African Journal of Biotechnology Vol. 10(55), pp. 11784-11791, 21 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2654
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Enhanced citric acid production by *Aspergillus niger* EB-3 mutant using an inert solid support in molasses medium

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Accepted 20 May, 2011

***Aspergillus niger* EB-3 was used to enhance citric acid production through optimization of nutritional parameters using banana stalk as carrier/inert support in solid state fermentation (SSF) of sugarcane molasses. The effects of moisture level, nitrogen sources, incubation time, metabolic inhibitors and metal complexing chemicals were studied on citric acid production in SSF. *A. niger* EB-3 mutant could produce 97.52 mg/ml citric acid with sugar utilization of 104.07 ± 1.98 mg/ml in 96 h by optimizing moisture level (60%) and nitrogen source (0.2% w/w ammonium nitrate). Addition of different surfactants to the medium increased fungal biomass, sugar utilization and citric acid production and Tween-80 was the best surfactant that caused maximum enhancement in citric acid yield at 0.2% concentration. The metabolic inhibitors like naphthol, resorcinol and cresol also enhanced citric acid production when used in lower concentrations, and cresol was the most effective inhibitor at 0.4% (w/w) level. Addition of metal complexing compounds also stimulated citric acid production by *A. niger* EB-3 and EDTA was the most effective metal chelator at 0.4% (w/w) level. Under optimum conditions, 112.42 mg/ml citric acid was produced with sugar utilization of 121.84 mg/ml.**

Key words: Citric acid, hyperproducting mutant, *Aspergillus niger* EB-3, optimization, metabolic inhibitors, metal complexing compounds.

INTRODUCTION

Citric acid is a necessary constituent of various food preparations, pharmaceuticals, synthetic biodegradable detergents, cosmetics, alkyd resins and many other products. Currently, it is commercially produced by submerged (liquid state) fermentation (SmF) of starch or sucrose based media (sucrose or glucose syrups) using *Aspergillus niger* (Lofty et al., 2007; Barrington and Kim, 2008). *A. niger* is being used commercially for citric acid production and it remains the organism of choice for commercial production because it produces more citric acid per time unit. Recently, a wide range of citric acid production has been reported in response to different levels of nutrient supplementation (Bari et al., 2009; Immandi et al., 2008). The main advantages of using *A. niger* are its ease of handling, its ability to ferment a variety of cheap raw materials and high yields (Soccol et al., 2006; Immandi et al., 2007). The yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source (Papagianni, 2007). Organic wastes like apple peels and pomace, grape pomace, banana stalks, sugar cane bagasse, and sugar cane and beet molasses have been used to produce citric acid (Wang, 1998; Ngadi and Correia 1992; Gutierrez et al., 1999; Dhandayuthapani et al., 2008). The production of citric acid using cheap carbon source from agro-industrial byproducts provides considerable combined benefit of waste material management as well as reduction of citric acid production cost (Kumar et al., 2003; John et al., 2006). The major physico-chemical parameters which influence the growth of *A. niger* and its production of citric acid on a solid substrate are moisture content, particle sizes, nutrient sources, incubation temperature, pH and

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Abbreviation: SSF, Solid state fermentation.

inoculum size (Lee and Yun, 1999; Ellaiah et al.,2004; Lofty et al., 2007; Bari et al., 2010). The accumulation of citric acid is strongly affected by fermentation conditions and higher production of citric acid was recorded by the optimization of fermentation process conditions besides optimization of media, (Lofty et al., 2007).

