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KINETICS OF CITRATE OVER-PRODUCTION BY AN *ASERGILLUS NIGER* MUTANT RABt-10

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Abstract

The present study deals with the kinetic investigation of the effect of initial sugar concentration and rate of citrate overproduction by a mutant strain of *Aspergillus niger* RABt-10 developed after UV treatment. The maximal production of citric acid (66.15±1.1 g/l) during the course of study was achieved using initial sugar concentration, 150 g/l while the time course was optimized at 168hrs after inoculation. All the kinetic parameters *i.e.*, product and growth yield co-efficients (Yp/s, Yp/x and Yx/s in g/g), volumetric rates (Qp, Qs and Qx in g/g cells/h) and specific rate constants (qp, qs and qx in g/g/h) were highly significant.

Introduction

Citric acid fermentation is one of the main industrial fermentation processes and has always been considered as a most sophisticated technology (Roehr, 1998). Citric acid is one of the most important bulk-produced organic acids (Seaton & Wales, 1994). It is produced by fermentation using specific moulds, mostly strains of *Aspergillus niger.* Effect of various fermentation conditions and the biochemistry of citric acid by surface, submerged and solid state fermentation and continuous culture techniques have been studied by using mutant strains of *Aspergillus niger* (Gupta & Sharma, 1994; Grewal & Kalra, 1995). The final yield of citric acid in fermentation by *Aspergillus niger* is strongly dependent on the type and concentration of carbon source (Kubicek, 1998). Cane molasses is the most desirable fermentation medium for this process. In the present investigation, the effects of initial sugar concentration and rate of citrate bio-production were analyzed by studying kinetic relations. The purpose of the present investigation was to develop an economical process for hyper-production of citric acid on the basis of kinetic study.

Materials and Methods

Organism and culture maintenance: The mutant strain of *Aspergillus niger* RABt-10 used in the present study has previously been developed and screened from various available cultures in Biotechnology Research Centre of Government College University, Lahore, Pakistan by the treatment of ultraviolet irradiation (1.6×10^2 J/m^2/S) for different time intervals (5-45 min). The culture was maintained on sterilized potato dextrose agar medium (Diced potato 200 g/l, Dextrose 20 g/l and Agar 15 g/l), pH 4.5 and stored at 4°C in the refrigerator. All the culture media, unless other wise stated, were sterilized in autoclave at 15-lbs/inch^{2}pressure (121°C) for 15 min.

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Pre-treatment of cane molasses: Cane-molasses obtained from Kamalia sugar mills was used for the present study. Cane-molasses contains water 20%, sugar contents 62%, non-sugar contents 10%, and inorganic salts (ash contents) 8%, making a blackish homogenous liquid with high viscosity. Ash contents include ions such as Mg, Mn, Al, Fe and Zn in variable ratio (Prescott & Dunn's, 1987). Sugar content was diluted to about 25% sugar level. The molasses solution, after adding 35 ml of 1N H_2SO_4 per litre, was boiled for half an hour, cooled, neutralized with lime-water (CaO) and was left to stand over night for clarification (Panda *et al.*, 1984).

Inoculum preparation: Conidia from 3-5 days old slant culture were used for inoculation. The conidial suspension was prepared in sterilized 0.005% Monoxal O.T (Dioctyl ester of sodium sulfosuccinic acid). Ten ml of solution of Monoxal O-T was added to each slant having profuse conidial growth on its surface. Inoculating needle was used for breaking the clumps of conidia and test tube was shaken vigorously to obtain homogeneous mixture of conidial suspension. The number of conidia was counted with the help of Haemocytometer Slide-Bridge. The conidial suspension contained about $2.5x10^6$ conidia.

Fermentation technique: Submerged fermentation technique in 250 ml Erlenmeyer flasks was employed to investigate the optimum conditions for maximal production of citric acid by *Aspergillus niger*. Twenty-five ml of clarified cane molasses with 15% sugar level (initial pH 6.0) was taken in each of the 250 ml Erlenmeyer flasks. The flasks were inoculated with 1.0 ml of conidial suspension after sterilization. One ml of 200 ppm potassium ferrocyanide, $K_4Fe(CN)_6$ was added at the time of inoculation when the medium was hot. The flasks were then incubated at a rotary incubator shaker (Gallenkemp PLC, UK) at 30°C for 7 days. The shaking speed was kept at 160 rpm. After incubation, fermented broth was filtered and filtrate was used for the estimation of citric acid and residual sugar contents.

Analytical techniques: Residual sugar was estimated gravimetrically by DNS method (Tasun *et al.*, 1970). A UV/V is double beam scanning spectrophotometer (Model: CECIL CE-7200, UK) was used for measuring colour intensity. The culture medium was filtered through weighed Whatmann filter paper No. 44. The mycelium left upon the filter paper folds were used to determine the dry cell mass according to the method of Haq & Daud (1995). Anhydrous citric acid was estimated spectrophotometrically, using pyridine-acitic anhydride method, as reported by Marrier & Boulet (1958). For determining kinetic parameters of batch fermentation process, the procedures of Pirt (1975) were adopted.

