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CITRIC ACID FERMENTATION OF HYDROLYSED RAW STARCH BY *ASPERGILLUS NIGER* IIB-A6 IN STATIONARY CULTURE

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Abstract

In the present investigation, better citric acid production (23.87±2.75g/l) was obtained with sweet potato starch hydrolysate at an initial sugar concentration of 200 g/l by *Aspergillus niger* IIB-A6. The product formation kinetic parameters viz., Q_p (0.1 g/l/h) and q_p (0.02 g/g/h) were higher for sweet potato when compared to maize starch hydrolysate. Incubation period (264h), initial pH (3.0), volume of fermentation medium (50 ml/250 ml Erlenmeyer flask) and inoculum size (2.0 %) were also optimized. The optimal citric acid production was obtained when methanol (1.5 %, v/v) as a stimulant was added into the medium 24 h after the inoculation. Different metal complexing agents such as ethylenediamine tetra aceticacid (EDTA) and potassium ferrocyanide were used to reduce heavy metal ions during the fermentation process. The maximum amount of citric acid (45.90±4.20g/l) was achieved when 200 ppm of $K_4Fe(CN)_6$ was added into the medium just before inoculation under hot conditions. EDTA however, showed insignificant results.

Keywords: Citric acid, *Aspergillus niger*, IIB-A6 stationary culture

1. Introduction

Citric acid is used as acidulate, flavour enhancer, preservative, antioxidant and stabilizer. There are three principle methods which are available for microbial production of citric acid i.e., surface culture, submerged and solid-state fermentation (Kristiansen *et al.*, 1999). The surface culture technique is a conventional method but it is still being extensively employed. Although it is labour intensive but the energy requirements are less compared to the submerged or solid-state fermentation. One of the prerequisites for abundant citric acid production is *Aspergillus niger* (Maddox and Brooks, 1998) and has dominated others both in laboratory and industrial scale. Citric acid fermentation by *A. niger* was successfully carried out with crude carbon sources such as starch hydrolysates or incompletely refined sucrose. It has been observed that starch hydrolysate gives better yield of citric acid (Wayman and Mattey, 2000). The initial sugar concentration has been found important to determine the amount of citric acid and other organic acids produced in the culture broth. Normally strains of *A. niger* need a fairly higher initial sugar concentration in the medium (Grewal and Kalra, 1995).

The optimal incubation period for the maximum citric acid production varies both with the organism and fermentation conditions. Attempts have been made to decrease fermentation time period by altering the cultural conditions (Rojas *et al.*, 1995). An appropriate initial pH is critical for successful fermentation process and it varies from strain to strain (Hess *et al*., 2000). The stimulatory effect of methanol permits its application in the commercial production of citric acid. The alcohol addition reduces mycelial growth, inhibits sporulation and increases the efficiency of the fermentation. *A. niger* needs a variety of divalent trace elements such as Fe^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2} and Mg^{+2}, etc. for growth and citric acid production (Majolli and Aguirre, 1999). Potassium ferrocyanide reacts with the heavy metals causing their precipitation. It removes not only metals of vegetative influence but also some of

the microelements. Therefore, the concentration of these heavy metals should be regulated for the optimal fungal growth (Walish *et al*., 1983). The present study deals with the citric acid fermentation of hydrolysed raw starch by *Aspergillus niger* IIB-A6 in stationary culture.

2. Materials and methods

Organism and culture maintenance

Aspergillus niger strain IIB-A6 was obtained from the available stock culture of Biotechnology Research Centre, GCU Lahore. The culture was maintained on potato dextrose agar (PDA) slants, pH 5.6. The slants were inoculated by transferring small amount of conidia by an inoculum needle and incubated at 30°C in an incubator (3-5 days) for maximum sporulation. The slant cultures were stored at 4°C in a cold-cabinet.