There is an immense need to investigate the factors responsible for low citric acid yields and to develop strategies to increase yield and reduce production cost (Milson and Meers, 1985). Such process developments may be approached either by improving product formation properties (example yields, titers) of naturally occurring microbes through metabolic engineering, or by developing hyper-producing mutants and standardization of media and fermentation conditions. Sugar cane molasses has high sugar and metal ions content that inhibit the growth of *A. niger*. *A. niger,* however, 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). In liquid state fermentation of molasses medium, the biosynthesis of citric acid by *A. niger* is inhibited by higher concentrations of metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+}, etc.) in LSF. Whereas, in solid state fermentation (SSF), the inhibition caused by high sugar and metal ions concentrations is negligible (Pintado et al., 1998; Gutierrez-Rozas et al., 1995). In SSF using a lignocellulosic inert solid support material, most of the metals ions are adsorbed by the solid matrix. The metal complexing reagents like potassium ferro cyanide and EDTA can bind metal ions and metabolic inhibitors like naphthol, and resorcinol decrease the formation of side products including acetic acid, ethanol, methanol, etc. and hence increased citric acid synthesis during fermentation.

In continuation of our previous paper (Javed et al., 2010) on development of hyperproducing mutants of *A. niger* EB-3, this manuscript reports on the enhanced citric acid production by the selected ethidium bromide treated mutant *A. niger* EB-3, through optimization of some fermentation and nutritional parameters.

MATERIALS AND METHODS

All the chemicals were of analytical grade and were mainly purchased from Sigma-Aldrich-Fluka, Gemany. All the flask experiments were run in three replicates (n = 3) and all analyses were run in triplicate. The results have been presented as mean ± S.E. in tables and S.E. values have been shown as error bars in figures.

Substrate preparation

Lignocellulosic fruit waste banana stalk was used as carrier/inert support material for SSF of molasses medium. Banana stalk collected from fruit market of University of Agriculture, Faisalabad, Pakistan was cut into pieces, oven dried (60°C), grounded to 2 mm particle size and stored in airtight plastic jars to avoid moisture. Sugarcane molasses containing 47.8% (w/v) total sugar content obtained from Shakarganj Mills, Jhang was used as carbon and energy source.

Fungal culture and inoculum preparation

The culture of *A. niger* EB-3 mutant developed in Industrial Biotechnology Laboratory, University of Agriculture, Faisalabad, Pakistan was grown on potato dextrose agar (PDA) medium slant (Javed et al., 2010). Vogel's medium (100 ml) without agar was prepared in Erlenmeyer flask and adjusted to pH 4 using 1 M NaOH / 1 M HCl solutions. The pH was checked with the help of pH meter (WTW- Inolab. 720). The medium were sterilized (121°C) in autoclave (Sanyo, MLS-3020 U, Japan) for 15 min. After cooling at room temperature, the spores of *A. niger* from PDA slants were transferred into the flask aseptically in laminar air flow (Dalton, Japan). The inoculated medium was incubated at 35°C in a shaking incubator (120 rpm) for 72 h to get homogenous spore suspension containing 1×10^8 spores/ml.

Mutagenesis by ethidium bromide treatment

Spores of *A. niger* were treated with 1 mg/ml ethidium bromide for different time periods (30 to 180 min). After treatment, the kill/survival curve for EB treated spores was prepared and time of exposure giving 0.4×10^3 CFU/ml was selected. Spore dilutions (0.1 ml) from chemically treated mutants were spread on PDA media containing 2% triton X-100 as colony restrictor (Khattab and Bazaraa, 2005). The plates were covered with aluminum foil and placed in incubator at 30°C for 3 to 7 days. A dose producing 82% kill, by making 3log kill curve proved to be the best (Javed et al., 2010).

Selection of mutants

The ethidium bromide treated mutants were selected using 2-deoxy, D-glucose as selective marker. Colonies of yellow brown color (producing citric acid over Petri plate) were selected. Five colonies were picked and grown on slants for further selection of best mutant. Colonies that turned to yellow brown color were selected and five colonies picked from these plates were grown on slants for further selection.

Solid state fermentation

Banana stalk (5 g) was taken in triplicate 250 ml Erlenmeyer flasks. Molasses solution (10% w/v) was added to get 60% (w/w) moisture level (except in optimizing moisture level). pH was adjusted to 4.0 using 1 M NaOH / 1 M HCl solutions and the flasks were sterilized (121°C) in autoclave for 15 min. After cooling to room temperature, the flasks were inoculated with 2 ml of homogenous spore suspension of *A. niger* EB-3 and subjected to still culture fermentation at optimum temperature (35°C) for 96 h (unless otherwise mentioned) in a still culture incubator (Sanyo MIR-254, Japan).

