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Isolation of *Aspergillus niger* Strains from Soil and their Screening and Optimization for Enhanced Citric Acid Production Using Cane Molasses as Carbon Source

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ABSTRACT

Citric acid is one of the most important metabolic products produced commercially by fermentation using moulds, specifically, strains of *Aspergillus niger*. It is widely used in food and pharmaceutical industry. To enhance its microbial production by fermentation, it is important to search for new strains with more production and their optimization in shake flask using ferrocyanide treated cane molasses as carbon source. Present study describes the isolation, screening and strain improvement of *Aspergillus niger* for enhanced citric acid production in shake flask using ferrocyanide treated cane molasses as carbon source. One hundred and ninety-seven cultures were obtained from different soil samples by serial dilution method using malt extract agar medium. One hundred and twenty nine cultures were isolated out of one hundred and ninety-seven by qualitative analysis (dye method). The best citric acid producing strain was selected by shake flask method using cane molasses. Among all the cultures examined, the strain *Aspergillus niger* GCB-117 gave maximum production of citric acid i.e; 14.17 g/l. This culture was further optimized for cultural conditions i.e; sugar concentration, pH and incubation period. The maximum citric acid was obtained at sugar concentration 150 g/l (14.17 g/l), pH-5.5 (20.3 g/l) and incubation period 168h (20.3 g/l). This study will be a milestone towards commercial production of citric acid on large industrial scale utilizing cane molasses in Pakistan. Moreover the best strain can further be improved by modern mutation techniques and recombinant organisms.

KEYWORDS: A. niger, Citric acid, Cane molasses, Isolation and Screening, Food industry

1. INTRODUCTION

The citric acid is an important commercial product. It was first isolated from lemon and has since been known as a natural plant substance present in many citrus fruits. It is responsible for tort taste of various fruits in which it occurs e.g., lemons, limes, figs, oranges, pineapples, pears and goose-berries. Citric acid has its applications in food, pharmaceutical and biochemical industries. The worldwide demand is generally satisfied by fermentation process. Its global production has reached to 1.7 million tons per year and its annual increasing growth rate is 5% (Kana et al., 2012). The largest amount of citric acid is consumed in food industry using almost 70% of the total production, followed by about 12% in the pharmaceutical industry and 18% for other applications (Rodrigues et al., 2013).

Several microorganisms have been evaluated for citric acid production such as fungi, bacteria and yeast. Various bacteria have been found to accumulate citric acid in their culture medium (Ohmori and Ikeno, 1973; Takayama and Adachi, 1974). The various fungi, which have been found to accumulate citric acid in their culture media, include strains of *Aspergillus niger, Aspergillus awamori, Penicillium restrictum, Trichoderma viride, Mucorpiri formis*and *Yarrowia lipolytica* (Arzumanov et al., 2000). However, *A. niger*is considered as the organism of choice for the production of citric acid because of the fact that this organism has the capacity to utilize varieties of substrates due to its well-developed enzymatic system (Munshi et al., 2013).

Approximately 80% of the world demand for citric acid is supplied through submerged fermentation by the filamentous fungus *A. niger* (Bigelis and Arora, 1991). A submerged process appears to be highly desirable and many articles and patents have appeared in the literature (Kubicek and Roehr, 1977; Obaidi and Berry, 1979; Fiedurek et al., 1996; Vandenberghe et al., 1999). Advantages of submerged method include the choice of a wide range of fermentation substrates usable in the process like glucose, sucrose and cane or beet molasses (Papagianni et al., 1999).

