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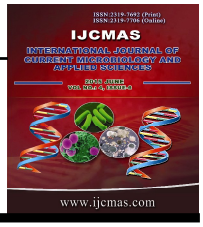

Original Research Article

Acidic pre-treatment of sugarcane molasses for citric acid production by *Aspergillus niger* NG-4

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Keywords

-Citric acid, *Penicillium restrictum*, *Aspergillus niger*

A B S T R A C T

The present study deals with the pre-treatment of sugar cane molasses for the enhanced production of citric acid by *Aspergillus niger* NG-4. For this purpose, different acids such as H_2SO_4, HNO_3 and HCl were used. Their level varied from 0.5-2.0 N and added in the production medium during the time of clarification. Among all the acids, the maximum amount of citric acid (40.0 g/l) was produced when 1.0N H_2SO_4 pre-treated cane molasses was used as a substrate, which is about 2.5, fold higher than the control (15.8 g/l citric acid). The kinetic parameters such as growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ in g/g), volumetric rates (Q_p, Q_s, Q_x in g/l/h) and specific substrate rates (q_p, q_s in g/g cells/h) of the research work were also undertaken. The value of Qp (0.134 g/l/h) is highly encouraging ($p \leq 0.05$).

Introduction

Citric acid obtained through the microbial fermentation is considered synthetic while that of present in fruits is referred to as natural (Ranya *et al*., 1999; Karklins *et al*., 2001). It is responsible for the tart taste of various fruits e.g., lemons, limes, oranges, pineapples, pears and gooseberries. It can be extracted from the citrus fruit juice by adding calcium oxide to form calcium citrate followed by recovery through the addition of sulphuric acid (Bizek *et al*., 1992; Karklins *et al*., 1996; Honda *et al*., 2011). It is one of the most important bulk-produced organic acids (Wieizorek and Brauer, 1998; Vanags and Viesturs, 2001). It is non-toxic and easily oxidized in the human body (Ma 2000). It can be used industrially for food, confectionary, beverages, to flavour the drinks, jam, jellie and pharmaceuticals. Its uses depend on three properties acidity flavour and salt formation. It adjusts the pH and chelates the trace metals. These and many other uses have placed greater stress on increasing the citric acid production (Kato *et al*., 1999). The worldwide demand for citric acid is about 6.0×10^5 tons per year (Karaffa and Kubicek, 2003). Citric acid has been produced on industrial scale by the fermentation of carbohydrates, initially exclusively by *A. niger* but in recent times

many microorganisms have been evaluated for the citric acid production including bacteria e.g., *Bacillus licheniformis, B. subtilis, Brevibacterium flavum, Arthrobactor paraffinens & Corynebacterium spp.* (Gomez *et al*., 1991), fungi e.g., *Aspergillus niger, A. awamori, A. foetidus, Penicillium restrictum, Trichoderma viride* & *Mucor pyriformis* (Kubicek *et al*., 1994) and yeasts e.g., *Candida lipolytica, C. intermedia, C. citrica* & *Saccharomyces cerevisiae* (Rymowicz *et al*., 1993; Kamzolova *et al*., 2003). However, *A. niger*, a filamentous fungus remained the organism of choice for citric acid production (Arzumanov *et al*., 2000; Wang *et al*., 2015). Some 4.5×10^5 tons of citric acid is being produced per year largely by the *A. niger* fermentation (Li *et al*., 2013).

The morphology of filamentous microorganisms during citric acid fermentation varies from round pellets to free long filaments depending upon the cultural conditions and strain genotype (Papagianni *et al*., 1998). All growth forms have their own characteristics regarding growth kinetics, nutrient consumption and broth toxicity (Allen and Robinson, 1990). The mycelium of *A. niger* is generally short and have branches with swollen tips. The effects of various cultural conditions and the rates of citric acid production by surface (Drysdale and McKay, 1995), submerged (Kirimura *et al*., 2000) and solid-state fermentations have been studied. Although surface culturing is still being used, most of the newly built citric acid plants have adopted submerged fermentation, a more sophisticated technology (Watanabe *et al*., 1998). The submerged citric acid fermentation process is labour intensive but gives higher production rates and uses less space (Grewal and Kalra, 1995). The present studies are concerned with the pretreatment of cane molasses for citric acid production by *A..niger* NG-4. All the optimizations for citric acid fermentation were carried out in 250 ml shake flasks. The clarified cane molasses, used as the basal fermentation media was obtained after treatments with four different concentrations of nitric acid, sulphuric acid, hydrochloric acid.

