Journal of Food Technology 1 (4): 157-159, 2003

Nitrogen Limitation for Citrate Accumulation by Yarrowia Lipolytica NRRL-143 in Shake Flask

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Abstract: In the present investigation, the effect of various nitrogen sources and their concentration on citrate fermentation by Yarrowia lipolytica NRRL 143 was studied and the results were analyzed kinetically. Of the three nitrogen sources i.e NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$, Ammonium nitrate in 0.3 % concentration gave maximum production of citric acid during the course of study i.e. 31.12 ± 0.1. The product and growth yield coefficients such as Yp/s and Yp/x were found to be quite significant.

Key words: Cane molasses, citric acid, ammonium chloride, ammonium nitrate and ammonium sulphate

Introduction

Citric acid has been produced using various fungi since 1917 and by yeast since the 1960's. (Anastassiadis *et al.*, 2002). A variety of yeasts have been studied for the production of this acid. But Yarrowia lipolytica has been the organism of choice. Many of the modern pilot projects prefer yeasts on filamentous fungi including *Aspergillus* species (Crolla and Kennedy, 2001). The concentration of nitrogen constituents has a profound effect on the yield of citric acid. The nitrogen requirement of citric acid production is generally met by the addition of inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $NaNO_3$, KNO_3 and urea etc. The type of the nitrogen source and its concentration affects the performance of the organism considerably e.g., ammonium sulphate prolongs the vegetative growth, while ammonium nitrate favours the shorter period of vegetative growth (Naguchi and Bando, 1960; Gupta *et al.*, 1976). In general, a high concentration of nitrogen leads to vegetative growth and delays the onset of production phase (Prescott and Dunn, 1987). In the present work, the effect of NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$ on citric acid accumulation by Yarrowia lipolytica NRRL-143 was studied and the results were analyzed kinetically.

Materials and Methods

Organism and Culture Maintenance: Yarrowia lipolytica strain NRRL-143 used in the present study, was obtained from North Regional Research Laboratories (USA). The yeast culture was maintained on sucrose-yeast extract peptone agar medium in a cool cabinet at 4°C.

Pre-treatment of Cane-molasses: Sugar-cane molasses obtained from Kamalia Sugar Mills was pre-treated according to the method of Panda *et al.*, 1984.

Inoculum Preparation. Yeast cells from 2-3 days old slant culture were used as an inoculum. The inoculum was prepared in sterilized 0.005 % monoxal O.T (Di-ocetyl ester of sodium sulfo succinic acid). Ten ml of monoxal O.T solution was added to each slant having profuse cellular growth on its surface. The test tube was shaken vigorously to obtain homogenous mixture of cell suspension and then the suspension was aseptically transferred to a sterilized test tube. Each ml of yeast cell suspension contained 1.2×10^7 cells. The cell count was made on a Haemocytometer Slide Bridge (Precicolor, HBG, Germany).

Fermentation Technique: Submerged fermentation technique was employed in the present study. Twenty-five ml of clarified cane molasses was taken in each of the 250ml cotton plugged Erlenmeyer flasks. The flasks were sterilized in an autoclave at 15 lb/in^2 (121°C). One ml of the yeast cells suspension was transferred to each flask. The flasks were then rotated at orbital shaking incubator (Model: 10X400.XX2C, SANYO, Gallenkamp PLC, UK) at 30°C for 6 days. All the experiments were run in triplicates.

Assay Methods: Anhydrous citric acid was estimated spectrophotometrically by pyridine and acetic-anhydride method (Marrier and Boulet, 1958). Dry cell mass was determined by filtering the culture medium through preweighed Whatman filter paper No.1. The filtrate was used for the estimation of citric acid and residual sugar contents. The yeast cells were thoroughly washed with tap water and dried at 105°C, overnight (Haq and Daud, 1995).

Sugar was estimated gravimetrically by DNS method after Tasun *et al.* (1970). A double beam UV/Vis scanning Spectrophotometer (Model: CECIL CE-7200, 7000-Series, Aquarius, UK) was used for

measuring colour intensity at 546 nm.

