Short Communication

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STUDY OF SOME KINETIC PARAMETERS FOR CITRIC ACID BIOSYNTHESIS BY *ASPERGILLUS NIGER* MUTANT NG - 110 USING SHAKE FLASK TECHNIQUE

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Citric acid can be produced from various microorganisms such as bacteria, filamentous fungi and yeast by applying various fermentation techniques. Because of its high solubility, palatability and low toxicity, citric acid has now become one of the most commonly used acids. Approximately, 75% of this compound is used as food acidulate and 12% in pharmaceutical industry (Haq *et al* 2001). Various fungi have been evaluated for citric acid production but best one for abundant citric acid production is *Aspergillus niger* (Maddox and Brooks 1998). The present study is concerned with the effect of pH and various concentrations of $K_4Fe(CN)_6$ and K_2HPO_4 on citric acid bio-production and their kinetic analysis.

Organism and inoculum preparation. The mutant strain of *Aspergillus niger* NG - 110 has been screened for citric acid accumulation from various available cultures in Biotechnology Research Centre of Government College University, Lahore, Pakistan developed 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. Conidial inoculum was used in the present study. Conidia from 3 - 5 days old slant culture were used for inoculation. The conidial suspension was prepared in sterilized 0.005% Monoxal O.T. (Dioctyle ester of sodium sulfosuccinic acid). One ml of the suspension contained 1.5×10^7 conidia. The count was made on a haemocytometer slide bridge under microscope.

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Fermentation technique. Submerged fermentation technique in 250 ml Erlenmeyer flasks was employed to investigate the optimum conditions for maximal production of citric acid. Twenty-five ml of clarified cane molasses with 15% sugar level (initial pH 6.0) was taken in each of the flasks. After sterilization, the flasks were cooled at room temperature and inoculated with 1.0 ml of conidial suspension. The flasks were then incubated at a rotary incubator shaker (Gallenkamp PLC, UK) at 30°C for 7 days. The shaking speed was kept at 200 rpm. After incubation, fermented broth was filtered through pre-weighed Whatman filter paper No.44 to remove the fungal mycelia and filtrate was used for the estimation of citric acid and residual sugar contents.

Analytical techniques. The filtrate was analysed for the estimation of residual sugar gravimetrically by DNS method (Tasun *et al* 1970) and citric acid anhydrous was estimated spectrophotometrically using pyridine-acetic anhydride method as reported by Marrier and Boulet (1958) whereas, for the calculation of dry cell mass, mycelia were thoroughly washed with tap water and dried at 105°C for two hours (Haq and Daud 1995).

Any increase or decrease in the pH greatly reduced citric acid biosynthesis. It might be due to that at lower pH the ferrocyanide was more toxic for the growth of mycelium in molasses medium. This has been reported by Pessoa *et al* (1984) whereas, a higher pH leads to the accumulation of oxalic acid. Fermentation medium with initial pH 6.0 resulted in maximum citric acid production (65.20 ± 0.2 g / l). Any increase or decrease in the phosphate quantity reduced citric acid production due to improper growth of mould mycelia. A high concentration of phosphate in the fermentation medium promotes more growth and less acid production (Khan *et al* 1970).The sugar consumption and mycelial dry weight were 93.50 ± 2.0 and 16.00 ± 0.3 gl / l, respectively. The percentage yield of citric acid on the basis of sugar fermented was 69.73%. Figure 1 shows the comparison of specific growth rate of NG - 110 (µg / h) for citric acid production.

Potassium ferrocyanide concentration. Effect of addition of different concentration of potassium ferrocyanide (50 - 300 ppm) on citric acid production by *Aspergillus niger* NG - 110 from molasses was investigated in shake flask. The fermentation medium containing 200 ppm potassium ferrocyanide showed the maximum citric acid production (69.3 ± 0.8 g / l). The sugar consumption and mycelial dry weight were 83.5 $\pm$ 4.0 and 25.3 ± 0.4g / l, respectively. The percentage yield of citric acid on the basis of sugar consumed in the medium containing 200 ppm $K_4Fe(CN)_6$ was 80.99%. A decrease in citric acid production was observed, when the concentration of potassium ferrocyanide was increased or decreased from

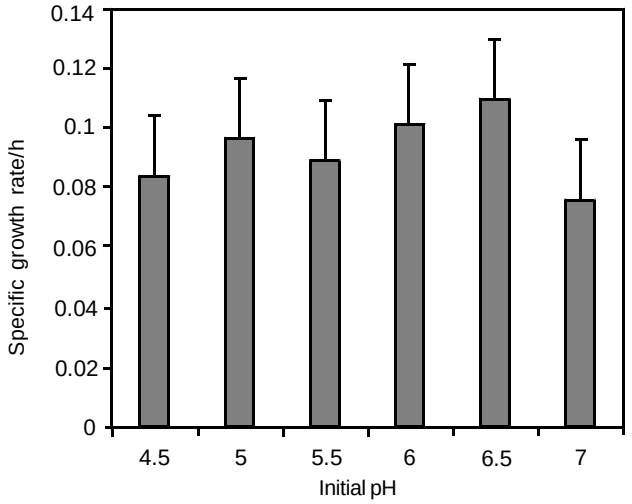

Fig 1. Comparison of specific growth rate for citric acid production at various initial pH.