Materials and Methods

Organism

A. niger strain NG-4 was obtained from the stock culture of *Biotechnology Research Centre, Department of Botany, Government College University, Lahore*. It was maintained on 3.9 % potato dextrose agar (pH 5.6) slants. The potato dextrose agar medium was prepared by dissolving 39.0 g of PDA in approximately 800 ml of distilled water and raising the final volume upto 1000 ml. This was cooked for 10-15 min while constant stirring until a clear solution formed. The pH was maintained at 5.6 by 0.1N HCl or 0.1 NaOH. Approximately 5.0 ml of this medium was poured into the individual test tubes. The tubes were cotton plugged and sterilized in an autoclave at 15.0 Ibs/in^2 pressure (121°C) for 15 min. After sterilization, the test tubes were kept in a slanting position (at an angle of about 30°) to increase the surface area. The PDA slants were then inoculated by transferring a small amount of *A. niger* conidia from the culture provided and incubated at 30°C (4-6 days) for maximum sporulation. The cultures were stored at 4°C in a lab cool (Sanyo, Japan), for further studies.

Composition of cane molasses

The composition of cane molasses depends on the climatic factors, variety and maturity of cane as well as the processing conditions (Wolfram and Binkley, 1953). Consequently, considerable variations may be found in the nutrient contents, flavour,

colour and viscosity. The typical nutrient analysis of cane molasses used in the present study is variable (Table 1).

Molasses clarification

Cane molasses obtained from Kamalia Sugar Mills, Pvt. Ltd. (Kamalia, Pakistan) was pre-treated. Different acids i.e., H_2SO_4, HNO_3 and HCl were used for molasses pre-treatment. Their level was varied from 0.5, 1.0, 1.5 and 2.0 N. For each trial, 15 ml of the cane molasses was diluted upto 100 ml with distilled water. Then 5.0 ml of the acid was added and placed in a water bath at 90±2°C for 1 h. After cooling at room temperature, the medium was neutralized with lime (CaO) and left to stand overnight. Two layers were formed, the upper shiny black and lower yellowish brown due to the presence of trace metals. The clear supernatants were diluted to desired sugar level.

Preparation of conidial inoculum

Conidial inoculum was used in the present study. Ten millilitre of sterilized 0.005 % (w/v) diocetyl ester of sodium sulfo succinic acid (Monoxal O.T.) was added to a 3-5 day old slant culture having profuse conidial growth on its surface. An inoculum needle was used to break the conidial clumps. The tube was shaken vigorously to obtain a homogenous mixture of the conidial suspension. The conidial density was measured on a haemocytometer (Neubauer Precicdor HBG, Germany) after Sharma (1989). The conidial count in 1.0 ml of inoculum was calculated to be 1.2×10^6 conidia.

Fermentation technique

Twenty-five millilitre of the clarified cane molasses medium containing 150 g/l sugar at pH 6.0 was added into individual 250 ml cotton plugged conical flasks. The flasks were autoclaved at 15.0 lbs/in^2 pressure for 15 min. After cooling at room temperature, the flasks were inoculated with 1.0 ml of the conidial suspension and incubated at 30ºC in a rotary shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm for 168 h. The ingredients of the flasks were then filtered and the filtrate was used for the estimation of citric acid and residual sugar content.

Assay methods

Sugar estimation

The estimation of total reducing sugars (as glucose) is based on the dinitrosalicylic acid (DNS) method after Miller (1959) and Ghose & Kostick (1970). A double beam UV/VIS-scanning spectrophotometer (Model: Cecil-CE 7200-series, Aquarius, UK) was used for measuring the % transmittance. The sugar concentration in culture filtrate was estimated by diluting the filtrate a hundred times. Two millilitres each of the DNS reagent and dilute culture filtrate were added into a test tube. The tube was placed in a boiling water bath for 5 min. After cooling the contents of test tube at room temperature, the mixture was diluted to 20.0 ml with distilled water. A blank was run in parallel replacing 2.0 ml of the dilute filtrate sample with distilled water. The % transmittance was estimated at 546 nm on a spectrophotometer and the sugar concentration was determined from standard curve.