Preparation of conidial inoculum

The conidial suspension was prepared by adding 10 ml of sterilized 0.005 % (w/v) sodium salt of bis-2-ethylhexyl sulfosuccinate to a 4-6 day old slant culture of *A. niger* having profuse conidial growth on its surface. A sterile wire-loop was gently used to break the conidial clumps and shaken vigorously to make a homogeneous suspension.

Preparation of starch hydrolysate

Sweet potatoes obtained from local market were cut into small pieces after washing and peeling. The pieces were blended in distilled water to form a homogenous mixture and placed at 4^{o}C. The starch settled down at the bottom was separated from liquid and oven dried at 60^{o}C, overnight. Maize starch was obtained from the local market. A starch solution of 250g/l was prepared and autoclaved. To liquefy starch, alpha amylase (2.0 U/ml) was added and heated at 95^{o}C in a water bath for 15 min. For saccharification, amyloglucosidase (2.0 U/ml) was added and heated at 55^{o}C while constant stirring for 4h. The reducing sugars were determined by dinitrosalicylic acid (DNS) method (Miller 1959).

Fermentation technique

Surface culture technique was employed for the production of citric acid by *A. niger*. Fifty millilitre of the fermentation medium containing starch hydrolysate 150, NH_4NO_3 2.5, KH_2PO_4 1.0, $MgSO_4$. $7H_2O$ 0.25 and $CaCl_2$ 0.5 was transferred to 250 ml conical flasks and cotton plugged. The flasks were autoclaved at 15 psi (121°C) for 15min. Sterilized ferrocyanide (free conc. 200 ppm) was added to each flask aseptically while the medium was hot. After cooling at room temperature, the flasks were inoculated with 1.0 ml of the conidial suspension (1.35×10^6 conidia). The addition of methanol (1.0 ml of 1.5 %) was made 24h after the inoculation and incubated at 30°C for 264h. All the experiments were run parallel in triplicates.

Assay methods

The mycelial morphology was determined on an aliquot extended on the petriplates and dry cell mass was determined according to Haq and Daud (1995). The sugar contents were estimated after Miller (1959). Citric acid was estimated following pyridine-acetic anhydride method (Marrier and Boulet, 1958). The ferrocyanide concentration was estimated by colorimetric method (Marrier and Clark, 1962). The kinetic parameters for citric acid production were studied according to the procedures of Pirt (1975).

Statistical analysis

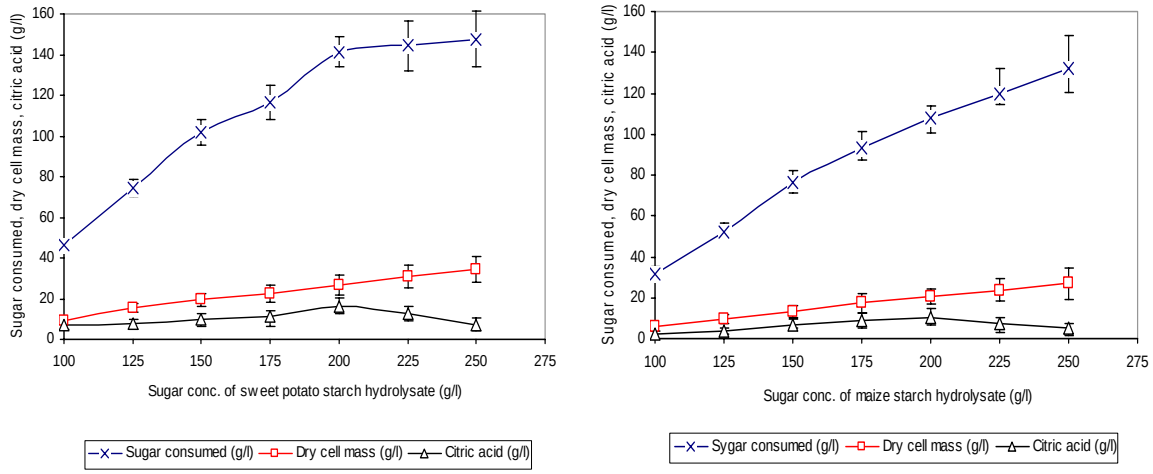
Treatment effects were compared by the protected least significant difference method (Spss-10, Version-4.0, USA) after Snedecor and Cochran (1980). Significant difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (p) value.