Analytical

After 96 h, 100 ml distilled water was added to the flasks and the contents were shaken for 30 min at 150 rpm for citric acid extraction. The extracts were filtered through Whatman filter paper No.1 and centrifuged at 10,000 rpm (Eppendorf, USA) for 10 min. Citric acid was analyzed in a reaction mixture containing 1 ml of culture supernatant and 1.30 ml pyridine (Marrier and Boulet, 1958). The mixture was added in the test tube and swirled briskly.

Then 5.7 ml of acetic anhydride was added into test tubes. Test tubes were placed in water bath at 32 ± 0.25°C for 30 min. The absorbance was measured at 405 nm on a spectrophotometer (T-60, PG Instruments, UK). Standard curve was constructed using different concentrations of citric acid. Total sugars were estimated calorimetrically using dinitorsalysylic acid (DNS) as coupling reagent (Tasun et al., 1970).

Optimization of fermentation parameters

Fermentation and nutritional parameters were optimized under preoptimized conditions (Javed et al., 2010) of pH (pH 4.0), temperature (35°C) and inoculum size (2 ml). The classical method of optimization was adopted; varying one parameter at a time and maintaining the preoptimized ones at optimum level.

Moisture level

Varying volumes of 10% molasses solution of pH 4.0 were added to banana stalk to adjust the media to varying initial moisture levels (30 to 80%, w/w). The media were adjusted to initial optimum pH of 4.0, sterilized, inoculated with optimum volume of inoculum (2 ml) and incubated at optimum temperature (35°C) under still culture conditions.

Nitrogen supplements

To study the effect of nitrogen supplementation on citric acid production, different nitrogen sources (ammonium sulfate, peptone, yeast extract and ammonium nitrate) were added to the SSF medium at varying levels (0.1 to 0.6%, w/w) and fermentation was carried out under optimum conditions of pH, temperature, inoculum size and moisture content.

Fermentation/incubation time

All the previous experiments were carried out for fixed time period of 96 h. To study the effect of incubation period, the optimum banana stalk media moistened with molasses solution were incubated for different time periods under optimum conditions. Triplicate flasks were harvested after 24, 48, 72, 96 and 120 h.

Effect of surfactants/detergents

Surfactants/detergents increased the surface area for microbial growth in SSF. Different surfactants including Tween-80, Tween-20 and SDS were used at varying levels (0.1 to 0.6%, w/w) to study their effect on fungal growth, sugar utilization and citric acid production in SSF of molasses medium under optimum conditions

Effect of metabolic inhibitors

Effects of varying concentrations (0.1 to 0.5%, w/w) of organic metabolic inhibitors like naphthol, resorcinol, cresol and benzaldehyde were investigated to increase citric acid synthesis by inhibiting the formation of side products including acetic acid, ethanol and methanol.

Effect of metal complexing compounds

Different chemical compounds like potassium ferrocyanide, sodium monofluoroacetate, activated charcoal and trilon (Sod. EDTA) were added in varying concentrations (0.1 to 0.5%, w/w) into banana stalk medium to promote microbial growth and citric acid production by complexing with metal ions in molasses.

RESULTS AND DISCUSSION

Selection of mutant

A. niger EB-3 (treated for 120 min) showing best growth in the presence of selective marker was selected as the best ethidium bromide treated mutant. Colonies turning to yellow brown color were selected and five colonies picked from these plates were grown on slants for further selection on the basis of maximum citric acid production.

Optimization of parameters

Moisture level

Effect of different moisture level on citric acid production by *A. niger* EB-3 is shown in Figure 1. Citric acid concentration increased by increasing moisture level from 30 to 60% and decreased, thereafter. The highest value of citric acid (69.81 ± 1.63 mg/ml) and sugar consumption (86.85 ± 1.18 mg/ml) were achieved at 60% moisture level.