Estimation of dry cell mass

The dry cell mass was determined by filtering the culture broth through a pre-weighed Whattman filter paper No. 44. Mycelia were thoroughly washed with tap

water and dried in an oven (Model: 1442A, Memmert, Germany) at 105ºC for 2 h following Haq and Daud (1995). The filtrate was used for further analysis. The mycelial morphology was determined on an aliquot extended on the petri plates followed by the pellet diameter (Moreira *et al*., 1996).

Estimation of organic acid

Total acid

The total acid (oxalic acid, fumaric acid, malic acid, succinic acid, etc) was estimated by titrating 10.0 ml of diluted culture filtrate against 0.1 N NaOH. Phenolphthalein was used as an indicator.

$$\% \text{ Total acid} = \frac{\text{Titre x normality of alkali x equivalent weight of acid}}{\text{Volume of sample x 1000}} \times 100$$

Equivalent weight of the acid is 70. The total acid was reported in g/l.

Citric acid

Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method (Marrier and Boulet, 1958; Watanapokasin *et al*., 2007). The diluted culture filtrate (1.0 ml) along with 1.30 ml of pyridine was added into a test tube and swirled briskly prior to 5.70 ml of acetic anhydride addition. The test tube was placed in a water bath at 32±0.5ºC for 30 min. The optical density was measured at 405 nm using a spectrophotometer. The citric acid concentration of the sample was estimated from a reference (run parallel, replacing 1.0 ml of the culture filtrate with distilled water). The % citric acid (w/v) was determined following Suzuki *et al*. (1996).

$$\% \text{ Citric acid} = \frac{\text{Citric acid}}{\text{Sugar used}} \times 100$$

Kinetic parameters

For determining the kinetic parameters of batch fermentation process, the procedures of Pirt (1975) and Lawford & Roseau (1993) were adopted.

Statistical analysis

Treatment effects were compared by the protected least significant difference method and one-way ANOVA (Spss-9, version-4) after Snedecor and Cochran (1980).

Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (<p>) values.

Results and Discussion

Pre-treatment of cane molasses with HNO_3

The data of Table 2 shows the effect of pre-treatment of cane molasses with different concentrations of HNO_3 (such as 0.5 N, 1.0 N, 1.5 N, 2.0 N) for citric acid production by *A. niger* strain NG-4. HNO_3 was added in the production medium during the time of molasses clarification.

The maximum amount of citric acid (36.5 g/l) was produced when 0.5 N HNO_3 pre-treated cane molasses was used as a substrate that is 2.2 fold higher than the control (16.5 g/l citric acid). There is a gradual decrease in citric acid production at 1.0 N, 1.5 N and 2.0 N pretreated molasses. The sugar consumed was 133.5 g/l while dry cell mass was 52.0 g/l. Mycelial morphology was in the form of large round pellets.

Pre-treatment of cane molasses with H_2SO_4

The effect of pre-treatment of cane molasses with different concentrations of H_2SO_4 (such as 0.5 N, 1.0 N, 1.5 N, 2.0 N) for citric acid production by *A. niger* strain NG-4 was undertaken (Table 3). The acid concentrations were added in the production medium during the time of molasses clarification. The maximum amount of citric acid (40.0 g/l) was produced when 1.0 N H_2SO_4 pre-treated cane molasses was used as a substrate which is 2.5 fold higher than the control (15.8 g/l citric acid). The other concentrations, however, resulted in less production of citric acid in the broth. Sugar consumption and dry cell mass were 93.6 and 44.2 g/l, respectively. Mycelial morphology was in the form of dumpy mass.

Pre-treatment of cane molasses with HCl

The effect of pre-treatment of cane molasses with different levels of HCl (i.e., 0.5 N, 1.0 N, 1.5 N, 2.0 N) for citric acid production by *A. niger* strain NG-4 was also carried out (Table 4). Acid concentrations were added in the fermentation medium during the time of molasses clarification. Maximum amount of citric acid (33.0 g/l) was produced when 1.0 N HCl pre-treated cane molasses was used as a substrate. Control gave 18.0 g/l citric acid. Other acid concentrations, however, resulted in the decreased production of citric acid. The optimal sugar consumption and dry cell mass were 94.5 and 34.0 g/l, respectively. Viscous mycelial morphology was observed in the fermented culture broth.

