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Citric Acid Fermentation by *Aspergillus niger* NG-110 in Shake Flask

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Abstract: The present study was conducted on the optimization of cultural conditions for the production of citric acid by mutant strain of ***Aspergillus niger*** NG-110 by using shake flask technique. Different cultural conditions such as alcohol (1% methanol) and calcium chloride (2%) were optimized for enhanced citric biosynthesis. The results showed that maximum amount of anhydrous citric acid (81.21±0.2 gl^{-1}) was obtained after 168 h inoculation, with a sugar consumption of 92.20±3.5 gl^{-1}. The dry weight of mycelia was 20.40±0.2 gl^{-1}. On the basis of comparison of kinetic parameters namely the product and growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$), volumetric rates (Qp) and specific rate constants (q_p), the mutant strain of ***Aspergillus niger*** NG-110 was a faster growing organism and had the ability to hyper produce citric acid.

Key words: Citric acid, fermentation, *Aspergillus niger*

Introduction

Citric acid is ubiquitous in nature and exists as an intermediate in the citric acid (Kreb's) cycle when carbohydrates are oxidized to carbon dioxide (Rajoka ***et al.***, 1998). It is solid at room temperature, melts at 153°C and decomposes at higher temperature into other products. 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). Several microorganisms have been evaluated for citric acid production such as fungi, bacteria and yeast. Sikander ***et al.*** (2002) has dominated others both in laboratory and industrial scale. Cane-molasses is a desirable raw material for citric acid fermentation because of its availability and relative low price molasses is a very complex mixture of many components and attempts have been made to relate the presence of toxic components to fermentation performance.

Pakistan has not fared well in all fields of science, but it has made some progress in the area of biotechnology. Being an agricultural country, it has vast resources of biomass for their conversion into useful products through the application of fermentation technology. Substantial amounts of cereals and other crops are grown in the country for the purpose of food and other products. Some of the by-products include broken rice, damaged wheat, different types of molasses and corn steep liquor etc. In this study attempts have been made to utilize "cane molasses", as a cheap and abundantly available raw substrate for the enhanced production of citric acid. The objective of this study was to describe the kinetics of citric acid fermentation

by ***Aspergillus niger*** through optimization of the cultural conditions in a laboratory scale stainless stirred fermentor.

Materials and Methods

A mutant strain of ***Aspergillus niger*** was obtained from the available stock culture of Biotechnology Research Labs., Government College, Lahore. Cane molasses obtained from Kamalia Sugar Mills was clarified according to the method of Panda ***et al.*** (1984).

Inoculum preparation

The conidial inoculum was used in this study. Conidia from 3-5 days old slant culture were used as inoculum. The conidial suspension was prepared in sterilized 0.005% Monoxal O.T. (Di-Octyl Ester of sodium sulfosuccinic acid) solution. Monoxal O.T solution was added to each slant having profuse conidial growth on its surface. The test tube was shaken vigorously for breaking the clumps of conidia and the conidial suspension was aseptically transferred to sterilized test tube. The number of conidia was counted with the help of Haemocytometer. The conidial suspension contained about 2.5×10^{6} conidia ml^{-1}.

Fermentation technique

Submerged fermentation technique was employed. Cane molasses obtained from Chunian Sugar Mills were used for citric acid fermentation after clarification. The sugar content of molasses was about 50% w/w. The molasses solution, after adding 35 ml l^{-1} of 1N H_2SO_4 was boiled for 1 h, cooled, neutralized with lime-water and was left to stand overnight for clarification. The clear supernatant was diluted to 15% sugar levels. Twenty-five ml of the clarified cane molasses containing 15% sugar (pH 6.0) was taken in 250 ml cotton plugged conical flask. The flasks were sterilized in autoclave and cooled at room temperature. One ml of the conidial inoculum was transferred to each flask. The flasks were then rotated at the rotary incubator shaker (model GLSC-AF-199-10 Pak) at 30°C for 168 h. The fermented broth was filtered and was used for the estimation of sugar and citric acid. All the experiments were run parallel in triplicates.