3. Results

The maximum citric acid production (15.81±2.60 g/l) was obtained with sweet potato starch hydrolysate containing initial sugar concentration of 200 g/l which is 1.5 fold higher than maize starch hydrolysate **(Fig.1).** In (**Fig.2)** is depicted the comparison of sweet potato and maize starch hydrolysates during time course study (24-360 h) on citric acid production by *A. niger* IIB-A6. The maximum citric acid production (23.87±2.45g/l) from sweet

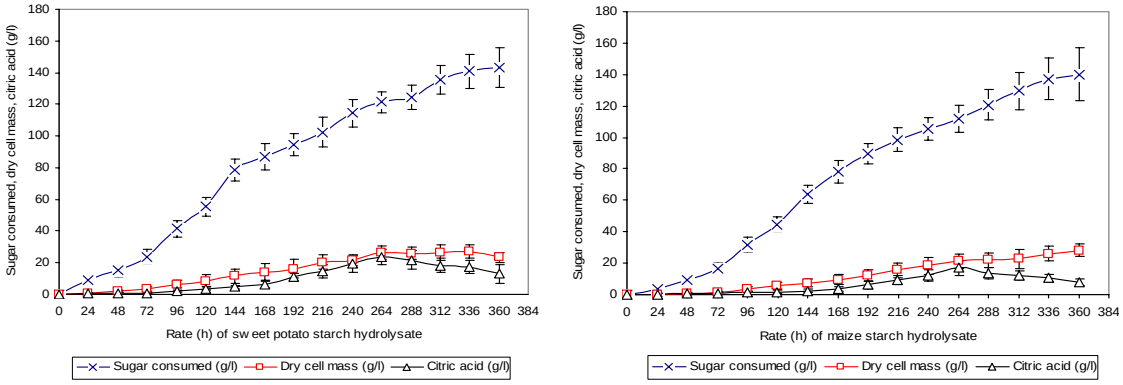
potato starch hydrolysate was achieved 264h after incubation which is 1.41 fold higher than maize starch hydrolysate. The sugar consumption and dry cell mass were 121.3±3.10 and 26.02±1.63g/l, respectively. **(Table-1)** shows the kinetic parameters during time course study by using different sugar concentrations of sweet potato and maize starch hydrolysates. The maximum growth in terms of specific growth rate (μ) was only marginally different in both hydrolystes containing different sugar concentrations. When substrate consumption parameters were monitered, the volumetric substrate uptake rate Q_s (0.46g/l/h), volumetric cell formation rate Q_x(0.1g cells/l/h) and specific substrate uptake rate q_s(0.46g/g cells/l/h) showed enhancement in sweet potato starch hydrolysate containing 200g/l sugar concentration over to other levels.

Fig. 1. Comparison of sweet potato and maize starch hydrolysates on citric acid production containing different sugar concentrations by *A. niger* IIB-A6*



*Fermentation period 240 h, incubation temperature 30°C, initial pH 3.5.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤0.05.

Fig. 2. Comparison of sweet potato and maize starch hydrolysates during time course study on citric acid production by *A. niger* IIB-A6 *



*Sugar concentration 200 g/l, initial pH 3.5, incubation temperature 30°C.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤0.05.