Rate of citric acid fermentation

The rate of citric acid fermentation by a strain of *A. niger* NG-4 was investigated in shake flask. 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. Further increase in the incubation period resulted in increased citric acid production. However, maximum production (57.0 g/l.) was achieved, 168 h, after inoculation. The sugar consumption and dry cell mass were 93.50 and 14.58 g/l, respectively. 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 (Figure 1). The values for Yp/s, Yp/x, Qp and qp were more significant after 144 h of incubation than all other time periods, for citric acid production.

In the present study, the effect of pre-treatment of cane molasses with different acids i.e., H_2SO_4, HNO_3 and HCl on citric acid production by *A. niger* strain NG-4 was undertaken. Their level varied from 0.5-2.0 N. The acid concentrations were added in the production medium during the time of molasses clarification. Among all the acids, the maximum amount of citric acid (40.0 g/l) was produced when 1.0N H_2SO_4 pre-treated cane molasses was used as a substrate, which is about 2.5, fold higher than the control (15.8 g/l citric acid). It might be due to that H_2SO_4 provides available sulphate ions, which facilitate the optimal growth and also it hydrolyses the sucrose into simpler sugars particularly glucose while the other acids may have toxic inhibitory effects on the mycelial growth due to the presence of Cl^- or NO_3 ions in the medium (Xu *et al*., 1989). The H_2SO_4 concentrations other the optimal, however, resulted in less production of citric acid in the broth. Sugar consumption and dry cell mass were 93.6 and 44.2 g/l, respectively.

Table.1 Typical nutrient analysis of cane molasses

Components	Concentration range (g/l)
Water	170 - 250
Sucrose	300 - 400
Dextrose (Glucose)	40.0 - 90.0
Levulose (Fructose)	50.0 - 120.0
Ash contents	70.0 - 150
Nitrogenous compounds	20.0 - 60.0
Non-nitrogenous compounds	20.0 - 80.0
Waxes, sterols and phospholipids	1.0 - 10.0

Table.2 Pre-treatment of cane molasses with HNO3 for citric acid production by the A. niger strain NG-4 in shake flask

Conc. of HNO_3	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	102.5±3.2	42.0±2.2	16.5±3.1	Viscous
0.5	133.5±4.2	52.0±2.2	36.5±3.1[a]	Large pellets
1.0	137.6±3.5	40.6±3.0	34.2±2.5[ab]	Mixed pellets
1.5	121.5±3.6	38.5±2.7	31.0±2.2[b]	Fine pellets
2.0	142.8±6.2	37.4±2.5	26.5±3.6[c]	Fine pellets

Sugar concentration, 150.0 g/l; Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6.0. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table.3 Pre-treatment of cane molasses with H2SO4 for citric acid production by the *A. niger* strain NG-4 in shake flask

Conc. of H_2SO_4	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	98.2±3.1	43.5±2.8	15.8±3.1	Viscous
0.5	72.5±2.8	47.0±3.1	38.5±4.2[b]	Dumpy mass
1.0	93.6±3.2	44.2±1.4	40.0±1.9[a]	Dumpy mass
1.5	84.0±3.7	55.5±2.6	33.5±1.9[b]	Large pellets
2.0	75.5±4.2	48.5±3.0	31.4±2.4[cd]	Mixed pellets

Sugar concentration, 150.0 g/l; Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6.0. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table.4 Pre-treatment of cane molasses with HCl for citric acid production by the *A. niger* strain NG-4 in shake flask

Conc. of HCl	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	101.5±3.2	38.0±2.5	18.0±2.1	Viscous
0.5	105.5±2.8	37.6±2.4	30.5±1.2[ab]	Viscous
1.0	94.5±2.2	34.0±2.0	33.0±1.2[a]	Viscous
1.5	89.4±3.1	37.2±2.8	26.8±2.0[c]	Fine pellets
2.0	73.0±1.2	12.8±1.8	22.1±1.6[cd]	Gelatinous

