Xu, D.B.; Madrid, C.P.; Rohr, M.; Kubicek, C.P.:** The influence of type and concentration of the carbon source on production of citric acid by Aspergillus niger. Appl. Microbiol. Biotechnol. 30 (1989) 553–558
8. **Kovats, J.:** Studies on submerged citric acid fermentation. Acta Microbiol. Pol. 9 (1960) 275–287
9. **Jaehoon, C.; Young, J.Y.:** Effect of ammonium ion concentration and application to fed-batch culture for over production of citric acid. J. Ferment. Bioeng. 72 (1991) 106–109
10. **Dawson, M.W.; Maddox, I.S.; Brooks, J.D.:** Evidence for nitrogen catabolite repression during citric acid production by Aspergillus niger under phosphate limited growth conditions. Biotechnol. Bioeng. 33 (1989) 1500–1504