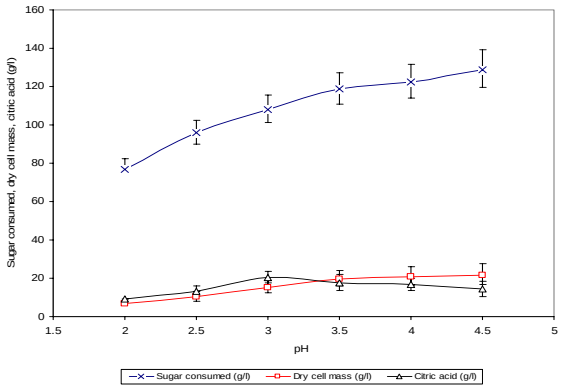
Table-1. Kinetic parameters of citric acid production by *A. niger* IIB-A6 at different sugar concentrations of sweet potato and maize starch hydrolysates*

Kinetic parameters	Sugar concentration (g/l)					
	Sweet potato starch			Maize starch		
	175	200	225	175	200	225
Specific growth rate μ (h^{-1})	0.08	0.10	0.11	0.06	0.08	0.1
Substrate consumption parameters						
Q_s (g/l/h)	0.44	0.46	0.54	0.35	0.42	0.45
q_s (g/g cells/l/h)	0.41	0.46	0.48	0.32	0.42	0.50
Q_x (g cells/l/h)	0.08	0.1	0.11	0.06	0.08	0.1
Citric acid formation parameters						
Q_p (g/l/h)	0.045	0.1	0.046	0.032	0.064	0.026
q_p (g/g cells/h)	0.008	0.02	0.008	0.006	0.012	0.005

*Q_s=g substrate consumed/l/h, q_s =g substrate consumed/g cells/h, Q_x=g cells formed/l/h, Q_p=g citric acid produced/l/h, q_p=g citric acid produced/g cells/h. The value differs significantly at $p \leq 0.05$.

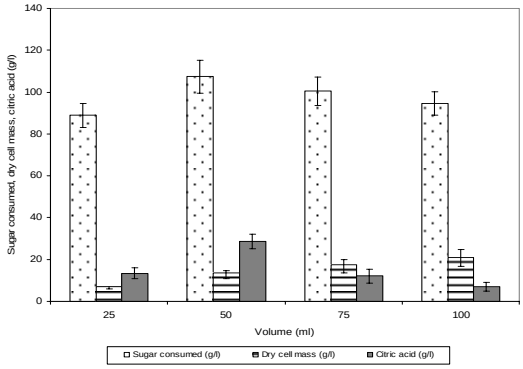
The maximum citric acid production (20.60±1.70 g/l) was obtained at pH 3.0. The sugar consumption and dry cell mass were 107.96±1.56 and 15.27±0.89 g/l, respectively **(Fig. 3 and 4)** exhibits the effect of different volumes (25-100 ml/250 ml flasks) of fermentation medium on citric acid production by *A. niger* IIB-A6 in stationary culture. The citric acid production (13.43±2.53g/l) was lower at 25ml volume. The maximum citric acid production (28.66±1.76g/l) was obtained when 50 ml of the medium was used. The effect of different alcohols (methanol, ethanol and butanol; 1.0 %, v/v) on citric acid production by *A. niger* IIB-A6 was studied **(Fig.5a).** The maximum citric acid production (36.80±2.54 g/l) was obtained with methanol. The effect of different concentrations of methanol (0-2.0 %) and its addition at different time intervals was also studied **(Fig. 5b c).** The maximum amount of citric acid (40.23±2.05 g/l) was produced when 1.5% methanol was added into the medium 24h after inoculation.

Fig. 3. Effect of different initial pH on citric acid production by *A. niger* IIB-A6*



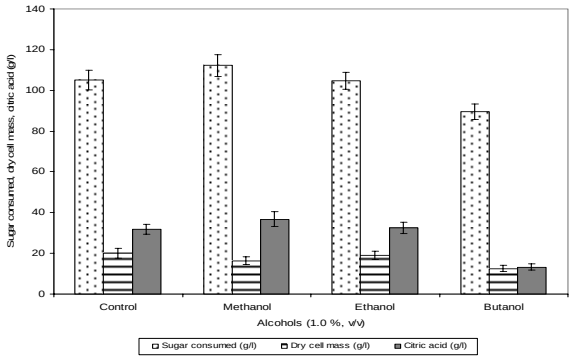
*Sugar concentration 200 g/l, fermentation period 264 h, incubation temperature 30°C.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Fig. 4. Effect of different initial volume of fermentation medium on citric acid productionby *A. niger* IIB-A6*