The growth of *A. niger* in the molasses medium is sensitive to ferrocyanide when added during the exponential growth phase (Ogawa and Fazeli, 1976; Walish et al., 1983). The nitrogen requirement of citric acid production is generally met by the addition of inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$,

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KNO_3 and urea (Usami and Takatomi, 1958; Singh et al., 1998). However, the type of nitrogen source and its concentration affect the performance of fungus considerably. The concentration of phosphate in the fermentation medium also has a profound effect on the amount of citric acid produced (Pera and Callieri, 1997). A high concentration of phosphate promotes more growth and less acid production (Khan et al., 1970). In citric acid production the use of methanol and ethanol as stimulant was found to enhance the yield of citric acid (Moyer, 1953; Manonmani and Sreekantiah, 1987; Saha et al., 1999). Among both the most important of these is methanol (Haq, P.B. and Daud, 1995; Pazouki, et al., 2000). The temperature also plays an important role in the production of citric acid. Temperature between 25-30ºC was usually employed for culturing of *A. niger.*

Citric acid fermentation is also very sensitive to pH (Roskosu, and Anenil, 1980). The maximum production of citric acid 19.5% was obtained at pH 5.4 in molasses medium. The appropriate pH is important for the progress and successful termination of fermentation. The optimum pH requirement for maximum citric acid production also depends on the *A. niger* strain used (Pessoa et al., 1984).

In Pakistan, sugarcane is grown in the three zones and is an important and the second largest cash crop of Pakistan grown over an area of 963,000 hectares with an average yield of 47 tons/hectare (Ather et al., 2009). Normally molasses contains about 46% total sugars, 3.0% crude protein, 0.0% fat with 79.5° brix and are used in animal feed as well as for the production of alcohol through fermentation. As it contains high amount of sugars (46%), it must not be considered as a waste material instead researchers should make efforts to produce value added products from this cheaper source of carbohydrates/sugars (Rashid and Altaf, 2008).

Pakistan being an agricultural country is producing both cane and beet molasses more than $1.5x10^6$ tons annually and it is cheap raw material for citric acid production. Citric acid is one of the most useful organic acids in pharmaceuticals and food industries and its worldwide demand is increasing day by day. So, the development of this technology would be highly beneficial. The present work, therefore, is concerned with the isolation and screening of different strains of *A. niger* from different habitats and its improvement by UV irradiation for enhanced citric acid production in shake flask.

2. MATERIALS AND METHODS

A. Isolation of Organisms

One hundred and ninety seven strains of *A. niger* were isolated from different soil samples by the serial dilution method (Clark, et al., 1958). Soil samples were collected from different areas in sterilized polythene bags. The soil suspension was prepared by dissolving one gram of soil in 250 ml conical flask containing 100 ml of sterilized distilled water. The flask was rotated at orbital shaking incubator (Model: 10X400.XX2.C, SANYO, Gallenkamp, PLC, UK) (200 rpm) for 10 minutes to homogenize the soil suspension. Different dilutions such as 10^{-4}-10^{-6} were prepared from this stock solution in sterile distilled water under aseptic conditions. One milliliter of this dilute suspension was then transferred to individual petri plates containing malt extract agar medium. The malt extract agar medium was prepared by dissolving 20.0 g of malt extract in approximately 900 ml of distilled water. The pH of the medium was maintained at 4.8 by 0.1 N HCl or 0.1 N NaOH. The agar (20.0g) was then added. After raising the volume up to 1000 ml, the medium was heated for about 10 min to obtain a homogeneous mixture.

The petri plates were gently rotated clockwise and anticlockwise to facilitate a uniform spreading of soil suspension. The petri plates were then incubated at 30±1ºC in an incubator (Model: MIR-153, Sanyo, Japan) for 3-4 days. The young *A. niger* colonies were picked up and transferred to potato dextrose agar (PDA) 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 up to 1000 ml. This was heated for 10-15 min while constant stirring until a clear solution formed. The pH was maintained at 5.6 by 0.1 N HCl or 0.1 N NaOH. About 4.5 to 5.0 ml of this medium was poured into individual test tubes. The tubes were cotton plugged and sterilized in an autoclave at 15.0 lbs/in^2 pressure (121°C) for 15 min. After sterilization, the test tubes were kept in slanting position (at an angle of about 30º) to increase the surface area.

The PDA slants were then inoculated by transferring a small amount of *A. niger* conidia from the petri plates and incubated at 30°C (4-6 days) for maximum sporulation. The cultures were stored in a cool lab (Model: MPR-1410, SANYO, Japan) at 4°C for further studies.