Assay methods

Residual sugar was estimated gravimetrically by DNS method (Tasun ***et al.***, 1970). A UV/vis 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 mycelia left upon the filter paper folds were used to determine the dry cell mass according to the method of Haq and Daud (1995). Anhydrous citric acid was estimated spectrophotometrically, using pyridine-acitic anhydride method, as reported by Marrier and Boulet (1958). For determining kinetic parameters of batch fermentation process, the procedures of Pirt (1975) were adopted.

Results

Rate of citric acid fermentation

The rate of citric acid fermentation by a strain of ***Aspergillus niger*** NG-110 was investigated in shake flask (Table 1). The fermentation was carried out from 24-242 h. After 24 h of incubation, the amount of citric acid produced was 10.50 g l^{-1}. Further increase in the incubation period resulted in increased citric acid production. However, maximum production (57.0±0.4 g l^{-1}) was achieved, 168 h, after inoculation. The sugar consumption and mycelial dry weight were 93.50±3.5 and 14.58±0.3 g l^{-1}, respectively. The percentage yield of citric acid on the basis of sugar used was 60.96%. The mycelial morphology was mixed mycelium. Further increase in incubation period did not show any enhancement in citric acid production. Hence optimum time for citric acid production was 168 h, after inoculation. 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 (Fig. 1-3). The values for $Y_{p/s}$, $Y_{p/x}$, Qp and qp were more significant after 144 h of incubation than all other time periods, for citric acid production.

Effect of addition of different concentrations of potassium ferrocyanide

Effect of addition of different concentrations of potassium ferrocyanide (50-300 ppm) on citric acid fermentation by a strain of ***Aspergillus niger*** NG-110 was investigated in shake flask (Table 2). Potassium ferrocyanide was added to the sterilized fermentation medium just before inoculation when the medium was hot. The fermentation medium containing 200 ppm potassium ferrocyanide showed the maximum citric acid production (69.30±0.8 g l^{-1}). The sugar consumption and mycelial dry weight were 83.50±4.0 and 25.30±0.4 g l^{-1}, respectively. The percentage yield

Table 1: Rate of citric acid fermentation by a mutant strain of ***Aspergillus niger*** NG-110 in shake flask

Incubation period (h)	Mycelial dry wt. (g l^{-1})	Sugar consumption (g l^{-1})	Citric acid (g l^{-1})	Mycelial Morphology
24	6.6±0.2	55.0±2.0	10.5±0.1	Elongated mycelium
48	9.5±0.1	70.0±2.5	21.0±0.1	Round pellets
72	11.0±0.2	80.5±2.0	24.5±0.2	Small round pellets
96	11.5±0.2	85.0±4.0	31.0±0.2	Large round pellets
120	12.3±0.2	91.2±5.5	42.0±0.2	Small round pellets
144	13.4±0.5	92.3±5.0	48.0±0.2	Small round pellets
168	14.5±0.3	93.5±3.5	57.0±0.4	Mixed mycelium
192	16.7±1.2	99.7±2.9	42.0±0.5	Gelatinous mass
216	16.2±1.0	104.4±3.4	35.0±0.2	Dumpy mass
242	17.9±1.1	110.3±4.0	39.7±0.4	Dumpy mass

Initial sugar concentration, 150 g l^{-1}; Temperature, 30°C; Initial pH, 6; Potassium, ferrocyanide, 200 ppm. ± indicates standard error of means among the three parallel, replicates. The values differ significantly at $P < 0.05$

of citric acid on the basis of sugar consumed in the medium containing $K_4Fe(CN)_6$ (200 ppm) was 80.99%. A decrease in citric acid production was observed, when the concentration of potassium ferrocyanide was increased or decreased from 200 ppm. The level of $K_4Fe(CN)_6$ at 200 ppm concentration was found to be optimum for citric acid production. Product and growth yield coefficients as kinetic parameters were also studied for citric acid using different concentration of potassium ferrocyanide (Fig. 4). The values for $Y_{p/s}$ and $Y_{p/x}$ at 50ppm $K_4Fe(CN)_6$ were found to be more significant than the others.