Results and Discussion

Rate of citric acid fermentation: The optimum time of incubation for maximal citric acid production varies both with the organism and fermentation conditions (Kubicek, 1998). The maximum yield of citric acid (57.00±0.4 g/l) was achieved, 168 hrs after incubation. The sugar consumption and mycelial dry weight were 93.50±3.5 and 14.58±0.3 g/l, respectively. Further increase in incubation period did not show any enhancement in citrate production. It might be due to decrease in amount of available nitrogen in fermentation medium, the age of fungi, the presence of inhibitors produced by

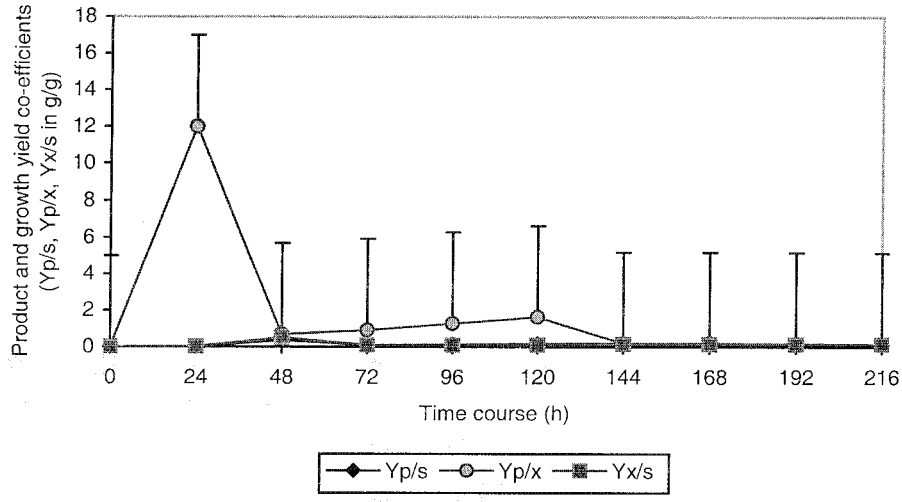

Fig. 1. Comparison of product and growth yield coefficients for citric acid fermentation.

Kinetic parameters
Yp/s = g citric acid produced /g substrate consumed, Yp/x = g citric acid produced /g cell formed, Yx/s = g cell formed/g substrate consumed, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

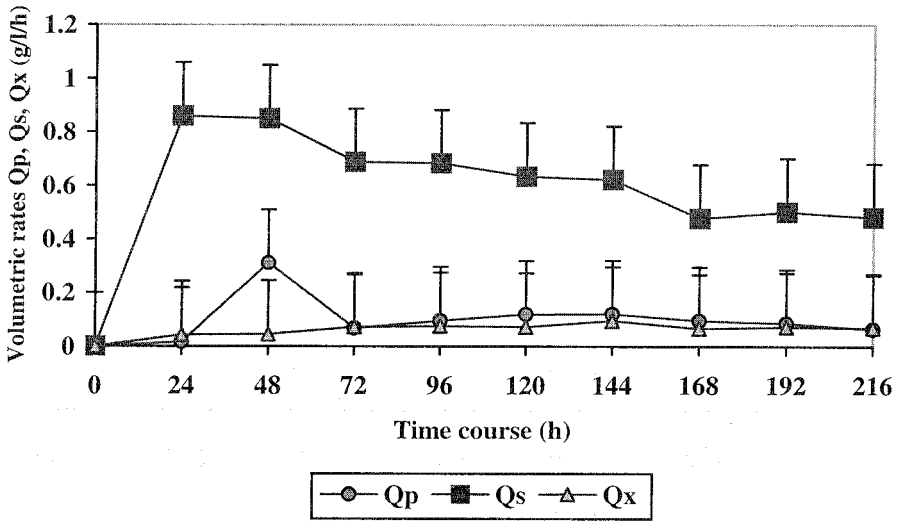

Fig. 2. Comparison of volumetric rates for citric acid fermentation.

Kinetic parameters
Qp = g citric acid produced /l/h, Qs = g substrate consumed /l/h, Qx = g cell formed /l/h, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$

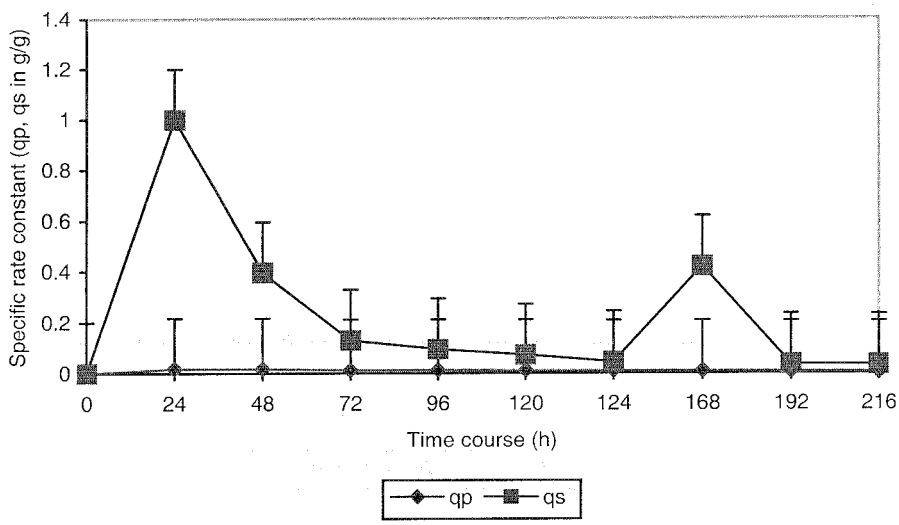

Fig. 3. Comparison of specific rate constants for citric acid fermentation.