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

200 ppm. Product and growth yield coefficients as kinetic parameters were also studied for citric acid at potassium ferricyanide concentration of 200 ppm (Fig 2).

Dipotassium hydrogen phosphate concentration. The effect of addition of different concentrations of K_2HPO_4 ranging from 0.15% - 0.30% w/v, on citric acid fermentation by *Aspergillus niger* NG - 110 in shake flask was studied. The addition of K_2HPO_4 in the fermentation medium resulted in maximum citric acid production (81.21± 0.2 g / l) of K_2HPO_4. Sugar consumption and mycelial dry weight were 92.20 ± 3.5 and 20.40 ± 0.2 g / l, respectively. The percentage yield of citric acid on the basis of sugar used was 88.11%. The mould growth was in the form of small round pellets, observed in the fermented broth. Product and growth yield coefficients as kinetic parameters were also studied for citric acid production using different concentrations of dipotassium hydrogen phosphate (Fig 3). The values for Yp/s and Yp/x (g / g) at 2 g / l K_2HPO_4 were found to be significant.

The kinetic parameters such as growth yield coefficients (Y p/s and Y p/x in g / g), were also undertaken. The mutant strain of *Aspergillus niger* NG - 110 showed improved values for Y p/s and Y p/x. Similar kind of work has also been reported by Pirt (1975). Maximum growth in terms of specific growth rate (μ / h) was only marginally different during growth of mutant *A. niger* NG - 110 on 150 g / l carbohydrates in molasses at 30°C (than 32°C or 165 g / l sugar). Therefore, when the culture was monitored for Y p/s and Y p/x, there was a significant enhancement in these variables at optimal nutritional conditions.

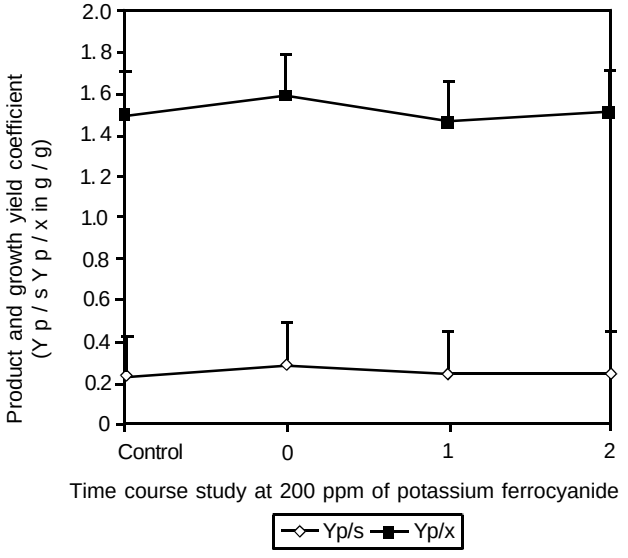

Fig 2. Comparison of product and growth yield coefficients for various time intervals at potassium ferrocyanide concentration of 200 ppmn.

Kinetic parameter: Y p/s = g citric acid produced/g substrate consumed; Y p/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 $p < 0.05$.

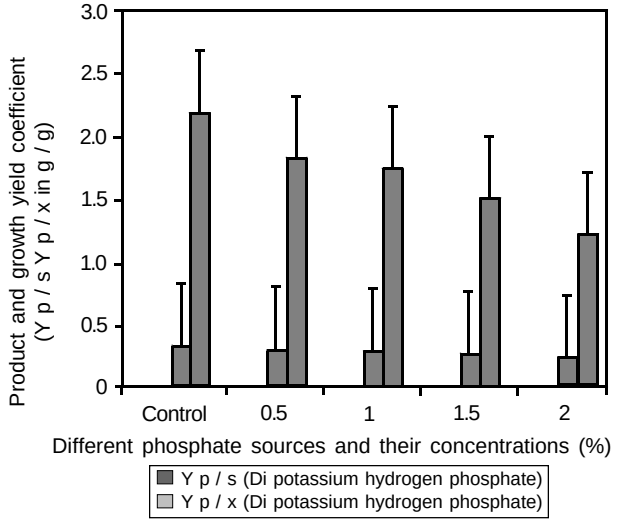

Fig 3. Comparison of product and growth yield coefficients for citric acid production.

Kinetic parameter: Y p/s = g citric acid produced/g substrate consumed; Y p/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 $p < 0.05$.

This indicated that the mutant strain used in the current studies is a faster growing organism and has the ability to overproduce citric acid without additional replacements. The study is directly substantiated with the findings of Rajoka *et al* (1998). Maximum values for Y p/s, were several folds improved

over the previous workers (Pirt 1975; Roehr 1998; Kamal *et al* 1999).

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