Journal of Advances in Biological and Basic Research 01[01] 2015
www.asdpub.com/index.php/jabbr e-ISSN-2454-6097

Original Article

Alternative pre-treatment of raw molasses by metal complexing agents for citric acid productivity by *Aspergillus niger*

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Keywords:
Sugar cane molasses,
Aspergillus niger, Citric acid

Abstract

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, the acids and metal complexing agents were added in the molasses medium, alternatively prior to heating at 90°C for 1 h. Among them, the maximum amount of citric acid (53.2 g/l) was produced when the ratio between H_2SO_4+$K_4Fe(CN)_6$ was maintained at 1.0:250 for the pre-treatment of cane molasses which is approximately 3.1 fold higher than the control (17.0 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 ($p \leq 0.05$).

1. Introduction

Citric acid has been produced on industrial scale by the fermentation of carbohydrates, initially exclusively by *Aspergillus niger*. The process is very sensitive to the concentration of these metals in the fermentation media. Therefore, the concentration of these heavy metals should be decreased well below that required for the optimal fungal growth [1,2]. Since the concentration of trace element, effect citric acid production profoundly, various technology have been used to minimize the concentration of these metals in fermentation media. Potassium ferrocyanide reacts with many heavy metals, causing their precipitation. It removes not only metals of vegetative influence but also some of the microelements necessary for mycelium growth. Therefore its addition to molasses has to be strictly regulated [3,4]. The optimum amount of ferrocyanide depends on molasses type and ranges from 200-1000 mg/dm^3 of medium. This has been used to develop a quick method of optimal ferrocyanide dosage in molasses media [5]. Ferro cyanide is normally added before and after sterilization or the total amount can be added after sterilization. Citric acid yield was decreased with the increase of ash content in the medium. Among all the main molasses minerals (K, Na, Ca), calcium is the most inhibitor of citric acid fermentation [6]. Other chemicals that are used for reducing the metal content of molasses are the chelating agents such an EDTA activated charcoal [7,8] and polyethylene amine [9]. EDTA reacts with metals of I & II valency at pH 2 with metals of III valency at pH 4-5 and with multivalent metals at pH 1. Ca and Mg ions gave trilon soluble salts and they are not removed from solution.

The present studies are concerned with the pretreatment of cane molasses for citric acid production by *A. niger* NG-4. The clarified cane molasses, used as the basal fermentation media was obtained after alternative treatments of potassium ferrocyanide and EDTA. Product and growth kinetic parameters were further evaluated to gain an insight into the batch fermentation process.

2. Materials and Methods

2.1 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 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 [10]. Consequently, considerable variations may be found in the nutrient contents, flavour, colour and viscosity (Table 1).

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

2.2 Alternative pretreatment of molasses by metal complexing agents

Cane molasses obtained from Kamalia Sugar Mills, Pvt. Ltd. (Kamalia, Pakistan) was pre-treated. In these pre-treatments, the acids and metal complexing agents were added alternatively prior to heating at 90°C for 1 h.

2.3 Preparation of conidial inoculum

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 conidial density was measured on a haemocytometer (Neubauer Precicdor HBG, Germany) after Sharma [11] (1989). The conidial count in 1.0 ml of inoculum was calculated to be 1.2×10^6 conidia.

2.4 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.

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2.5 Assay methods

2.5.1 Sugar estimation

The estimation of total reducing sugars (as glucose) is based on the dinitrosalicylic acid (DNS) method after Miller (1959). 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 the standard.

2.5.2 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[12] (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[13].

2.5.3 Estimation of citric acid

The total acid was estimated by titrating 10 ml of diluted culture filtrate against 0.1 N NaOH. Phenolphthalein was used as an indicator. Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method. 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).

2.6 Kinetic parameters and statistical analysis

For determining the kinetic parameters of batch fermentation process, the procedures of Pirt[14] (1975) and Lawford & Roseau[15] (1993) were adopted. Treatment effects were compared by the protected least significant difference method and one-way ANOVA (Spss-9, version-4) after Snedecor and Cochran [16] (1980). Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (<p>) values.

3. Results

The effect of combined pre-treatment of cane molasses with HNO_3+$K_4Fe(CN)_6$ at different ratios was also performed for citric acid production by *A. niger* strain NG-4 (Table 2). HNO_3+$K_4Fe(CN)_6$ were alternatively added in the production medium during the time of molasses clarification. The maximum amount of citric acid (44.5 g/l) was produced when the ratio between HNO_3+$K_4Fe(CN)_6$ was maintained at 1.5:250 for the pre-treatment of cane molasses which is 2.4 fold higher than the control (18.5 g/l citric acid). The other ratios, however, resulted in less production of citric acid in the broth. Optimal sugar consumption and dry cell mass were 131.0 and 46.54 g/l, respectively. Mycelial morphology was in the form of dumpy mass.