Effect of different alcohols and their concentrations

Table 3 shows the effect of different alcohols, such as methanol, ethanol and butanol, and their concentrations (0.5-1.5%) on the rate of citric acid fermentation by mutant strain of ***Aspergillus niger*** NG-110 in shake flasks. The production of citric acid was found to be maximum (73.29±0.2 g l^{-1}) when methanol at the level of 1% was added to the fermentation medium. At this stage the % yield of citric acid was 83.28% along with sugar consumption 88.0±3.0 g l^{-1} and dry cell mass 14.07±0.2 g l^{-1}. There was a decrease in the yield by increasing the concentration of

Table 2: Effect of addition of different conc. of $K_4Fe(CN)_6$ on citric acid fermentation by a mutant strain of *Aspergillus niger* NG-110 in shake flask

Conc. of $K_4Fe(CN)_6$ (ppm)	Mycelial dry wt. (g l^{-1})	Sugar consumption (g l^{-1})	Citric acid (g l^{-1})	Mycelial Morphology
50.00	14.7±0.4	57.3±3.5	18.5±0.2	Intermediate size pellets
100.00	19.2±0.3	72.5±3.0	27.9±0.4	Gelatinous
150.00	22.5±0.2	89.7±3.5	52.3±0.6	Small rounded pellets
200.00	25.3±0.4	83.5±4.0	69.3±0.8	Mixed Mycelial
250.00	25.7±0.4	85.4±0.2	65.7±0.4	Small rounded pellets
300.00	28.4±0.2	92.7±0.2	62.8±0.2	Dumpy mass

Sugar concentration, 150 g l^{-1}; Fermentation period, 168 h; Temperature, 30°C; Initial pH, 6.0.

Table 3: Effect of different alcohols and their concentrations on citric acid fermentation by mutant strain of *Aspergillus niger* NG-110, using molasses based medium in shake flask

Alcohols	Concentrations (%)	Sugar consumption (g l^{-1})	Dry cell mass (g l^{-1})	Anhydrous citric acid (g l^{-1})	Mycelial Morphology
Control	0	97.0±2.0	16.5±0.2	70.9±0.2	Mixed pellets
Methanol	0.50	105.2±3.5	14.0±0.2	69.3±0.2	Small pellets
	1.00	88.0±3.0	14.0±0.2	73.2±0.2	Small pellets
	1.50	112.7±2.5	16.3±0.2	70.5±0.3	Small pellets
Ethanol	0.50	108.3±3.2	19.4±0.1	49.6±0.2	Large pellets
	1.00	95.0±2.5	14.9±0.2	53.4±0.2	Large pellets
	1.50	102.0±2.5	15.7±0.2	52.0±0.2	Round pellets
Butanol	0.50	112.0±2.5	19.9±0.5	36.4±0.2	Intermediate pellets pellets and fusy mass
	1.00	97.6±2.5	15.3±0.2	41.4±0.2	Fusy mass
	1.50	109.2±3.8	16.2±0.2	40.1±0.4	Dumy mass

Initial sugar concentration, 150 g l^{-1}; Incubation period, 144 h; Temperature 30°C; Initial pH, 6. Potassium ferrocyanide concentration, 200 ppm

Table 4: Effect of different concentration of $CaCl_2$ on citric acid fermentation by a mutant strain of *Aspergillus niger* NG-110 in shake flask

$CaCl_2$ conc. (% w v^{-1})	Mycelial dry wt. (g l^{-1})	Sugar consumption (g l^{-1})	Citric acid (g l^{-1})	Mycelial Morphology
0.50	2.0±0.2	90.1±2.5	62.5±0.2	Small pellets
1.00	4.0±0.3	93.7±2.0	69.7±0.1	Rounded
1.50	3.3±0.2	95.4±2.4	74.1±0.2	Mixed pellets
2.00	2.9±0.2	98.0±2.0	80.7±0.2	Elongated Pellets
2.50	3.4±0.2	105.7±2.0	72.8±0.2	Elongated Pellets

Initial sugar concentration, 150.0 g l^{-1}; Fermentation period, 168 h; Initial pH, 6. Potassium ferrocyanide concentration, 200 ppm; Methanol, 1%; NH_4NO_3, 0.20%

methanol. The addition of other alcohols such as ethanol and butanol was, however, not significant. Hence 1% methanol was optimized for further studies. The comparison of product and growth yield coefficient for citric acid fermentation using different alcohols (Fig. 5). The more significant values for $Y_{p/s}$ and $Y_{p/x}$ (g g^{-1}) were recorded with methanol.