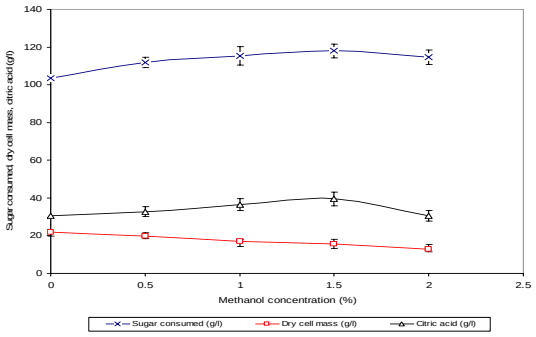
*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0,incubation temperature 30°C.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Fig. 5a. Effect of different alcohols on citric acid Production by *A. niger* IIB-A6*



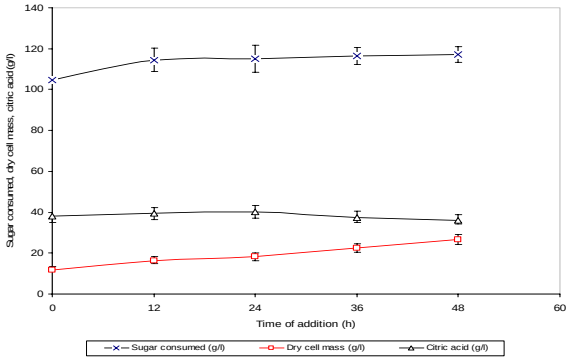
*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤ 0.05.

Fig. 5b. Effect of different methanol concentrations on citric acid production by *A. niger* IIB-A6*



*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤0.05.

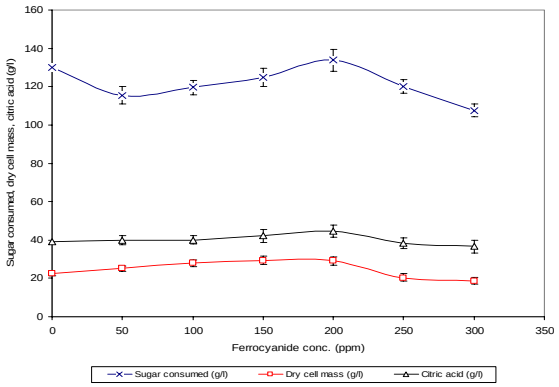
Fig. 5c. Effect of varying the time of methanol addition on citric acid production by *A. nige* IIB-A6*



*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C, methanol concentration 1.5 %.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤ 0.05.

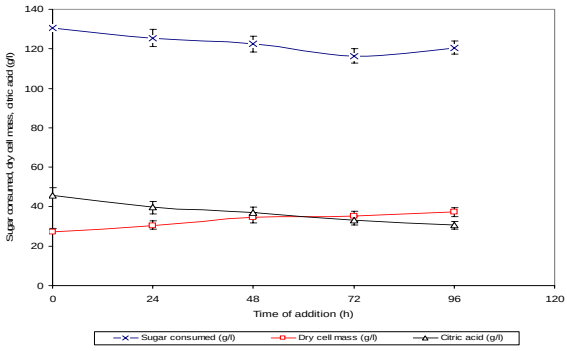
The effect of different concentrations of potassium ferrocyanide (50-300 ppm) and its addition at different time intervals (0-96 h) was studied by *A. niger* IIB-A6. A slight difference was observed in the production of citric acid by increasing the ferrocyanide concentration **(Fig. 6a).** The maximum citric acid (45.90±1.272 g/l) was produced when 200 ppm K_4 Fe $(CN)_6$ was added into the medium before inoculation, under hot conditions. The sugar consumption and dry cell mass were 130.7±2.44 and 27.22±2.17g/l, respectively **(Fig. 6b).** The effect of addition of different concentrations of EDTA (0-200 ppm) and its addition at different time intervals (0-96 h) was also examined **(Fig. 7).** The maximum citric acid (41.50±1.10 g/l) was produced when 50 ppm of EDTA was added into the medium prior to the time of inoculation, under hot conditions.

