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ISOLATION OF CITRIC ACID PRODUCING STRAINS OF *ASPERGILLUS NIGER* USING TANNIC ACID MEDIUM AND PRODUCTION PARAMETERS

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ABSTRACT

Thirty one strains of *Aspergillus niger* were isolated using a new approach which describes the selective isolation of *A. niger* using tannic acid medium. Twelve strains, selected after qualitative screening using Bromocresol green dye, were further screened quantitatively for citric acid production by submerged fermentation. Of all the strains, *A. niger* GCBT-7 gave the maximum citric acid production (8.27±0.95 g/l). The optimal production of citric acid by the isolate GCBT-7 (11.91+0.76.23 g/l) was achieved 168 h after the conidial inoculation, in the fermentation medium containing 150-g/l sugar. The initial pH of the medium was also optimized and a pH of 5.5 was found to be the best for citric acid production.

Keywords: Citric acid, fermentation, *Aspergillus niger*, Tannic acid, isolation, mollases.

INTRODUCTION

Citric acid is a non toxic, biodegradable and ubiquitous chemical (Ma, 2000). It is an intermediate of TCA cycle and finds extensive applications in food beverages, pharmaceuticals, paper and textile industries (Johnson 2003; Magnuson and Lasure, 2004). Wehmer (1893) was the first man who recognized citric acid as a microbial metabolite. Many microorganisms have been evaluated for the citric acid production including bacteria e.g., *Bacillus licheniformis, B. subtilis, Brevibacterium flavum, Arthrobactor paraffinens* and *Corynebacterium* species. (Edlaur *et al*., 1990; Gomez *et al*., 1991), fungi e.g., *Aspergillus niger, A. awamori, A. foetidus, Penicillium restrictum, Trichoderma viride* and *Mucor pyriformis* (Mattey and Allan, 1990; Kubicek *et al*., 1994; Gradisnik-Grapulin and Legisa, 1996; Tran *et al*., 1998) and yeasts e.g., *Candida lipolytica, C. intermedia, C. citrica* and *Saccharomyces cerevisiae* (Rymowicz *et al*., 1993; Skolov *et al*., 1996; Crolla and Kennedy, 2001; Archer 2001; Kamzolova *et al*., 2003). However, *A. niger*, a filamentous fungus remained the organism of choice for citric acid production. Today, most of the citric acid used in food and other industries comes from *A. niger* (Cameron *et al*., 2003).

Isolation and selection of a suitable strain is the step of prime important for the development of a successful fermentation process. When selecting a culture for citric acid fermentation, consideration must be given to its citric acid producing capacity, stability, amount of sporulation and mycelium production when grown in liquid media, resistance to other organisms, and tendency to degrade citric acid and ability to form other products (Perlman and Sih, 1960). Citric acid is a primary metabolite produced by *A. niger* during idiophase stage when growth drops and acid production becomes the main cellular activity. In order to force a microorganism to secrete primary metabolites, it must be grown under sub-optimal conditions. Cultural conditions for citric acid production by fungi vary from strain to strain and also depend on the type of process used (Kubicek and Roehr, 1977; Pera and Callieri, 1999).

In Pakistan sugar industries are mostly based on cane with exception of few using beet have been well established producing molasses in tons, annually. Bulk of the molasses is exported at throwaway prices and in return the expensive chemicals are imported to meet the demands. So it is the need of the hour to exploit our own resources to get the value added products through process optimization. The main objective of the present study is isolation and screening of citric acid producing strains of *Aspergillus niger* and optimization of cultural parameters. It is aims at the optimum exploitation of cane molasses to produce citric acid by using submerged fermentation.

MATERIALS AND METHODS

Isolation of organism

A relatively new method for the isolation of *A. niger* using tannic acid medium was used. Thirty one strains of *Aspergillus niger* were collected from different localities of District Lahore and nearby in sterilized polythene bags. A pinch of soil was added in the individual 100 ml conical flask containing 20 ml of tannic acid solution in addition to (g/l) $MgSO_4$, 0.5; K_2HPO_4, 0.1; KCl, 0.5; $NaNO_3$, 2.0; sugar, 100 (Knudson, 1913). The flasks were incubated at 30°C for 5-6 days and the potent strains were isolated on the basis of qualitative screening. Purified isolates of *A. niger* were identified after Onion *et al*. (1986) and maintained on potato dextrose agar slants at 4°C.