Effect of different concentrations of calcium chloride

The concentration of calcium chloride varied from 0.5-2.5% (Table 4). The production of citric acid was increased with the increase in the concentration of $CaCl_2$ and found to be optimum

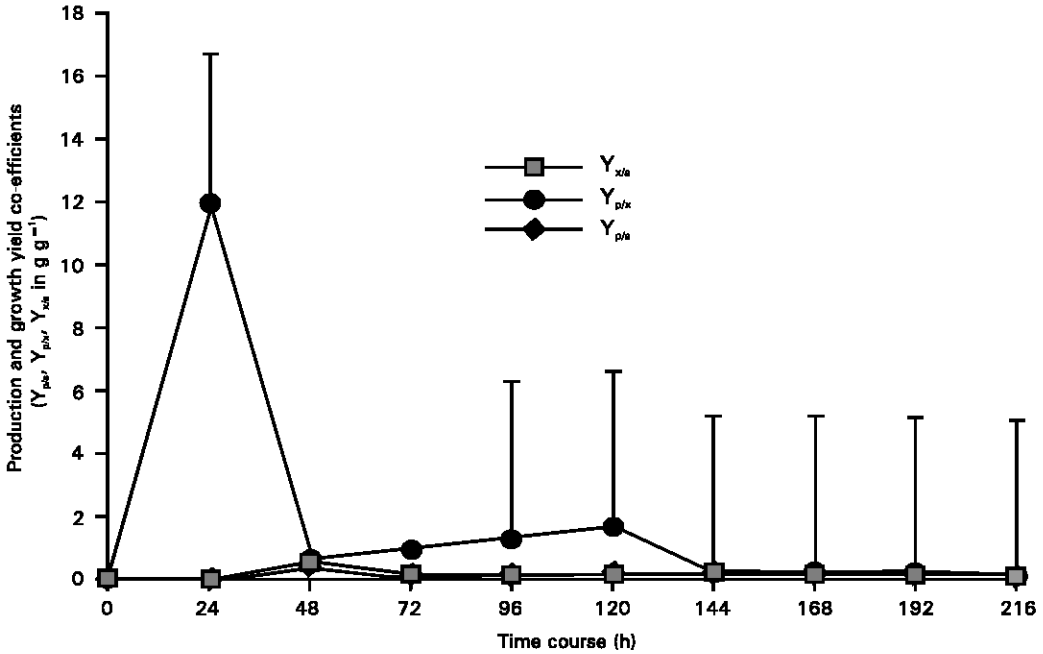

Fig. 1: Comparison of product and growth yield coefficients for citric acid fermentation Kinetic parameters, $Y_{p/s}$ = g citric acid produced/g substrate consumed, $Y_{p/x}$ = g citric acid produced/g cell formed, $Y_{x/s}$ = g cell formed/ g substrate consumed, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $P< 0.05$

when 2.0% $CaCl_2$ was added to the fermentation medium (80.76±0.2 g l^{-1}). The % yield of citric acid was 82.40% with sugar consumption 98±2 g l^{-1} while the dry weight of mycelia was 2.92±0.2 g l^{-1}. Further increase in the concentration of $CaCl_2$ reduced the secretion of citric acid from mycelia. Product and growth yield coefficients as kinetic parameters were also studied for citric acid using different sources of phosphate and their concentrations (Fig. 6). The values for $Y_{p/s}$ and $Y_{p/x}$ (g g^{-1}) at 1.0% KH_2PO_4 were found to be significant.