Screening

B. Dye Method

The qualitative screening of *A. niger* cultures were carried out on petri plates having Czapek-Dox agar medium (table 1). The medium was prepared by dissolving all of the ingredients except agar in approximately 900 ml distilled water. The pH was maintained at 6.0. The agar was then dissolved by heating the medium for about 15 min with

constant stirring. The volume was raised up to 1000 ml with distilled water and sterilized in an autoclave. About 10-12 ml of this medium was poured into individual sterile petri plates and allowed to solidify at room temperature. Approximately 0.5 ml of the conidial suspension was aseptically transferred to each of these petri plates. The plates were rotated clockwise and counter clockwise to spread the suspension uniformly and incubated at 30ºC for 3-5 days. Yellow zones due to acid hydrolysis were formed. On the basis of larger zones the best strains of *A. niger* were picked and transferred to the PDA slants. The cultures were incubated at 30ºC for 5-7 days for maximum sporulation. One hundred and twenty nine isolates of *A. niger* were selected for further screening by submerged fermentation in shake flasks.

Table 1: Composition of Czapek-Dox agar medium, pH = 6.0

Ingredients	Composition (g/l)	Ingredients	Composition (g/l)
Sucrose	30	$FeSO_4$	0.01
$NaNO_3$	2.0	Bromocresol green dye	40.0 ml (1.0 %, w/v)
K_2HPO_4	1.0	Agar	20.0
$MgSO_4.7H_2O$	0.5	Distilled water	1000 ml
KCl	0.5		

The composition contains bromocresol green dye which changes its color on production of citric acid and makes a clear circle around the *A. niger* colony. The relative size of the clear zone gives an indication of the intensity of the strain to produce citric acid.

Fermentation Method

The *A. niger* strains were further screened out for citric acid production by submerged fermentation technique in 250 ml Erlenmeyer flasks.

Inoculum Preparation

The conidial inoculum was used in the present study. Spore from 5-7 day old cultures were used for the inoculation. The conidial suspension was prepared by addition of 10 ml of sterilized 0.005 % (w/v) diocetyl ester of sodium sulfo succinic acid (Monoxal O.T.) solution to the slant culture having profuse conidial growth on its surface. A sterile wire-loop was gently used to break the conidial clumps. The tube was shaken vigorously to make homogeneous suspension. Conidial count was measured by measuring the density of conidia (number of conidia per unit volume) with Haemacytometer, Neubauer improved; precicdor HBG Germany, (Tiefe depth profondeur 0.100mm and 0.0025mm^2 area).

Fermentation Technique

Twenty five milliliter of the clarified sugarcane molasses containing 150 (g/l) sugar at pH 6.0 was added into individual cotton plugged 250 ml Erlenmeyer flasks. The flasks were autoclaved at 15.0 lbs/in^2 pressure and 121ºC for 15 min. After cooling at room temperature, the flasks were inoculated with 1.0 ml of the conidial suspension and incubated at 30°C in a rotary shaking incubator at 200 rpm for 168h. After 168h of fermentation the ingredients of the flasks 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.

C. Molasses Clarification

The cane molasses obtained from Pattoki sugar mills (Ltd) Pattoki of Pakistan, was clarified after Panda et al. (1984). About 250 g of molasses was diluted up to 800 ml with distilled water. Thirty five milliliter of 0.1 N H_2SO_4 were added into it and placed in a water bath at 90±2°C for about 1h. After cooling at room temperature, the medium was neutralized with lime $Ca(OH)_2$ and left to stand overnight after making the total volume 1000 ml with distilled water. Two layers were formed, the upper shiny black layer containing sugar and lower yellowish brown due to the presence of trace metals which was separated from the supernatant carefully and discarded.

ANALYSIS

Mycelial Cell Mass

The mycelial dry cell mass was determined by filtering the culture broth through a pre-weighed Whattman filter paper No. 44. The filtrate was used for further analysis and mycelium was thoroughly washed with tap water and dried in an oven (Model: 1442 A, Memmert, Germany) at 105°C for overnight to calculate the mycelial dry weight after Haq and Daud (1995).