Qualitative screening

The qualitative screening of *Aspergillus niger* cultures was carried out in petriplates containing Czapek-Dox agar medium (g/l: molasses sugar 30.0, $NaNO_3$ 2.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4$ 0.01, Bromocresol green dye 40.0 ml of 1.0 %, Agar 20.0, pH 6.0) incubated at 30ºC for 5 days. Cultures were selected on the basis of larger yellow zones formed due to acid production (Das and Ponty, 1980).

Quantitative screening

The *Aspergillus niger* isolates were further screened for citric acid production by submerged fermentation in 250 ml Erlenmeyer flasks which follows as:

Molasses clarification

The cane molasses was obtained from Kamalia Sugar Mills (Kamalia, Pakistan). Thirty-five millilitres of 0.1N H_2SO_4 were added to 1.0 l molasses medium and placed in water bath at 90°C for about one hour. After cooling at room temperature, the medium was neutralized with lime (CaO) and left overnight, for clarification. Two layers were formed, upper shining black (clarified molasses) was separated carefully while lower yellowish brown (due to the presence of trace metals) was discarded. The clear supernatant was diluted to 15.0 % sugar level (Panda *et al*., 1984).

Preparation of conidial inoculum

The conidial suspension was prepared by addition of 10 ml of sterilized 0.005 % (w/v) Monoxal O.T. (Bis-2-ethylhexyl sulfosuccinate sodium salt) solution to a 5 days old slant culture having profuse conidial growth on its surface. A sterile wire-loop was gently used to break the conidial clumps. The test tube was shaken vigorously to make homogeneous suspension. Conidial count was determined after Sharma (1989) using haemocytometer.

Fermentation technique

Twenty-five millilitre of the clarified cane molasses medium containing 150 g/l sugar at pH 6.0 was added into the individual 250 ml cotton plugged conical flasks. The flasks were autoclaved at 15.0 lbs/in^2 pressure (121°C) for 15 min. Sterilized ferrocyanide (free conc. 200 ppm) was added to each flask while the medium was hot. After cooling at room temperature, the flasks were inoculated with 1.0 ml (1.25×10^6 conidia/ml) of the conidial suspension and incubated at 30°C in a rotary shaking incubator at 200 rpm for 168 h. The addition of methanol to the fermentation flasks (1 %, v/v) was made 24 h after the spore inoculation. After fermentation the ingredients of the each flask were then filtered and the filtrate was used for the estimation of citric acid and residual sugar content. All the experiments were run parallel in triplicate.

Assay methods

Sugar estimation

The reducing sugars (as glucose) were estimated by method of Miller (1959) using the 3,5-dinitrosalicylic acid (DNS) and the % transmittance was measured at 575 nm on a spectrophotometer (Cecil 7200, UK).

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 and dried in an oven at 105°C for 2 h following Haq and Daud (1995).

Estimation of citric acid

Citric acid was determined after the method of Marrier and Boulet (1958). Added appropriately diluted culture filtrate (0.5 ml) along with 0.65 ml of pyridine into the individual test tubes and swirled briskly prior to 2.85 ml of acetic anhydride addition. The test tubes were incubated at 32±1°C for 30 min in a water bath and OD was measured at 420 nm. A control was run parallel, replacing 0.5 ml of the culture filtrate with distilled water.

RESULTS AND DISCUSSION

It is highly significant to isolate a strain with superior characters, such as enhanced acid production and increased rate of fermentation (Kristiansen *el al*., 1999). In the present research work tannic acid containing medium was used to isolate the strains of the *Aspergillus niger*. This relatively new approach for isolation of *A. niger* for citric acid fermentation was adopted because the medium containing 10 % of tannic acid allows only *A. niger, A.*

oryzae and *Penicillium* species to grow (Knudson, 1913). Thirty one isolates of *A. niger* were qualitatively screened using Bromocresol green dye (Fig. 1) and selected 12 strains on the basis of larger zone of hydrolysis and were further evaluated for citric acid production by submerged fermentation (Table 1). The amount of citric acid production by these isolates ranged from 0.85-8.27 g/l. Among all the isolates, GCBT-7 gave maximum citric acid i.e. 8.27±0.95 g/l. The dry cell mass and sugar consumption were 24.32±1.43 and 73.89±0.99 g/l, respectively.