Discussion

This study describes the rate of citric acid biosynthesis, effect of potassium ferrocyanide and their different concentration, effect of alcohols and their concentration, effect of $CaCl_2$ on citric acid production by a mutant strains of ***Aspergillus niger*** NG-110 obtained from Government College University Biotechnology Lab. A number of reports have been published on the production of citric acid by submerged mould culture technique (Obaidi and Berry, 1979; Fiedurek ***et al.***, 1996; Pazouki ***et al.***, 2000).

The optimum time of incubation for maximal citric acid production varies both with the organism and fermentation conditions. The maximum yield of citric acid (57±0.4 g l^{-1}) was achieved, 168 h after incubation. Further increase in incubation period did not enhance citric

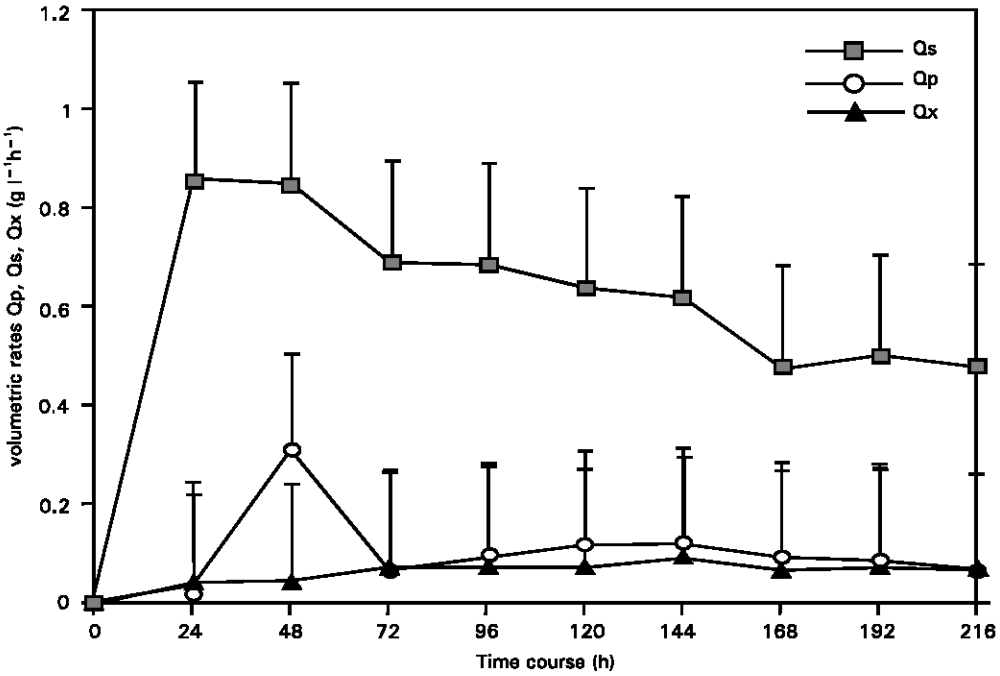

Fig. 2: Comparison of volumetric rates for citric acid fermentation, Kinetic parameters, Qp = g citric, acid produced l^{-1} h^{-1}, Qs = g substrate consumed l^{-1} h^{-1}, Qx=g cell formed l^{-1} h^{-1}, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at P = 0.05

acid 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 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. These findings are 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 h after incubation. Hence, the present findings are more encouraging as compare to Clark (1962) due to short incubation period.

Successful citric acid fermentation depends to a great extent on the control of concentration of trace elements (Fe^{++}, Cu^{++}, Zn^{++}, Mn^{++}, Mg^{++}). The effect of different concentrations of $K_4Fe(CN)_6$ on citric acid fermentation by a mutant strain of ***Aspergillus niger*** NG-110 was carried out in shake flask. The addition of $K_4Fe(CN)_6$ (200 ppm) at the time of inoculation when the medium was hot increased the citric acid production. The insoluble complexes of ferrocyanide with heavy metals acted as metal buffers in the fermentation medium, which made the metal ions available at concentration suitable for citric acid production. It was also due to the fact that it checked the mycelial growth and also inhibited the activity of enzyme