Sugar Estimation

The total reducing sugar was estimated by Dinitrosalicylic acid (DNS) method after Ghose and Kostick (Ghose and Kostick, 1970). A double beam UV/VIS-scanning spectrophotometer (Model: Cecil-CE 7200-series, Aquarius, UK) was used for measuring the % transmittance.

Estimation of Sugar in the Fermented Broth

The sugar concentration in fermented broth was estimated by diluting the filtrate appropriately. Two milli litres 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 measured at 546 nm on a spectrophotometer and the reducing sugar concentration was determined from standard curve.

Citric Acid Estimation

Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method (Marrier and Boulet, 1958).

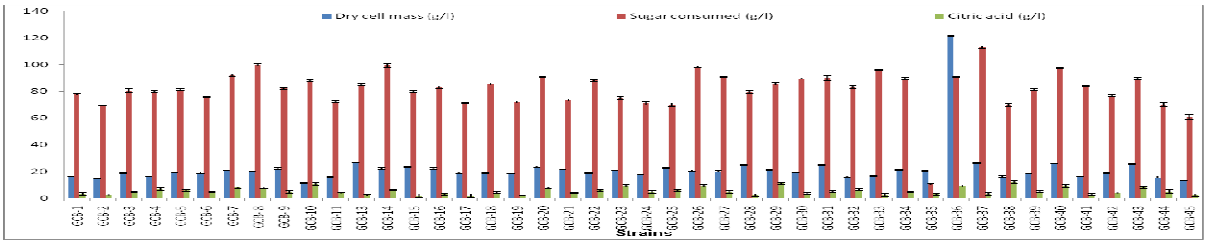
D. Estimation of Citric Acid in the Culture Filtrate

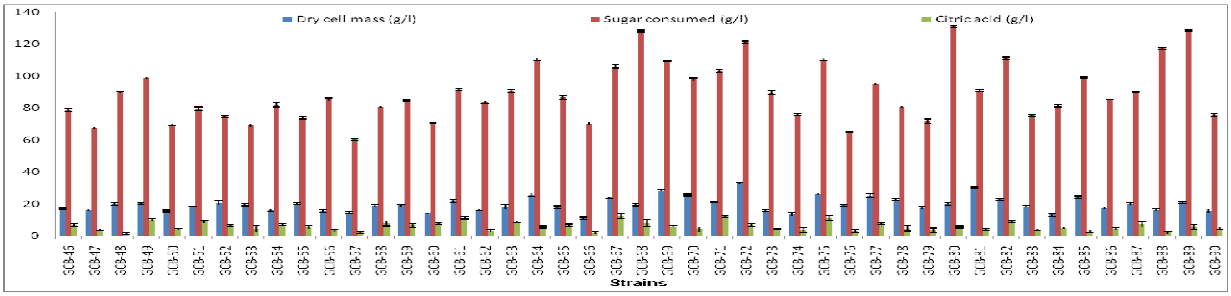
The diluted culture filtrate (1.0 ml) along with 1.30 ml of pyridine was added into a test tube and swirled the tube briskly prior to 5.70 ml of acetic anhydride addition. The test tube was placed in a water bath at 32±0.5°C for 30 min. The optical density was measured at 420 nm using a spectrophotometer. 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).

3. RESULTS

Isolation and Screening of *A. niger* Strains

The data of figure 1 shows the production of citric acid by submerged fermentation. One hundred and twenty nine different strains of *A. niger* were selected after qualitative screening by dye method and were evaluated for citric acid production. Citric acid production ranged from 1.56 to 14.17 g/l. The strain of *A. niger* GCB-117 was found to be the best producer of citric acid (14.17 g/l). Sugar consumption and mycelial dry weight were 86.2 and 19.87 g/l, respectively. The strain was identified according to Onion et al. (Onion, et al., 1986) and having the characteristics.
The colonies on malt extract agar medium spread rapidly. The mycelium was bright yellow in colour and produced dark brown conidial heads. The conidial head was globose. Phialids borne on the metullae were fairly uniform. The conidia were globose. The mould isolates were further sub grouped according to the productivity (table 2). Of all these strains tested, the culture number GCB-117 was selected for further optimization of cultural conditions e.g. sugar, pH and time course of fermentation.