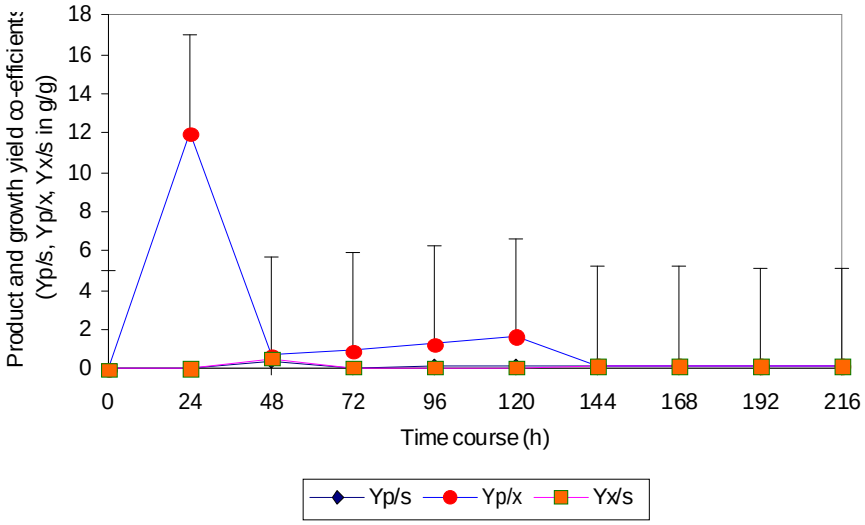
Sugar concentration, 150.0 g/l; Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6.0. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$

Table.5 Rate of citric acid fermentation by a mutant strain of *A. niger* NG-4 in shake flask

Incubation period (h)	Dry cell mass (g/l)	Sugar consumption (g/l)	Citric acid (g/l)	Mycelial morphology
24	6.64±0.2	55.00±2.0	10.5±0.1	Elongated mycelium
48	9.50±0.1	70.00±2.5	21.0±0.1	Round pellets
72	11.00±0.2	80.50±2.0	24.5±0.2	Small round pellets
96	11.58±0.2	85.00±4.0	31.0±0.2	Large round pellets
120	12.32±0.2	91.20±5.5	42.0±0.2	Small round pellets
144	13.47±0.5	92.30±5.0	48.0±0.2	Small round pellets
168	14.58±0.3	93.50±3.5	57.0±0.4	Mixed mycelium
192	16.79±1.2	99.78±2.9	42.0±0.5	Gelatinous mass
216	16.20±1.0	104.48±3.4	35.0±0.2	Dumpy mass
242	17.98±1.1	110.30±4.0	39.7±0.4	Dumpy mass

Initial sugar concentration, 150.0 g/l; Temperature, 30ºC; Initial pH, 6.0; Potassium ferrocyanide, 200 ppm. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$

Figure.1 Comparison of product and growth yield coefficients for citric acid fermentation



Yp/s = g citric acid produced /g substrate consumed
Yp/x = g citric acid produced /g cell formed; Yx/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 $p \leq 0.05$.

Mycelial morphology was in the form of dumpy mass. In a similar kind of study, Kristiansen *et al*. (1999) pointed out that the great advantage of the molasses clarification is to have a pure substrate or/and a substrate with minimum quantity of trace metals and contaminants.

The maximum yield of citric acid (57.0 g/l) was achieved, 168 h after incubation. Further increase in incubation period did not enhance citric 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 (Xie and West, 2009; Kutyła-Olesiuk *et al*., 2014). In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reaches maximum at the onset of stationery phase. This finding is 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, our finding is more encouraging as compare to Clark (1962) due to short incubation period.

The kinetic parameters such as growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ in g/g), volumetric rates (Q_p, Q_s, Q_x in g/l/h) and specific substrate rates (q_p, q_s in g/g cells/h) of the research work were also undertaken. The mutant strain of *A. 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 in h^{-1}) was only marginally different during growth of mutant *A. niger* GCB-47 on 150 g/l carbohydrates in molasses at 30°C (than 32°C or 165 g/l 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, methanol 1.0 %, NH_4NO_3 0.15 %, $CaCl_2$ 2.0 % K_2HPO_4 0.20 % and 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) and Jernejc & Legisa (2004). Maximum values for $Y_{p/s}$, Q_p and q_p were several folds improved over the previous workers (Pirt 1975; Rohr 1998).

Thus it can be concluded, many factors need to be considered by citric acid producers to obtain the economically favourable process. The design of culture media should base on the qualitative and quantitative requirements of nutrients, the interactions between substrates, the physical conditions and medium stability.

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