Kinetic parameters
qp = g citric acid produced / g cells/ h, qs = g substrate consumed /g cells/ h, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$

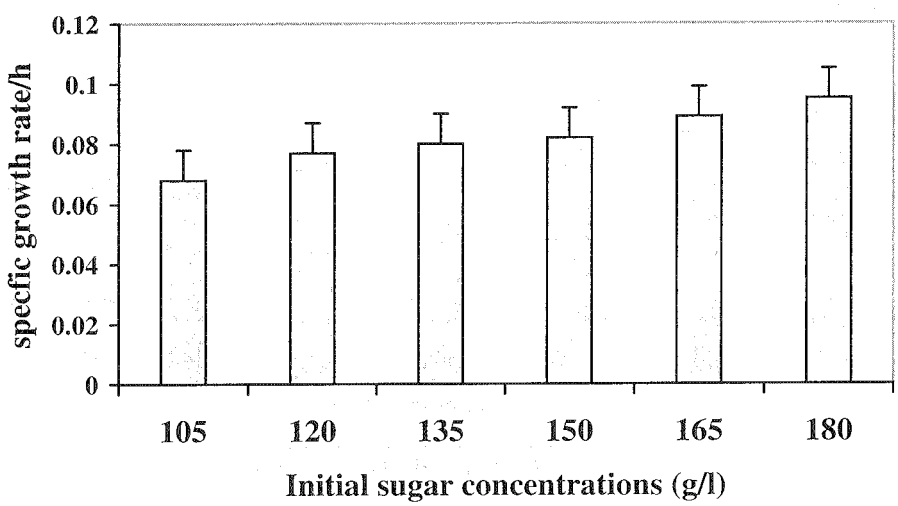

Fig. 4. Comparison of specific growth rate for citric acid fermentation.

Kinetic parameter
Specific growth rate, μ (h^{-1}) = g cell mass produced /l/h, Y error bars indicate the standard error of means among the three parallel replicates. The value differs significantly at $p \leq 0.05$.

fungi itself and the depletion of sugar contents. In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reaches maximum at the on set of stationery phase. This finding is in agreement with the observations of Vergano *et al.,* (1996) and Rajoka *et al.,* (1998). Clark (1962) obtained about 70% conversion of available sugar, 192 hrs after incubation. Hence, our finding is more encouraging as compared to Clark (1962) due to short incubation period. Different kinetic parameters such as product and growth yield coefficients (Y p/s, Y p/x, Y x/s), volumetric rates (Qp, Qs, Qx) and specific rate constants (qp, qs) were also studied (Figs. 1-3). The values for Yp/s, Yp/x, Qp and qp were more significant after 168 hours of incubation than all other time periods, for citric acid production.

Initial sugar concentration: The initial sugar concentration plays an important role in determining the amount of citric acid and also other organic acids produced by *Aspergillus niger* (Haq *et al.,* 2001). The present culture produced citric acid (66.15±1.1 g/l) in the medium containing 150.0 g/l sugars. The sugar consumption and mycelial dry weight were 118.88±2.0 and 14.12±0.4 g/l, respectively. The mycelial growth in the medium was in the form of small pellets and some fussy mass resulting in better agitation hence, improved aeration (or oxygen supply) of fermented broth. Further increase in concentration of sugar resulted in the gradual reduction of citric acid formation. It might be due to over growth of the mycelium, which resulted in increased viscosity and mass transfer limitations. A concentration higher than 15-18%, however, leads to greater amount of residual sugars, making the process uneconomical, while on the other hand a lower concentration of sugar leads to lower yield of citric acid due to accumulation of oxalic acid. Fig. 4 shows the comparison of specific growth rate, μ (h^{-1}) at different sugar concentration. The more significant value of the μ (h^{-1}) was recorded at 150g/l sugar level.

It would suggest that citric acid fermentation using *Aspergillus niger* is one of the novel examples of industrial fermentation. Kinetics of citric acid fermentation without including the necessary parameter other than mentioned above indicated that the mutant strain RABt-10 has the potential to hyper-produce citric acid and that the strain can be exploited on industrial scale after optimizing further nutritional parameters such as nitrogen, phosphorus, calcium sources etc. Furthermore, treatment by chemical mutagens like NTG (N-methyl N-nitro N-nitroso guanidine), EMS (Ethyl methane sulfonate) etc., would be beneficial in this regard.

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