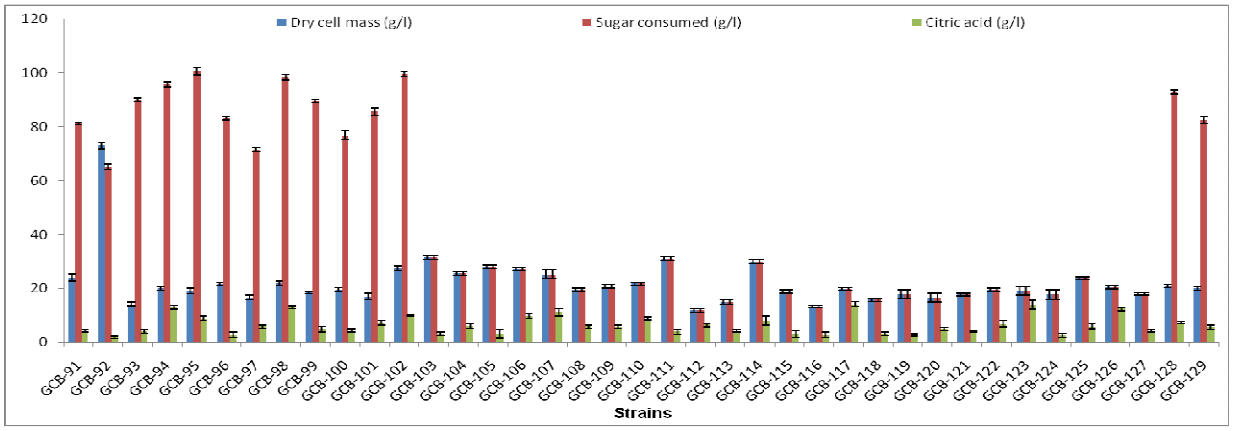


Figure 1(a,b,c): Comparison of isolated and screened *A. niger* strains for the production of citric acid in 50ml shake flask.

Initial sugar concentration 150 g/l, fermentation period 168h, Incubation temperature 30ºC, pH 6.0, potassium ferrocyanide concentration 200 ppm. All the values are the sum means of three parallel replicates. The mean difference is significant at the 0.05 level.

Table 2: Subgrouping of Citric Acid Producing Strains of *A. niger*

Number of Isolates	Range of Citric Acid (g/l)
65	1-4.9
50	5-9.9
14	10-14.17

Table 2: Subgrouping of Citric Acid Producing Strains of *A. niger.*

Range of citric acid production is shown in three groups along with the number of strains. The complete description is given in figure 1(a,b,c).

Effect of Sugar Concentrations on Citric Acid Fermentation by Strain of *A. Niger* GCB-117

The figure 2 shows the effect of different sugar concentrations on citric acid fermentation by isolated strain of *A. niger* GCB-117 in shake flasks. The maximum production of citric acid (14.17 g/l) was observed in the medium containing 150 g/l, initial sugar concentration. The sugar consumption and mycelial dry weight were 85.3 and 14.4 g/l, respectively. The mycelial growth in the medium was in the form of small pellets resulting in better agitation hence improved aeration (or oxygen supply) of fermented broth. Further increase in sugar concentration resulted in gradual reduction in citric acid production. The optimum level of sugar for fermentation was found to be 150 g/l and it was kept constant in further studies.