The data of Table 3 revealed the combined effect of pre-treatment of cane molasses with H_2SO_4+$K_4Fe(CN)_6$ at different ratios on citric acid production by *A. niger* strain NG-4. The alternative addition of H_2SO_4+$K_4Fe(CN)_6$ was made in the medium during the time of molasses clarification. The maximum amount of citric acid (53.2 g/l) was produced when the ratio between H_2SO_4+$K_4Fe(CN)_6$ was maintained at 1.0:250 for the pre-treatment of cane molasses which is 3.1 fold higher than the control (17.0 g/l citric acid). The other ratios, however, resulted in less production of citric acid in the broth. Optimal sugar consumption and dry cell mass were 87.0 and 31.6 g/l, respectively. Mycelial morphology was in the form of a viscous solution. Therefore, the alternative pre-treatment of H_2SO_4+$K_4Fe(CN)_6$ was optimized for molasses clarification and it was selected for hyperproduction of citric acid in shake flasks.

The effect of combined pre-treatment of cane molasses with HCl+$K_4Fe(CN)_6$ at different ratios was also performed for citric acid production by *A. niger* strain NG-4 (Table 4). HCl+$K_4Fe(CN)_6$ were alternatively added in the production medium during the time of molasses clarification. The maximum amount of citric acid (33.2 g/l) was produced when the ratio between HCl+$K_4Fe(CN)_6$ was maintained at 1.0:250 for the pre-treatment of cane molasses which is only 1.9 fold higher than the control (17.5 g/l citric acid), so, not encouraging. The other ratios, however, resulted in even less production of citric acid in the broth. Optimal sugar consumption and dry cell mass were 122.6 and 35.6 g/l, respectively. Mycelial morphology was in the form of mixed pellets.

The rate of citric acid fermentation by a strain of *A. niger* NG-4 was investigated in shake flask (Table 5). 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.

Table 2: Pre-treatment of cane molasses with HNO_3+$K_4Fe(CN)_6$ for citric acid production by the *A. niger* strain NG-4 in shake flask

HNO_3+$K_4Fe(CN)_6$ ratio	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	102.0±3.5	41.0±2.2	18.5±3.0	Viscous
0.5 : 250	113.3±2.6	38.5±2.0	36.5±2.1^{b}	Mixed pellets
1.0 : 250	121.5±3.0	45.6±3.5	41.2±1.5^{a}	Dumpy mass
1.5 : 250	131.0±1.6	46.5±2.3	44.5±2.2^{b}	Dumpy mass
2.0 : 250	126.0±3.4	41.5±3.2	38.2±3.0^{cd}	Dumpy mass

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 H_2SO_4+$K_4Fe(CN)_6$ for citric acid production by the *A. niger* strain NG-4 in shake flask

H_2SO_4+$K_4Fe(CN)_6$ ratio	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	99.5±4.1	42.5±2.2	19.5±3.4	Viscous
0.5 : 250	83.5±3.5	30.5±2.0	42.4±1.6^{b}	Viscous
1.0 : 250	87.0±3.8	31.6±3.1	53.2±2.2^{a}	Viscous
1.5 : 250	81.0±2.6	32.5±2.8	44.0±2.0^{b}	Mixed pellets
2.0 : 250	92.5±3.0	36.0±1.5	41.5±2.1^{cd}	Dumpy mass

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 $p \leq 0.05$.

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Table 4: Pre-treatment of cane molasses with HCl+$K_4Fe(CN)_6$ for citric acid production by the *A. niger* strain NG-4 in shake flask

HCl+$K_4Fe(CN)_6$ ratio	Sugar consumed (g/l)	Dry cell mass (g/l)	Citric acid (g/l)	Mycelial morphology
Control	98.0±3.0	41.0±3.2	17.5±2.4	Viscous
0.5 : 250	116.0±2.4	28.4±2.0	32.8±2.1[a]	Large pellets
1.0 : 250	122.6±2.0	35.6±3.1	33.2±1.5[ab]	Mixed pellets
1.5 : 250	138.6±2.8	34.5±2.8	26.0±2.2[b]	Fine pellets
2.0 : 250	130.0±3.1	32.4±3.6	23.5±2.6[cd]	Fine pellets