Fig. 6a. Effect of different concentrations of ferrocyanide on citric acid production by *A. niger* IIB-A6*



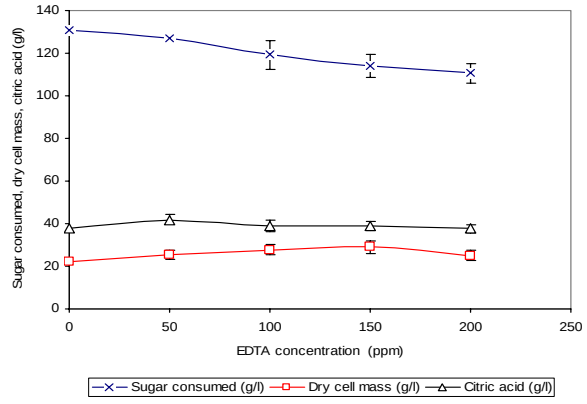
***Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C, methanol concentration 1.5 %.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at p≤0.05.**

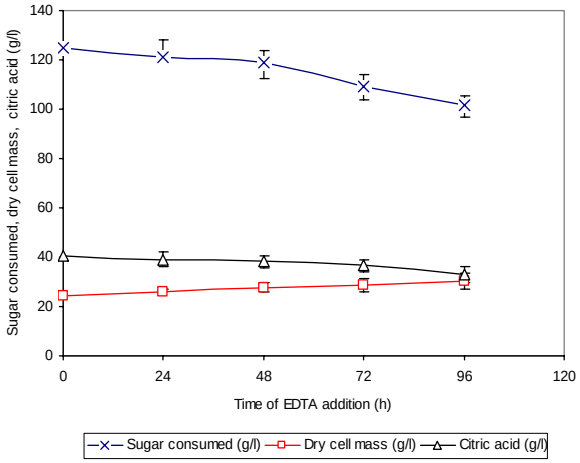
Fig 6b. Effect of varying the time of ferrocyanide Addition on citric acid production by *A. niger* IIB-A6*



*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C, methanol concentration 1.5 %, ferrocyanide conc. 200 ppm.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Fig. 7. Effect of different concentrations and arying the time of addition of EDTA on the production of citric acid by *A. niger* IIB





*Sugar concentration 200 g/l, fermentation period 264 h, initial pH 3.0, incubation temperature 30°C, methanol concentration 1.5 %.
Y-error bars indicate standard deviation (±SD) of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

4. Discussion

Citric acid is an important metabolite produced by fermentation with specific moulds, especially with *Aspergillus niger* (Hang and Woodams, 1998). Various carbohydrate materials may be used in citric acid production but only a few workers have used starch hydrolysate (Mourya and Jauhri, 2000) and got comparatively better results. The effects of different cultural conditions and nutritional requirements such as sugar concentration, rate synthesis, initial pH, volume of fermentation medium, type and size of alcohols and ferrocyanide concentration on citric acid production were studied. Sweet potato and maize starch containing different sugar concentrations (100-250g/l) were examined for citric acid production. A lower concentration of sugar leads to lower yield of citric acid as well as accumulation of oxalic acid (Kovats, 1960). In the present study, the maximum amount of citric acid (15.81± 1.60 g/l) was produced with sweet potato starch hydrolysate at a concentration of 200g/l which was 1.52 fold higher than maize starch hydrolysate. Gradual reduction in citric acid formation was observed when the sugar concentration of medium was further increased. It might be due to the over growth of mycelial cells which resulted in the increased viscosity of medium and hence, mass transfer limitations (Benuzzi and Segovia, 1996). An increase in dry weight of mycelia with the increase in the sugar concentration is in agreement with the work reported earlier by (Haq *et al.*, 2003).