Moisture content is one of the important factors that affect the performance of a solid state fermentation (Tran et al., 1998). Lower moisture level is advantageous in SSF because contamination chances in fermentation medium are reduced (Roukas, 2000). However, there is a lower limit below which *A. niger* may not produce citric acid. This may be due to higher osmotic pressure at lower moisture level (Kargi et al., 1985). Nagadi and Correia (1992) reported that in solid state fermentation, low moisture contents resulted in suboptimal product formation due to reduced mass transfer processes such as diffusion of solutes and gas to the cell during fermentation, while higher moisture content reduces the inter-particular space (Khosravi-Darani and Zoghi, 2008). The reason might be that these conditions do not provide the natural habitat which is unfavorable for citric acid production (Ellaiah et al., 2004). Thus, the optimum level of moisture is necessary to enhance the production (Bari et al., 2010).

Nitrogen source and concentration

Effect of varying concentrations (0.1 to 0.6%) of different nitrogen sources (ammonium sulfate, peptone, yeast extract and ammonium nitrate) was studied. All concentrations of ammonium sulphate, peptone and yeast extract were found to be inhibitory to fungal growth, sugar utilization and citric acid production (Table 1). The

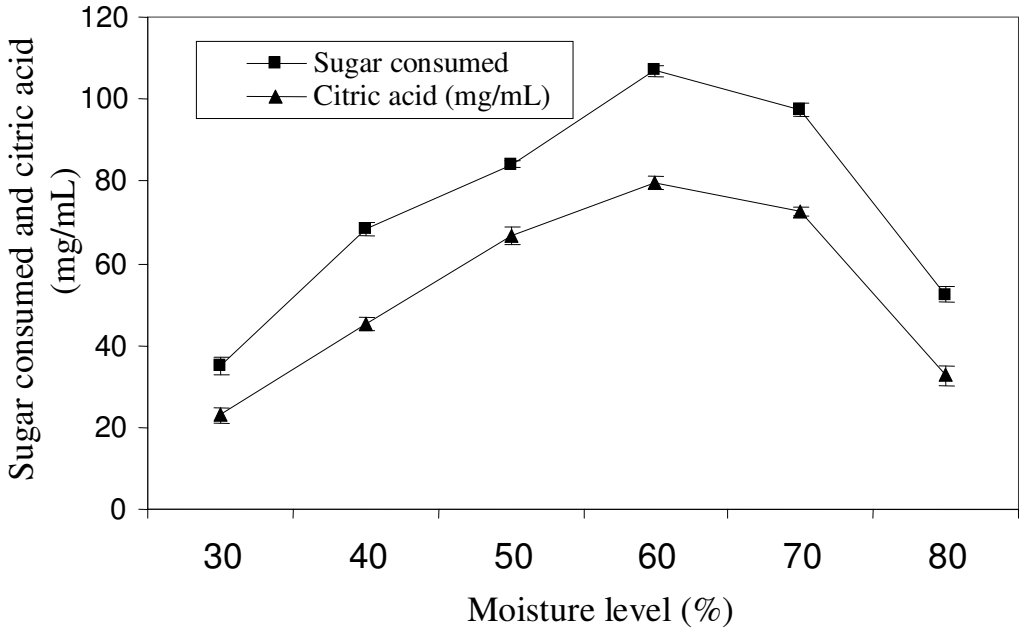

Figure 1. Effect of moisture level during citric acid production by *A. niger* EB-3 under optimum conditions.

Table 1. Citric acid production by *A. niger* EB-3 in SSF of molasses medium with varying concentrations of different nitrogen sources.