Results and Discussion

Effect of addition of different nitrogen sources such as NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl and their concentrations (0.20, 0.25, 0.30 and 0.35 %) on citric acid fermentation by *Yarrowia lipolytica* NRRL-143 was studied (Fig. 1). The maximum production of citric acid (31.12 ± 0.1 g l^{-1}) was achieved when NH_4NO_3 was added to the molasses medium. The sugar consumption and dry cell mass were 96.10 ± 3.5 and 14.50 ± 0.3 g l^{-1}, respectively. The percentage yield of citric acid on the basis of sugar used was 32.38 %. The addition of $(NH_4)_2SO_4$ and NH_4Cl, however, did not enhance citric acid production. Product and growth yield coefficients as kinetic parameters were also studied for citric acid using different sources of nitrogen and their concentrations (Fig 2). The values for Yp/s and Yp/x (g/g) at 0.20 % NH_4NO_3 were found to be significant than the others.

Nitrogen constituents have a profound effect on the yield of citric acid because the type of nitrogen source

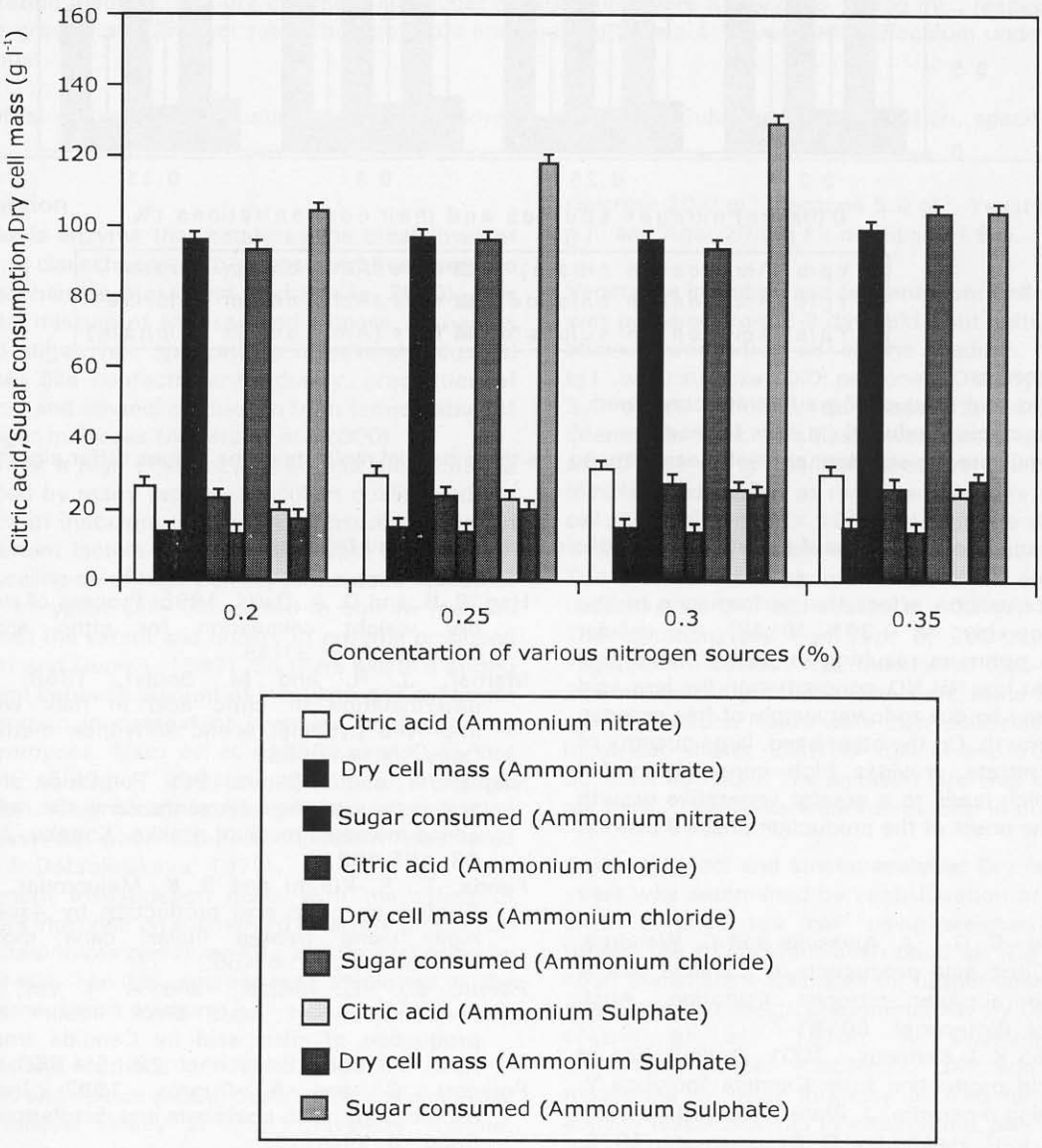
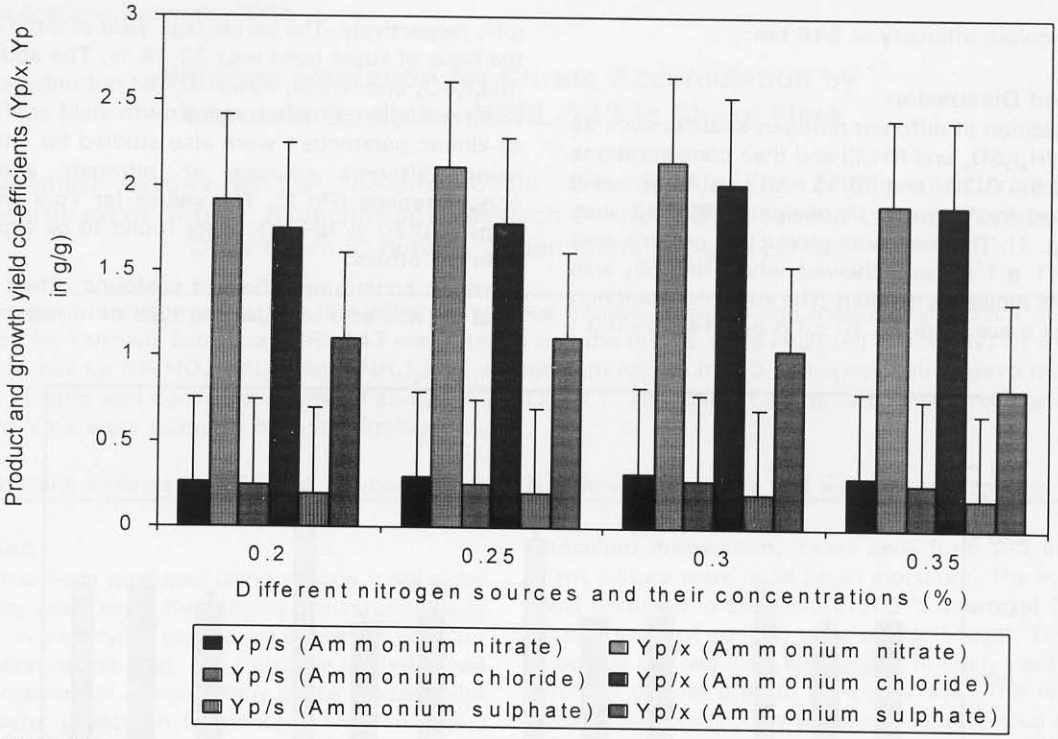


Fig 1: Comparison of the effect of various nitrogen sources and their concentration on citric acid fermentation by Yarrowia lipolytica NRRL-143



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

Fig 2: Comparison of product and growth yield coefficients for citric acid fermentation

and its concentration affect the performance of the yeast considerably. At 0.30% NH_4NO_3 the cellular growth was optimum resulting in greater citric acid production. At low NH_4NO_3 concentration the less acid production may be due to lower supply of free nitrogen for cellular growth. On the other hand, large quantity of ammonium nitrate provides high concentration of nitrogen, which leads to a greater vegetative growth and delays the onset of the production phase (Potvin *et al.*, 1988).

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