The rate of citric acid fermentation (24-360 h) was carried out using sweet potato and maize starch hydrolysates containing 200g/l sugar concentration. Increase in incubation period resulted in the increased citric acid production. The maximum amount of citric acid (23.87 ± 2.45g/l) was obtained 264h after inoculation by using sweet potato starch hydrolysate. In stationary culture, the production starts after a lag phase of approximately 2-3 days and reaches maximum at the onset of stationery phase (Vergano *et al.*, 1996). The incubation period beyond 264 h did not show any enhancement in citric acid production. It might be due to the decreased available nitrogen in fermentation medium, age of fungi; inhibitors produced by fungi itself and the depletion of sugar contents. This finding is in agreement with the observations of Rajoka *et al.*, (1998).

On the basis of a comparison of kinetic parameters namely the volumetric product formation rate (Q_p) and specific product

formation rate (q_p), sweet potato starch hydrolyste was found to be the best substrate for citric acid production. The findings suggested that sweet potato starch hydrolysate possesses enhanced ability for citric acid production compared to maize starch hydrolysate. The pH of the basal medium has a direct influence on mould metabolism (Roskosu and Anenil, 1980). The maximum citric acid production (20.60±1.70g/l) was obtained in the fermentation medium when pH was adjusted at 3.0. It might be due to proper cell division, appropriate nutrients supply and adjustment of fungus according to physiological conditions. Similar kind of work has also been reported by Pirt (1975). The study is directly substantiated by the findings of Rajoka *et al*., (1998). Maximum values for $Y_{p/s}$, were several folds improved over those of the previous workers (Pirt 1975; Benuzzi and Segovia, 1996; Kristiansen *et al.*, 1999).

The effect of different volumes (25-250 ml/250 ml Erlenmeyer flasks) of fermentation medium on citric acid production was also studied. The maximum citric acid production (28.66±2.20 g/l) was obtained when 50 ml of the medium was used (depth 1.5cm). At this volume of the medium, growth of cell mass was optimal due to a better oxygen supply, resulting in the increased citric acid production. In the present study, the maximum amount of citric acid (40.23±2.05g/l) was produced when methanol at 1.5 % (v/v) level was added into the medium 24 h after inoculation. It might be due to that methanol increased the permeability of cell membrane which resulted in a better citric acid excretion from mycelial cells (Ali *et al*., 2002). In addition, methanol markedly depressed cell proteins in the early stages of cultivation and also increased the enzyme metabolic activity (Pazouki *et al*., 2000). Addition of ethanol or butanol, however, did not enhance citric acid production.

A little enhancement in citric acid production over control was observed with EDTA. The maximum citric acid production (45.920±1.27 g/l) was achieved when 200 ppm (w/v) potassium ferrocyanide was added before inoculation, under hot conditions. It might be due to that the insoluble complexes of ferrocyanide with heavy metals acted as metal buffers in the medium, which made metal ions available at a concentration suitable for the optimal citric acid yield. In addition, the ferrocyanide ions check mycelial growth and inhibit aconitase activity (Shankaranand and Lonsane, 1993).

5. Conclusion

In a comparison between sweet potato and maize starch hydrolysates by *A. niger* IIB-A6 using stationary culture, the maximum amount of citric acid (23.87±2.27g/l) was obtained with sweet potato starch hydrolysate 264 after inoculation. The initial sugar concentration (200 g/l), pH (3.0), effect of alcohols (1.5 % methanol) and the effect of ferrocyanide (200 ppm $K_4Fe(CN)_6$) were also optimized. After the optimization of cultural conditions and nutritional requirements, an overall improvement of 3.45 fold in citric acid production was achieved, which is highly significant ($p \leq 0.05$). However, further studies on the effect of various levels of bis-2-ethylhexyl sulfosuccinate on citric acid production is prerequisite before scale up in a lab scale stationary shelf-bioreactor.

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