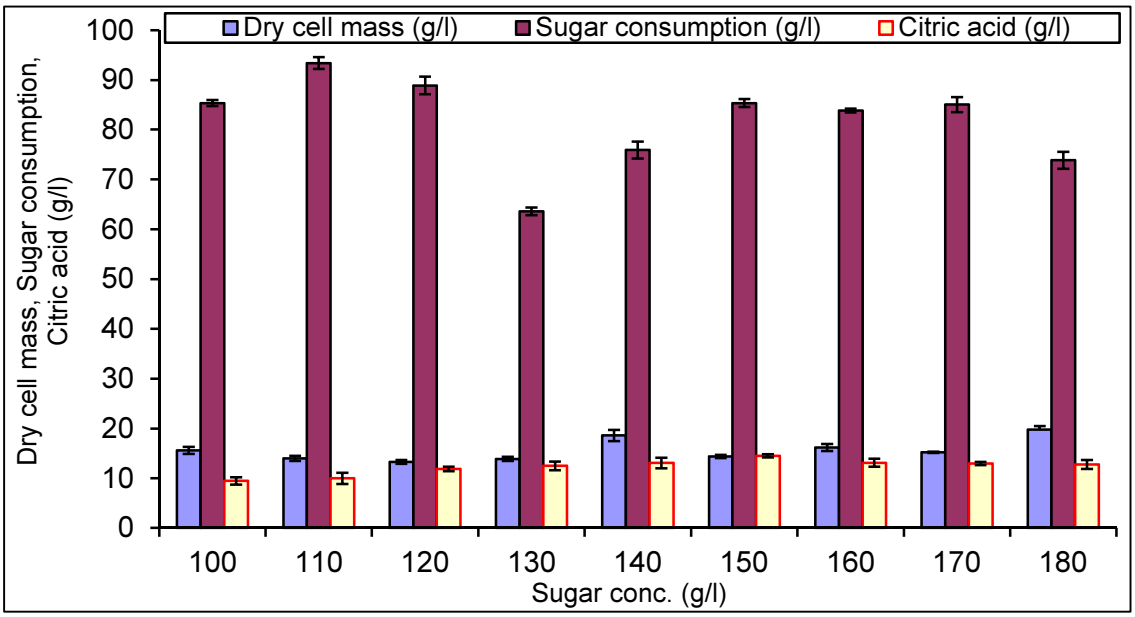


Figure 2: Effect of Different Sugar Concentrations on Citric Acid Fermentation by Isolated Strain GCB-117 of *A. niger* in Shake Flask.

Initial sugar concentration 150 g/l, Incubation temperature 30°C, Initial pH 5.5. All the values are sum means of three parallel replicates. Error bars are based on standard deviation (SD) among the replicates. The mean difference is significant at the 0.05 level.

Effect of Initial pH on Citric Acid Fermentation by Strain of *A. niger* GCB-117

The initial pH of the basal medium has great influence on the mould metabolism. Effect of initial pH of the basal medium on the production of citric acid, sugar consumption and mycelial dry weight by mould culture was investigated (figure 3). Fermentation medium with initial pH 5.5 resulted maximum citric acid production (20.3 g/l). The sugar consumption and mycelial dry weight were 82.3 and 16.3 g/l, respectively. Small pellets were observed in the fermented broth. When the pH was increased beyond 5.5, the production of citric acid was decreased gradually. Therefore, pH 5.5 was found to be an adequate, for citric acid fermentation.