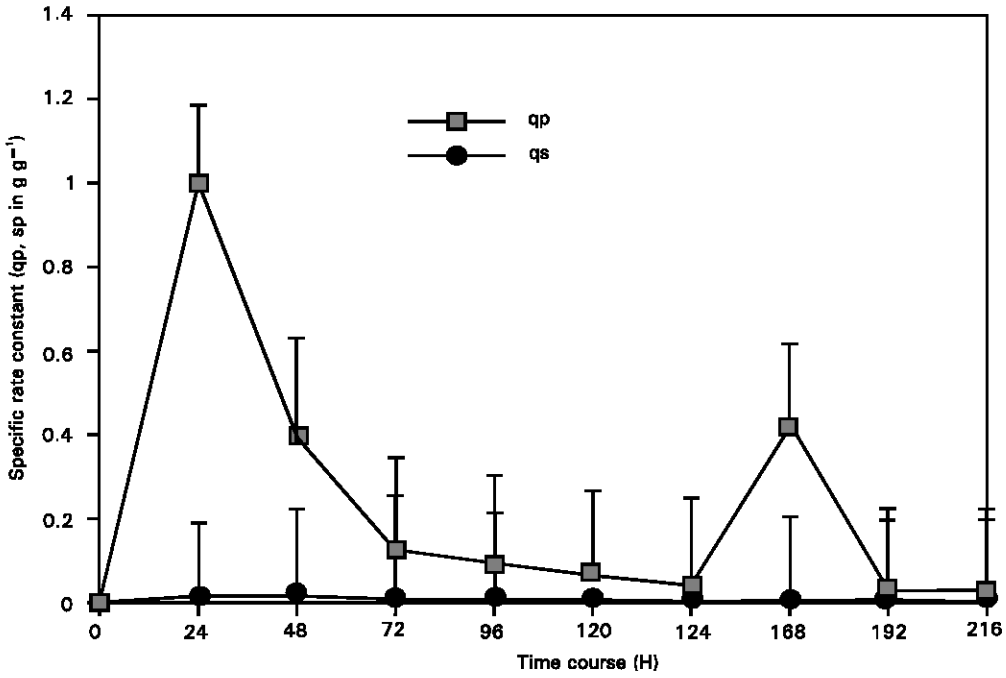

Fig. 3: Comparison of specific rate constants for citric acid fermentation, Kinetic parameters, qp = g citric acid produced g^{-1} cells h^{-1}, qs = g substrate consumed g^{-1} cells h^{-1}, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at P = 0.05

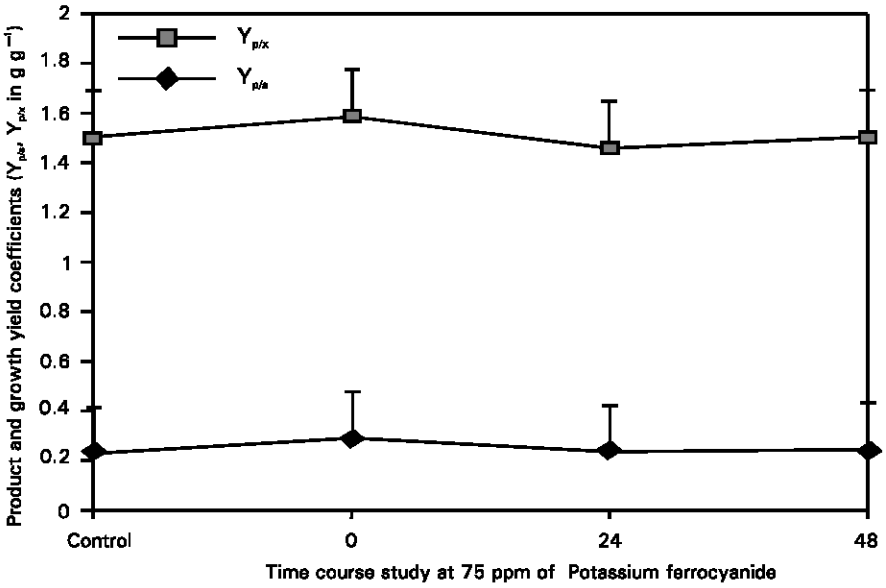

Fig. 4: Comparison of product and growth yield coefficients for citric acid fermentation