A successful process not only depends on appropriate strain selection but also on the optimization of fermentation parameters (George *et al*., 1999). Amount of carbon source is of prime importance in citric acid fermentation. A number of workers have reported molasses based medium for enhanced and consistent production of citric acid (Benuzzi and Segovia, 1996; Haq *et al*., 2004). The maximum citric acid was produced during present studies in the medium containing 150-g/l sugars (Figure 2). Further increase in the concentration of sugar resulted in the gradual reduction in citric acid production. It might be due to the over growth of the mycelium, which resulted in increased viscosity and mass transfer limitations. Increase in dry weight of mycelia with the increase in the sugar concentration is in agreement with the work reported earlier by Chaudhary and Pirt (1966). However, a lower concentration of sugar leads to lower yield of citric acid due to the accumulation of oxalic acid (Kovats, 1960).

Fig. 3 depicts the rate (24-240 h) of citric acid fermentation by *Aspergillus niger* GCBT-7. The maximum yield of citric acid was achieved, 168 h after incubation. Further increase in incubation period resulted in the decreased citric acid production. It might be due to the depletion of nitrogen in the fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself and the depletion of sugar contents. Moreover, the strains of *A. niger* may start to utilize citrate for growth when carbohydrate levels become low (Kontopidis *et al.*, 1995). Vergano *et al*. (1996) also reported the maximum production of citric acid after 7 days of the incubation.

The pH value of the culture medium is one of the important parameter for citric acid production and varies from strain to strain. A close energetic coupling relation exists between the citric acid production and pH regulation (Guebel and Torres, 2001). The effect of different initial pH of the medium (4.0-7.0) on citric acid production by *A. niger* GCBT-7 was investigated (Fig. 4). The fermentation medium with an initial pH 5.5 resulted in the maximum citric acid production. When the pH was decreased or increased other than 5.5, citric acid production was decreased gradually. It may be due to the fact that at a lower pH, the ferrocyanide ions are more toxic for mycelial growth in molasses medium (Pessoa *et al.*, 1984). A higher initial pH, however, may lead to the accumulation of oxalic acid in the medium (Shadafza *et al.*, 1976). Pessoa *et al*. (1984) reported the maximum citric acid production at pH 6.