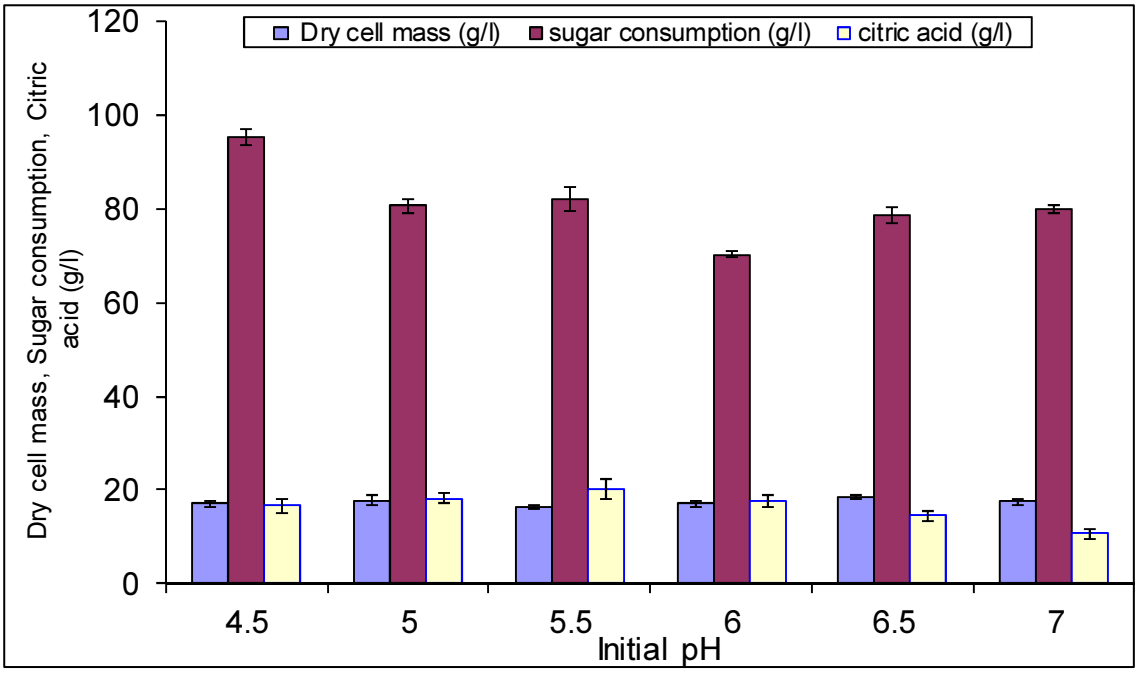


Figure 3: Effect of Different Initial pH on Citric Acid Fermentation by Strain of *A. niger* GCB-117 Shake Flask.

Initial sugar concentration 150 g/l, Incubation temperature 30°C, Initial pH 5.5. All the values are sum means of three parallel replicates. Error bars are based on standard deviation (SD) among the replicates. The mean difference is significant at the 0.05 level.

Time Course of Citric Acid Fermentation by Strain *A. niger* GCB-117

The rate of citric acid fermentation by *A. niger* GCB-117 was carried out in shake flask (figure 4). The fermentation was carried out from 24-216h. After 24h of incubation, the amount of citric acid produced was 3.2 g/l. Further increase in the incubation period resulted in increased citric acid production. However, maximum production (20.0 g/l) was achieved, 168h after inoculation. The sugar consumption and mycelial dry weight were 83.50 and 16.5 g/l, respectively. The mycelial morphology was small round pellets. Further increase in incubation period did not show any enhancement in citric acid production. Hence, optimum time for citric acid production was 168h, after spore inoculation.