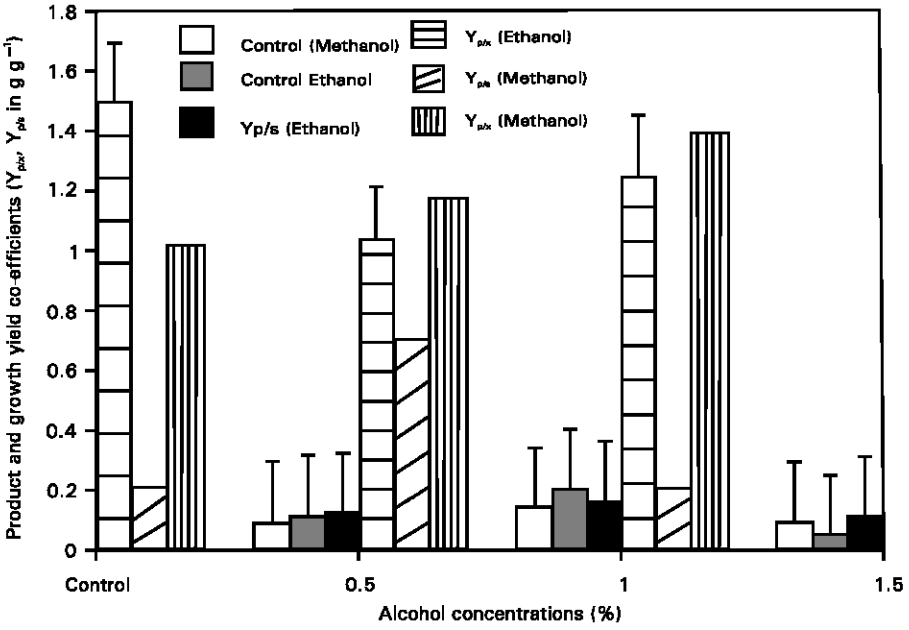

Fig. 5: Comparison of product and growth yield coefficients for citric acid fermentation Kinetic parameters:, $Y_{p/s}$ = g citric acid produced g^{-1} substrate consumed, $Y_{p/x}$= g citric acid produced g^{-1} cells formed, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $P < 0.05$

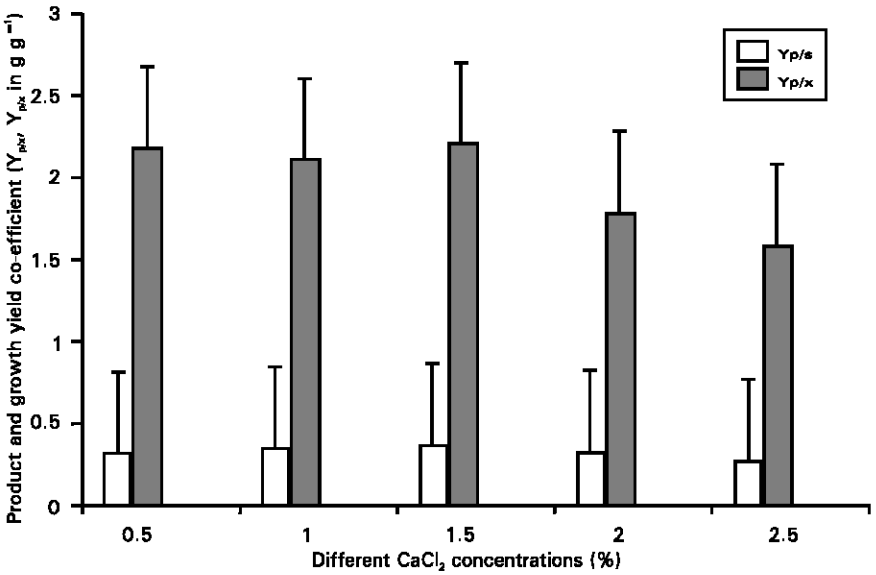

Fig. 6: Comparison of product and growth yield coefficients for citric acid fermentation, Kinetic parameters, $Y_{p/s}$= g citric acid produced g^{-1} substrate consumed, $Y_{p/x}$= g citric acid produced g^{-1} cells formed, Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $P < 0.05$

aconitase. Shankaranand and Lonsane (1993). Further increase in concentration of $K_4Fe(CN)_6$ resulted in the decreased citric acid accumulation. It might be due to toxic inhibitory effect of $K_4Fe(CN)_6$ on fungal growth and decreased sugar consumption. The optimum level of ferrocyanide in this study was 200 ppm.