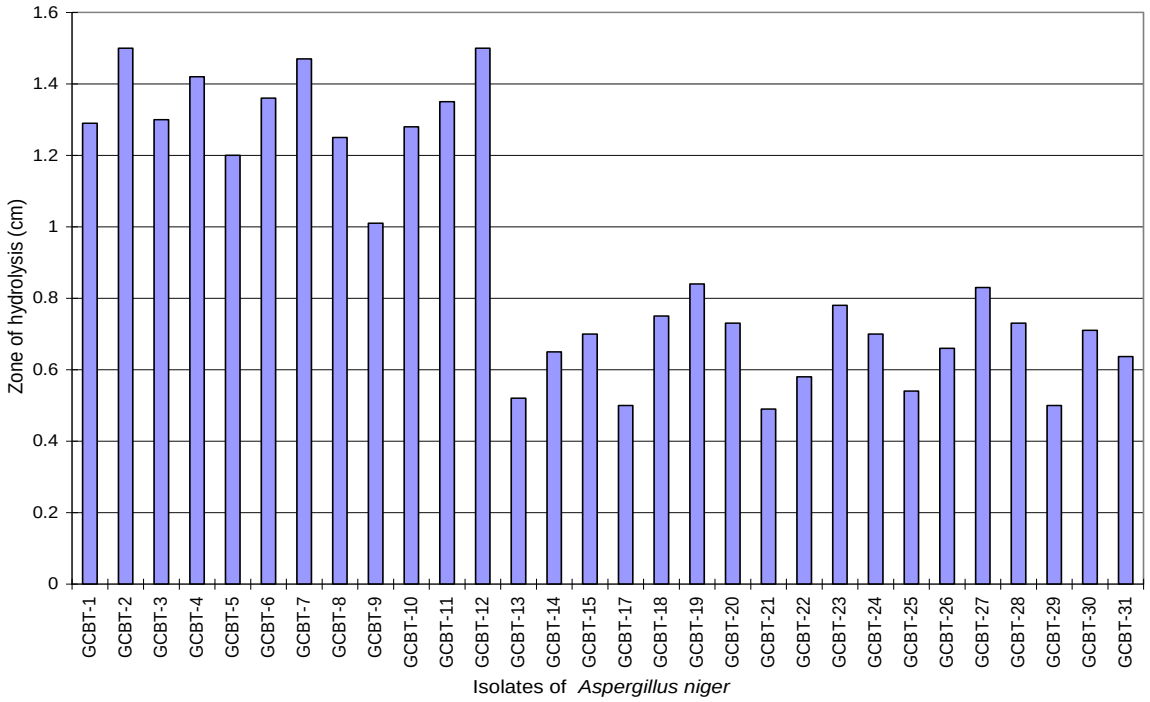
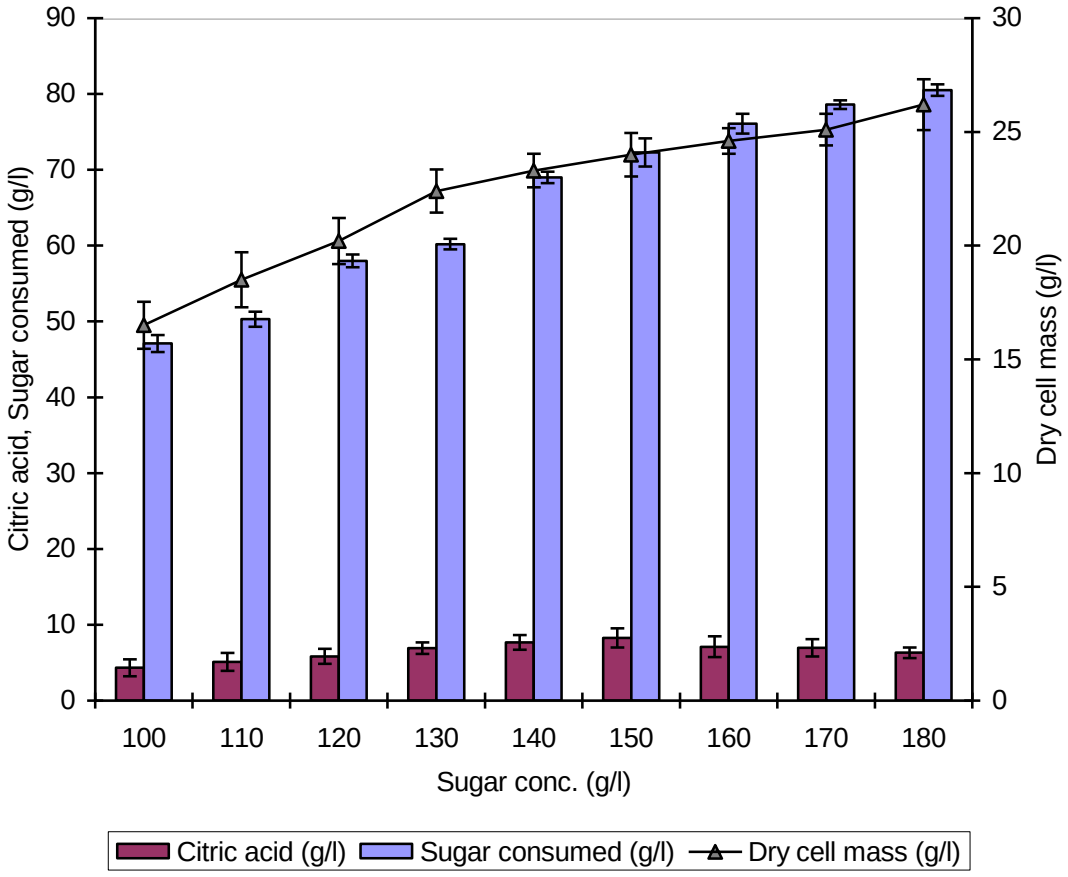


Fig. 1. Qualitative screening of *Aspergillus niger* isolates, using Bromocresol green dye.

Table 1. Screening of *Aspergillus niger* isolates for citric acid production, using cane molasses medium in shake flasks*