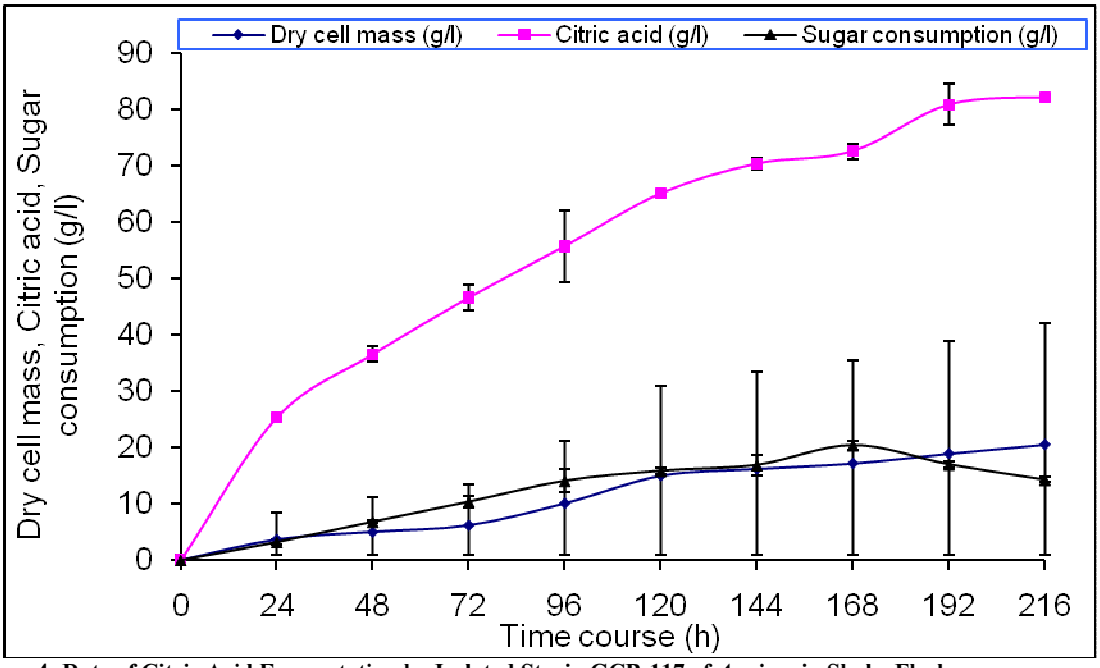


Figure 4: Rate of Citric Acid Fermentation by Isolated Strain GCB-117 of *A. niger* in Shake Flask.

Initial sugar concentration 150 g/l, Incubation temperature 30°C, Initial pH 5.5. All the values are sum means of three parallel replicates. Error bars indicates standard deviation (SD) among the replicates. The mean difference is significant at the 0.05 level.

4. DISCUSSION

Microorganisms have long been considered as harmful entities contributing towards diseases and food spoilage but also playing their role for the welfare of human being (Tortora et al., 2010). Presently, these are microorganisms are being widely used in food industry for production of a large number of fermented food products and at the same time these are also very helpful for conversion of food industrial wastes into value added useful products (Laufenberg, et al., 2003). This capacity of microorganisms to convert large and complex molecules into the simplest one depends upon the type of culture and the growth requirements which include both intrinsic and extrinsic environmental conditions (Lucas, et al., 2007). Selection of a suitable culture to convert the specific type of substrate into useful products thus plays a vital role in fermentation technology (Krishna, 2005). For the production of useful products at industrial level, attempts are being made by the researchers to identify the cheapest and easily available substrates to reduce the cost of production (John, et al., 2006; Farooq, et al., 2012). During primary screening, one hundred and twenty nine isolates were selected, out of one hundred and ninety seven by dye method. Secondary screening, using molasses medium in shake flask, isolated the strain of *A. niger* GCB-117 which gave better results of citric acid i.e; (14.17 g/l). The amount of sugar consumed and mycelial dry weight were 86.2 and 19.87 g/l respectively. The initial sugar concentration plays an important role in determining the amount of citric acid and also other organic acids produced by *A. niger* (Haq, et al., 2001). The present culture produced citric acid

(14.17 g/l) in the medium containing (150 g/l) sugars. Further increase in the concentration of sugar resulted in the gradual reduction of citric acid formation. It might be due to the over growth of the mycelia, which resulted in increased viscosity and mass transfer limitations. Similarly, Kovats (1960) pointed out that a concentration higher than 15-18 %, however, leads to greater amount of residual sugars, making the process uneconomical, while on the other hand a lower concentration <150 g/l of sugar leads to lower yield of citric acid due to the accumulation of oxalic acid.

Initial pH of the basal medium is very essential for the successful fermentation of citric acid (Yasser and Saad, 2012). Effect of different initial pH of the molasses medium on citric acid production was studied and maximum yield (20.3 g/l) was obtained when initial pH of the fermentation medium was 5.5. Any increase or decrease in the pH greatly reduced citric acid biosynthesis. It might be due to that at lower pH, the ferrocyanide was more toxic for the growth of mycelium in molasses medium. This has also been reported by Pessoa et al. (1982). A higher pH leads to the accumulation of oxalic acid as reported by Shadafza et al. (1976).

The optimal time of incubation for maximum citric acid production varies with both the organism and fermentation conditions (Kubicek, 1998). The maximum yield of citric acid (20.0 g/l) was achieved, 168h after incubation. Further increase in incubation period did not enhance citric acid production. It might be due to the decrease in amount of available nitrogen in the fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself, the depletion of sugar contents and heavy metals. In batch wise fermentation of citric acid, the production starts after a lag phase of one day and reaches maximum at the onset of stationary 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, 192h after incubation.

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