Nitrogen source concentration (%)	Peptone		Yeast extract		Ammonium nitrate		Ammonium sulphate	
	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)
0.1	96.21 ± 2.05	74.44 ± 2.76	104.21 ± 2.88	84.44 ± 3.04	102.51 ± 1.64	86.53 ± 2.13	102.21 ± 3.04	88.89 ± 3.25
0.2	85.86 ± 2.69	63.99 ± 2.46	99.86 ± 2.65	76.33 ± 3.16	106.07 ± 1.98	92.87 ± 2.72	101.86 ± 1.84	85.16 ± 2.79
0.3	80.41 ± 2.38	49.79 ± 1.34	85.61 ± 3.37	61.13 ± 2.43	86.19 ± 2.11	67.90 ± 2.34	93.41 ± 2.66	78.22 ± 2.82
0.4	73.24 ± 1.83	33.66 ± 2.38	78.24 ± 2.75	46.53 ± 2.65	78.24 ± 3.44	54.42 ± 2.77	91.84 ± 2.38	65.66 ± 1.95
0.5	66.34 ± 2.41	30.09 ± 1.77	65.34 ± 1.81	31.07 ± 2.41	69.63 ± 1.82	31.61 ± 2.94	86.34 ± 1.61	51.33 ± 2.44
0.6	61.19 ± 1.74	16.77 ± 1.93	63.19 ± 2.74	19.66 ± 1.77	59.79 ± 2.04	24.06 ± 1.85	67.19 ± 0.97	43.22 ± 2.41

*: pH 5.0; temperature, 35°C; inoculum size, 5 ml; moisture, 60% (w/w); incubation time, 96 h.

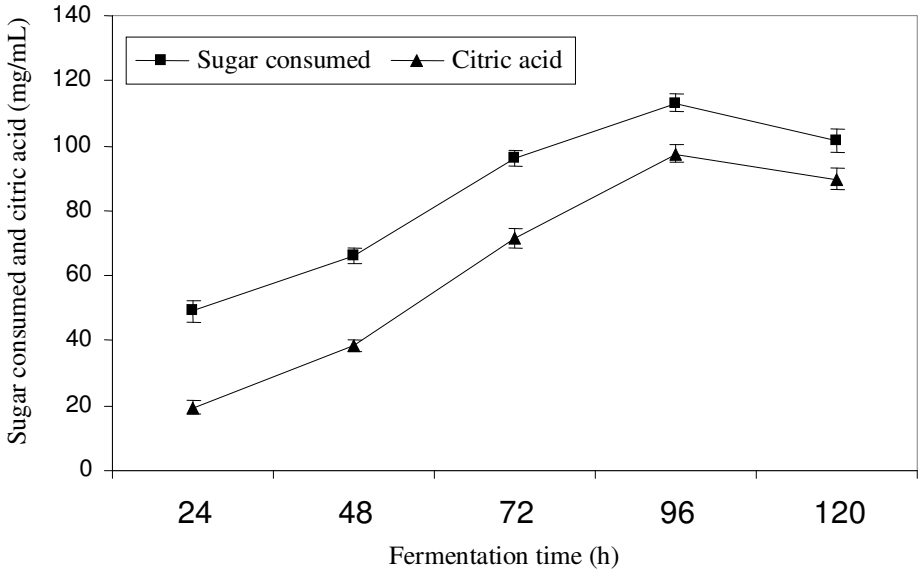

Figure 2. Effect of fermentation time during citric acid production by *A. niger* EB-3 under optimum conditions.

inhibitory effect was non-significant at lower concentrations but it became significant with increase in concentrations of nitrogen sources. However, ammonium nitrate was found to enhance the production of citric acid along with sugar utilization by *A. niger* EB-3. Maximum citric acid production (92.87 ± 2.72 mg/ml) and sugar utilization (104.07 ± 1.98 mg/ml) was observed with 0.2% ammonium nitrate. Higher ammonium nitrate levels in the medium decreased the production remarkably. Overall, the best nitrogen source was 0.2% (w/w) ammonium nitrate. Initial pH is very important in the citric acid fermentation and it can be maintained at very low level by using acid ammonium compounds like ammonium nitrate as nitrogen source (Ali et al., 2002; Roukas and Harvey, 1988).