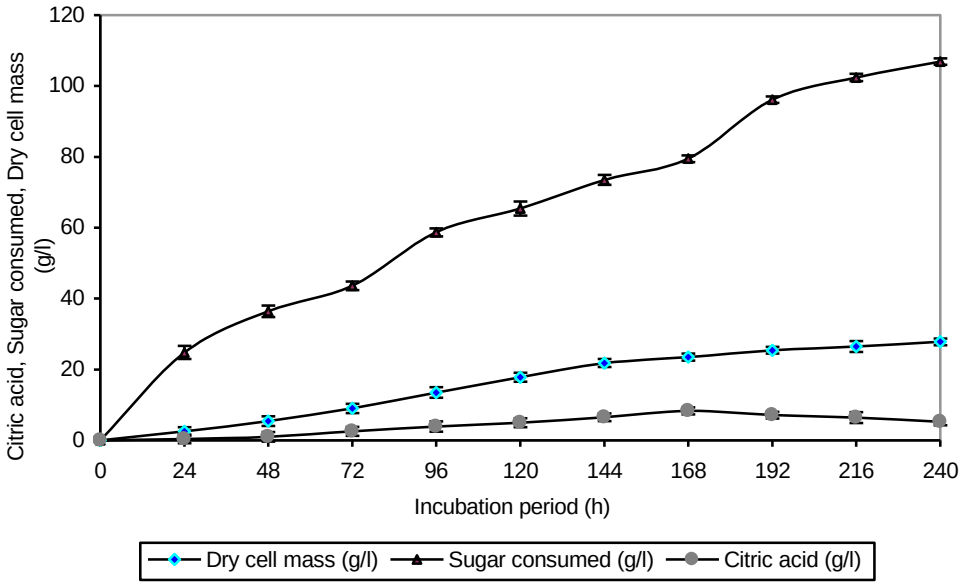
Isolates of *Aspergillus niger*	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
GCBT-1	17.48±0.90	76.43±0.847	4.47±0.942
GCBT-2	21.50±0.75	85.92±0.90	0.85±1.05
GCBT-3	30.52±1.18	89.16±1.07	2.98±1.25
GCBT-4	18.15±1.49	72.42±0.94	6.33±0.86
GCBT-5	24.83±1.13	81.40±0.70	3.03±9.15
GCBT-6	19.33±0.85	72.01±1.23	5.23±1.27
GCBT-7	24.32±1.43	73.89±0.99	8.27±0.95
GCBT-8	15.57±1.25	69.33±0.74	2.20±1.26
GCBT-9	10.95 ±1.48	70.43±1.07	1.63±1.12
GCBT-10	19.47±0.92	75.93±0.85	4.40±0.84
GCBT-11	25.56±0.87	85.20±0.92	6.23±0.79
GCBT-12	18.80±0.76	78.20±1.15	5.33±1.35
LSD	1.055	1.251	0.657

*Initial sugar conc. 150 g/l, fermentation period 168 h, initial pH 6.0, ferrocyanide ion conc. 200 ppm, incubation temperature 30ºC. The ± indicates standard deviation (±SD) among the three parallel replicates.



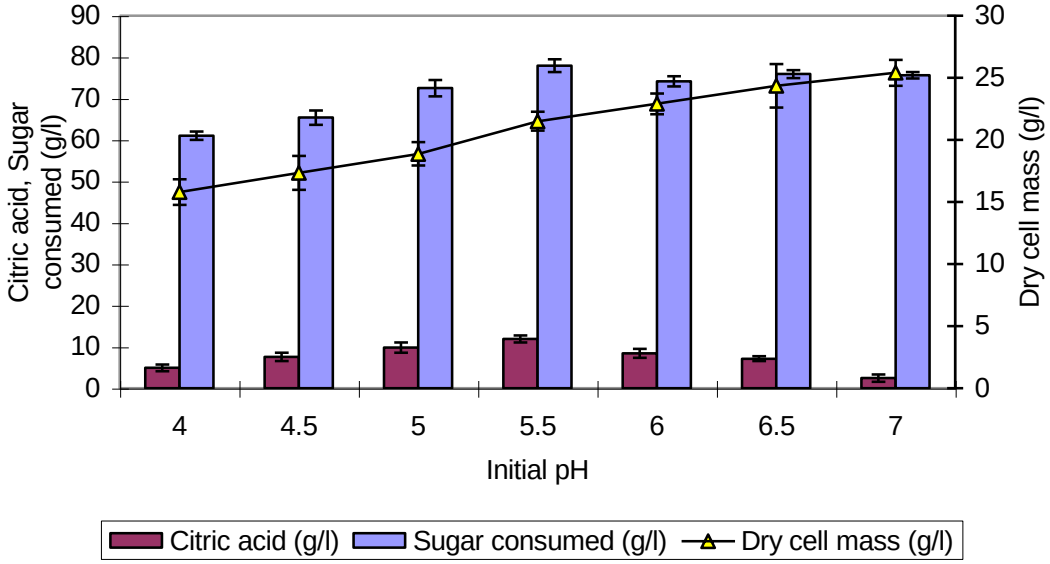
*Bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤0.05.

Fig. 2. Effect of different sugar concentrations on citric acid production by *Aspergillus niger* GCBT-7 in shake flasks*



*Bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤0.05.

Fig. 3. Time course study for citric acid production by *Aspergillus niger* GCBT-7 in shake flasks*



*Bars indicate standard deviation (±SD) among the three parallel replicates, which differ significantly at p≤0.05.

Fig. 4. Effect of initial pH of fermentation medium on citric acid production by *Aspergillus niger* GCBT-7 in shake flasks*

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