Different alcohols have stimulatory effect on the biosynthesis of the citric acid in the fermented broth. The effect of methanol on citric acid production by ***Aspergillus niger*** NG-110 was more efficient. It may be due to that methanol increases the permeability of cell membrane, resulting in better excretion of citric acid from mycelial cells. Similar kind of work has also been reported by Pazouki ***et al.*** (2000). Methanol at 1% level was found to be the most suitable for citric acid production. Methanol markedly depressed the cell protein in the early stages of cultivation and also increased the metabolic activity of the enzyme. From these facts, it was concluded that methanol induced enzyme activities to become suitable for citric acid fermentation in the early stages of the cultivation. Further increase in the level of methanol concentration results in the decreased production of citric acid because higher methanol concentration in the medium disturbs the fungal metabolism. Similar type of work has also been carried out by Haq ***et al.*** (2001). Addition of methanol after 24 h or later has not been found to be beneficial. Earlier it was suggested that methanol increases the tolerance of fungi to trace elements such as Fe^{+2}, Mn^{+2}, Zn^{+2} etc.

Calcium also plays an important role in citric acid fermentation with its effect on fungal growth, hyphal morphology and citric acid production. The addition of $CaCl_2$ to the fermentation

medium lowers the final biomass and increases the uptake of phosphate, sucrose and citric acid production (Pera and Callieri, 1997). Calcium chloride also reduces the biomass concentration and increases the volumetric productivity as well as specific production rate. The production rate, however, depends on the concentration and the type of organism. In this study the 1.5% level of $CaCl_2$ was found to be the best for citric acid production by ***Aspergillus niger***.

The kinetic parameters such as growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ in g g^{-1}), volumetric rates (Q_p, Q_s, Q_x in g l^{-1} h^{-1}) and specific substrate rates (q_p, q_s in g g^{-1} cells h^{-1}) of the research work were also undertaken. The mutant strain of ***Aspergillus niger*** NG-110 showed improved values for $Y_{p/s}$, $Y_{p/x}$, and $Y_{x/s}$. Similar kind of work has also been reported by Pirt (1975). Maximum growth in terms of specific growth rate (μ h^{-1}) was only marginally different during growth of mutant ***A. niger*** GCB-47 on 150 g l^{-1} carbohydrates in molasses at 30°C (than 32°C or 165 g l^{-1} sugar). However, when the culture was monitored for $Y_{x/s}$, Q_s and q_s, there was a significant enhancement in these variables at optimal nutritional conditions, i.e. incubation temperature 30°C, initial sugar concentration 150 g l^{-1}, methanol 1%, NH_4NO_3 0.15%, $CaCl_2$ 2.0% and K_2HPO_4 0.20% at an incubation period of 168 h (7 days). This indicated that the mutant strain used in the current studies is a faster growing organism and have 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 $Y_{p/s}$, Q_p and q_p were several folds improved over the previous workers Pirt (1975), Roehr (1998) and Kamal ***et al.*** (1999).

Citric acid production is mainly dependent on the type of strain and process used. The optimization of cultural conditions is necessary for higher yields of metabolites like citric acid. In this study, maximum amount of citric acid (81.24 g l^{-1}) was obtained, 168 h after inoculation. Calcium source (2.0% $CaCl_2$), potassium ferrocyanide (200 ppm K_4FeCN_6) and alcohol (1% methanol) were also optimized. From the studies it is concluded that a successful fermentation process depends on the medium composition, technology and nutritional parameters used. The value of specific rate constant (q_p = 0.426 g citric acid produced g^{-1} cells h^{-1}) is highly significant from industrial point of view.

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