Nitrogen is a constituent of many vital compounds including amino acids, basic proteins, enzymes, co-enzymes and nucleic acids. An optimum nitrogen concentration must be used to maintain proper C : N ratio in the optimum range because citric acid synthesis will decrease at both lower and higher nitrogen levels. Generally, microorganisms utilize more carbon than nitrogen and usually need a 20 to 30:1 ratio of C : N (Yadvika et al., 2004). With increase in nitrogen concentration, the production of new cells is increased, leading to increase in the flow of cytoplasm towards the new cells. Consequently, more and more aged cells become carbon stores due to nitrogen limitation and produce more citric acid (Kristiansen and Sinclair, 1979). Soccol et al. *(*2006) reported that the optimum concentration of nitrogen source required for citric acid fermentation was 0.1 to 0.4 g/L. High nitrogen concentrations increased fungal growth and sugar consumption but decreased the amount of citric acid produced.

Fermentation time

The results of citric acid production and sugar consumed by *A. niger* EB-3 at varying time periods is shown in Figure 2. The maximum citric acid (97.52 ± 2.65 mg/ml), and sugar consumption (113.12 ± 2.84 mg/ml) were noted after 96 h (4 days). Further increase in fermentation time did not improve citric acid production. It might be due to the age of fungus, depletion of sugar contents and decreased available nitrogen in fermentation medium. The optimal fermentation time for maximum citric acid production varies both with organism and fermentation conditions. According to Sikander et al. (2002), the production starts after a lag phase of one day and reaches maximum at the onset of stationary phase or late. Vergano et al. (1996) reported maximum yield of citric acid (64.12 g/L) 7 days after the inoculation. Haq et al. (2002) reported that 93.62 g/L citric acid was produced after 144 h of incubation. In line with our findings, Tran et al. (1998) and Yaykasli et al. (2004) also reported maximum citric acid production in SSF at the 4th day.

Effect of surfactants/detergents

It was noted that at lower concentrations (0.1 to 0.2) of SDS, Tween 20 and SDS enhanced citric acid production by *A. niger* EB-3 in SFF. There was a slight enhancement in citric acid produced in the flasks receiving 0.1% SDS and Tween-20. However, Tween-80 caused significant

Table 2. Citric acid production by *A. niger* EB-3 in SSF of molasses medium with varying concentrations of different surfactants under optimum conditions*.

Surfactant concentration (%)	Sodium dodecyl sulphate (SDS)		Tween-20		Tween-80	
	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)
0.1	111.84 ± 0.78	95.62 ± 1.89	104.47 ± 1.65	92.01 ± 0.92	105.64 ± 1.43	95.87 ± 1.71
0.2	96.42 ± 1.19	79.72 ± 0.79	105.21 ± 1.29	94.27 ± 0.84	115.14 ± 0.86	99.52 ± 1.58
0.3	69.41 ± 1.67	54.12 ± 0.86	109.42 ± 1.26	97.55 ± 1.25	98.54 ± 1.97	78.84 ± 1.49
0.4	45.81 ± 0.76	36.82 ± 1.83	86.41 ± 0.96	63.82 ± 1.06	94.65 ± 2.09	63.87 ± 1.33
0.5	31.98 ± 0.94	23.93 ± 1.34	73.54 ± 1.87	44.63 ± 1.81	69.47 ± 1.67	39.44 ± 1.15
0.6	28.15 ± 1.08	18.88 ± 1.14	56.84 ± 1.44	21.80 ± 1.18	40.45 ± 1.84	29.76 ± 0.77

*: pH 5.0; temperature, 35°C; inoculum size, 5 ml; moisture, 60% (w/w); ammonium nitrate, (0.2% w/w); incubation time, 96 h.

Table 3. Citric acid production by *A. niger* EB-3 in SSF of molasses medium with varying concentrations of different metabolic inhibitors under optimum conditions*.