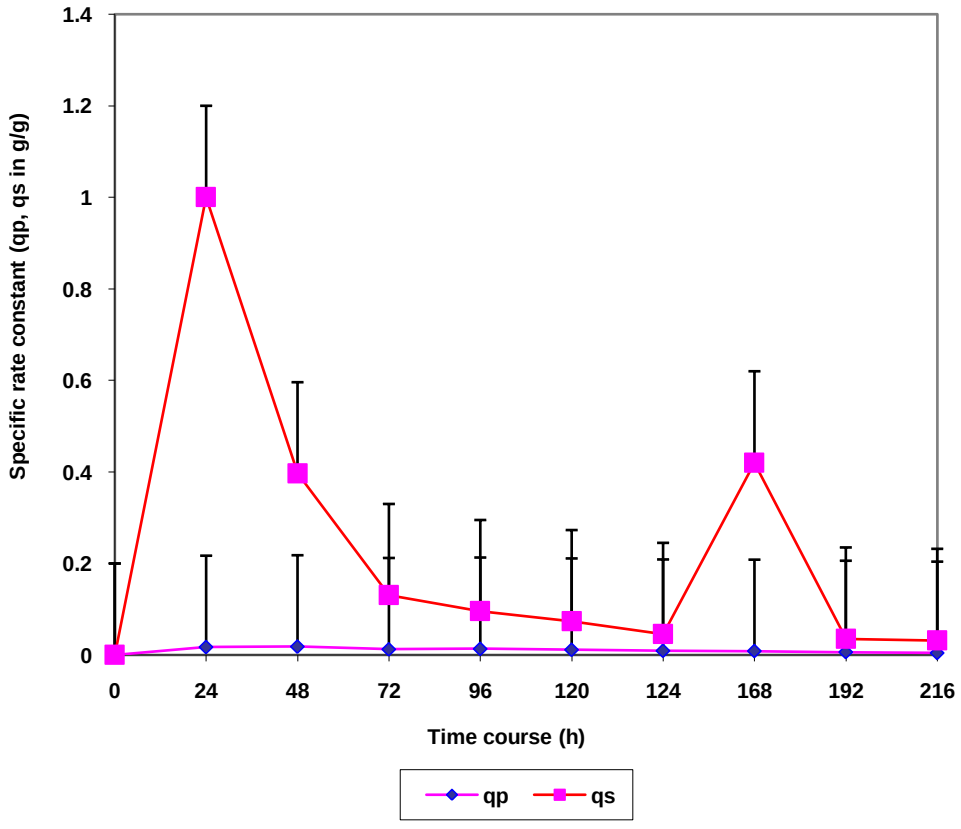
Sugar concentration, 150.0 g/l; Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6.0. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table 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 specific rate constants for citric acid fermentation



qp = g citric acid produced / g cells/ h
qs = g substrate consumed /g cells/ h
Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$

4. Discussion

In a particular study, the acids and metal complexing agents were added in the molasses medium, alternatively prior to heating at 90°C for 1 h. Among them, the maximum amount of citric acid (53.2 g/l) was produced when the ratio between H2SO4+K4Fe(CN)6 was maintained at 1.0:250 for the pre-treatment of cane molasses which is approximately 3.1 fold higher than the control (17.0 g/l citric acid). Similar, type of findings has also been carried out. The other ratios, however, resulted in less production of citric acid in the broth. Chaudhary and Pirt [17] (1969), however, optimized the addition of H_2SO_4+HCl for molasses pre-treatment and a better production of citric acid by *A. niger*. As in the present study, the alternative pre-treatment of H_2SO_4+$K_4Fe(CN)_6$ was optimized for molasses clarification, so, it was selected for the production of citric acid in shake flasks.

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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[18]. 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.* [19] (1996) and Rajoka *et al.[20]* (1998). Clark [21](1962) obtained about 70% conversion of available sugar, 192 h after incubation. Hence, our finding is more encouraging as compare to Clark [21](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 [14] (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*. [20] (1998). Maximum values for $Y_{p/s}$, Q_p and q_p were several folds improved over the previous workers [22,23].

From the results, it can be concluded that the reduction of heavy metal contents of molasses by chelating agents is of vital importance for improved citric acid production technology. The overall design of culture media is based on interactions between substrates, the physical conditions and medium stability. Further work is however needed, to improve the substrate consumption rate by isolating mutants which are resistant to higher concentrations of 2-deoxy D-glucose.

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