Metabolic inhibitor concentration (%)	Naphthol		Resorcinol		Cresol	
	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)
0.1	102.21 ± 1.64	96.43 ± 1.13	102.11 ± 1.57	96.43 ± 1.73	106.11 ± 1.64	98.43 ± 2.71
0.2	107.91 ± 1.98	99.91 ± 1.72	105.76 ± 1.51	97.91 ± 2.22	107.76 ± 2.08	97.91 ± 1.92
0.3	112.29 ± 1.11	102.29 ± 1.34	108.21 ± 2.11	99.29 ± 1.54	111.21 ± 1.91	99.29 ± 2.36
0.4	102.42 ± 1.44	94.42 ± 1.77	110.84 ± 1.64	102.42 ± 1.77	115.84 ± 1.79	106.42 ± 1.77
0.5	87.61 ± 1.82	22.61 ± 0.94	107.64 ± 1.12	92.61 ± 1.94	112.64 ± 1.23	98.61 ± 1.94

*: pH 5.0; temperature, 35°C; inoculum size, 5 ml; moisture, 60% (w/w); ammonium nitrate, (0.2% w/w); incubation time, 96 h, Tween-80, 0.2%.

increase in citric acid production and sugar consumption. Maximum citric acid production was 99.52 ± 1.71 mg/ml and sugar consumption was 115.14 ± 1.43 mg/ml with 0.2% level of this surfactant (Table 2). By increasing Tween-80 concentration beyond optimum, citric acid production decreased. Surfactants are well known surface active agents that are generally used to improve the surface area for microbial action and availability of nutrients to the microorganisms. The surfactants also stimulate the biodegradation of nutrients by increasing the solubility and dispersion of the compounds (Desai and Banat; 1997; Helmy et al., 2009), and enhance the citric acid release into the medium by cell rupturing after its production.

Effect of organic metabolic inhibitors

Effect of varying concentrations of naphthol, resorcinol and cresol on citric acid production by *A. niger* EB-3 in molasses based SSF medium was investigated. The results (Table 3) showed that naphthol, resorcinol and cresol all enhanced citric acid production when used in lower concentration range but the enhancing effect with varying concentrations was highly variable. Cresol used in the concentration of 0.4% gave maximum citric acid production (106.91 ± 1.92 mg/ml) and sugar utilization (115.76 ± 2.08 mg/ml). Citric acid formation showed a steady decline with higher concentrations of all the three compounds. Comparison of the effects of different metabolic inhibitors on citric acid accumulation by *A. niger* EB-3 clearly showed that cresol was the most

Table 4. Citric acid production by *A. niger* EB-3 in SSF of molasses medium with varying concentrations of metal complexing compounds under optimum conditions*.

Concentration (%)	Potassium ferrocyanide		Charcoal		Benzaldehyde		EDTA	
	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)	Sugar consumed (mg/mL)	Citric acid (mg/mL)
0.1	108.11 ± 2.94	94.76 ±1.43	105.11 ± 1.64	93.27 ±2.73	102.11 ± 1.64	86.65 ± 1.73	108.31 ± 1.64	104.76 ± 1.93
0.2	111.36 ± 2.18	97.91 ± 3.12	109.26 ± 2.18	96.79 ±2.12	111.86 ± 2.18	102.87 ± 1.19	112.96 ± 2.18	106.91 ± 1.82
0.3	114.21 ± 2.51	109.19 ± 2.94	112.21 ± 3.91	105.39 ± 2.54	116.21 ± 1.91	109.32 ± 2.54	118.21 ± 3.11	109.19 ± 2.54
0.4	101.84 ± 2.84	86.62 ± 2.97	115.64 ± 3.12	108.11 ± 3.44	117.84 ± 2.64	98.62 ±1.97	121.84 ± 2.69	112.42 ± 3.07
0.5	94.64 ±3.12	70.61 ±1.84	96.84 ± 2.64	77.62 ±1.97	97.64 ±1.12	74.61 ±1.44	106.64 ± 1.12	84.61 ±3.84

*: pH 5.0 ; temperature, 35°C; inoculum size, 5 ml; moisture, 60% (w/w); ammonium nitrate, (0.2% w/w); incubation time, 96 h, Tween-80, 0.1%; cresol, 0.4%.

enhancing aerobic metabolism inhibitor causing maximum enhancement in citric acid yield at 0.4% (w/w) level and supported *A. niger* growth.

Organic metabolic inhibitors inhibit further metabolism of produced citric. It is accumulated in the mitochondria in higher concentrations and is consequently pushed out into the cytosol through tricarboxylate transport system. Qureshi and Qadeer (1987) reported that there was slight increase in citric acid formation in the presence of phenol (20 ppm) and b-naphthol (20 ppm). Hydroquinone (30 ppm) and O-cresol (15 ppm) have also been previously found to enhance citric acid production in LSF. Increase in citric acid production may be due to either the direct effect of these compounds on fungal growth and metabolism, or due to the inhibition of enzymes involved in aerobic metabolism of citric acid in mitochondria (Qureshi and Qadeer, 1987).

Effect of metal complexing compounds

The effect of varying concentrations of potassium ferrocyanide, activated charcoal, benzaldehyde and EDTA (ethylenediaminetetra-acetic acid) was studied under optimum conditions. In case of potassium ferrocyanide, maximum citric acid production (109.19 ± 3.12 mg/ml) and sugar utilization (114 ± 2.18 mg/ml) was observed with the addition of this compound at 0.3% level. Further increase in the ferrocyanide concentration gave lower citric acid production (Table 4). Similarly, by the addition of activated charcoal, initially, the citric acid production increased and it was maximum (108.11 ± 2.54 mg/ml) with 0.4% charcoal with sugar utilization of 115.64 ± 3.91 mg/ml. Maximum citric acid formation (109.62 ± 2.54 mg/ml) and sugar utilization (116.21 ± 1.91 mg/ml) was noted with 0.3% benzaldehyde. With 0.4% benzaldehyde, the fungus utilized more sugar (117.84 mg/ml) but gave lower citric acid productivity (98 mg/ml). However, with 0.5% level, sugar utilization also decreased, suggesting fungal metabolic inhibition by higher concentration of benzaldehyde. EDTA was found to show the best citric acid production (112.42 ± 3.07 mg/ml) at 0.4% EDTA by utilizing 121.84 ± 2.69 mg/ml sugar. EDTA addition at lower and higher concentrations decreases citric acid synthesis by the fungus. Overall, EDTA proved to be the best metal ion complexing reagent at 0.4% (w/w) level in SSF of molasses medium for citric acid production under optimum process parameters.

The chemical compounds like EDTA, potassium ferrocyanide and benzaldehyde have metal ions chelating properties and form complexes with metal ions in molasses to promote microbial growth and citric acid production by minimizing the availability of metal ions (Asadi and Nikkahah, 2002). The stimulation by metal-chelating agents such as EDTA may be due to the modification of the trace-metals balance (Choudhary and Pirt, 1965). The observation that the Fe^{3+} : EDTA ratio should be kept low and that ferrocyanide does not stimulate unless iron is present in excess of that required for growth, suggests that there should be an unlimited supply of iron at a critical concentration which is controlled by ferrocyanide or other chelating agents. The stimulations by chelating agents differ markedly in the effect of the time of addition. Chelating agents can stimulate when added to the culture at any time up to 72 h from time of inoculation or possibly later. On the other hand, ferrocyanide stimulates only when added before 48 h, in early growth period (Choudhary and Pirt, 1966). According to Purohit and Daginawala (1986), ferrocyanide ion is sometimes added to chelate trace metals such as iron and zinc during autoclaving to reduce their availability to the microorganism and thereby promoting citric acid production. However, Lofty et al. (2007) reported that potassium ferrocyanide added at different concentrations had no effect or a slightly

increased productivity rate of citric acid.

Conclusions

In conclusion, banana stalk is a good carrier substrate for citric acid production by *A. niger* EB-3 in SSF of molasses medium. Citric acid production by the mutant *A. niger* EB-3 could be substantially enhanced from 62 to 112 mg/ml by optimization of the fermentation parameters and by the addition of a suitable surfactant, nitrogen source, metabolic inhibitor and metal ion complexing reagent.

ACKNOWLEDGEMENTS

The study was a part of a project funded by Higher Education Commission (HEC), Islamabad, Pakistan. The financial help by HEC is highly appreciated. The facilities and technical support rendered by High Tech Lab, UAF for citric acid analysis is highly appreciated.

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