ACIDIC PRE-TREATMENT OF SUGAR CANE MOLASSES FOR CITRIC ACID PRODUCTION BY *ASPERGILLUS NIGER* NG-4

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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, different acids such as H2SO4, HNO3 and HCl were used. Their level varied from 1.0N and added in the production medium during the time of clarification. Among all the acids, the maximum amount of citric acid (48.96 g/l) was produced when 1.0N HNO3 pre-treated cane molasses was used as a substrate, then H2SO4 1.0N (37.82g/l) , HCl 0.1N (21.6g/l). HNO3 is more reacted with sugar cane molasses as compare to H2SO4 & HCl.

Keywords: *Aspergillus niger*, Citric acid, molasses, *Penicillium* mold.

I. 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). 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). It is one of the most important bulk-produced organic acids (Wieizorek and Brauer, 1998). It is non-toxic and easily oxidized in the human body (Ma 2000). It can be used industrially for food, confectionary, and beverages, to flavour the drinks, jam, jellies and pharmaceuticals. Its uses depend on three properties acidity flavor 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 6.0×105 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). Some 4.5×105 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 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. The present studies are concerned with the pretreatment of cane molasses for citric acid production by *A..niger* NG-4. Citric acid is a weak organic tribasic acid. It occurs naturally in citrus fruits. In biochemistry, it is an intermediate in the citric acid cycle, which occurs in the metabolism of all aerobic organisms. It is used widely as an acidifier, as a flavoring, and as a chelating agent. (*Apleblat, Alexander (2014).* A citrate is a derivative of citric acid; that is, the salts, esters, and the polyatomic anion found in solution. An example of the former, a salt is trisodium citrate; an ester is triethyl citrate. Citric acid exists in greater than trace amounts in a variety of fruits and vegetables, most notably citrus fruits. Lemons and limes have particularly high concentrations of the acid; it can constitute as much as 8% of the dry weight of these fruits (about 47 g/L in the juices (Penniston KL et al., 2008). The concentrations of citric acid in citrus fruits range from 0.005 mol/L for oranges and grapefruits to 0.30 mol/L in lemons and limes. Within species, these values vary depending on the cultivar and the circumstances in which the fruit was grown.

Industrial-scale citric acid production first began in 1890 based on the Italian citrus fruit industry, where the juice was treated with hydrated lime (calcium hydroxide) to precipitate calcium citrate, which was isolated and converted back to the acid using diluted sulfuric acid. (FrankH Verhoff (2005)1893, C. Wehmer discovered *Penicillium* mold could produce citric acid from sugar. However, microbial production of citric acid did not become industrially important until World War I disrupted Italian citrus exports. In 1917, American food chemist James Currie discovered certain strains of the mold *Aspergillus niger* could be efficient citric acid producers and the pharmaceutical company *Pfizer* began industrial-level production. In this production technique, which is still the major industrial route to citric acid used today, cultures of *A. niger* are fed on a sucrose or glucose-containing medium to produce citric acid. The source of sugar is corn steep liquor, molasses, hydrolyzed corn starch or other inexpensive sugary solutions (*Lotfy* et al., 2007).

In 1977, a patent was granted to Lever Brothers for the chemical synthesis of citric acid starting either from aconitic or isocitrate/alloisocitrate calcium salts under high pressure conditions. This produced citric acid in near quantitative conversion under what appeared to be a reverse non-enzymatic Krebs cycle reaction. In 2007, worldwide annual production stood at approximately 1,600,000 tons (Berovic et al., 2007). The residues generated by this intense agricultural activity represent potential feedstock that could be inserted in diverse production chains instead of discarding them (Soccol and Vandenberghe 2003; Singhania et al. 2009). Agro-industrial wastes are interesting substrates for fermentative processes since they are easily available, rich in carbon and often represent a problem of disposal (Gassara et al. 2010). There are several publications describing bioprocesses to use the wastes such as hulls and bagasse as raw materials to produce ethanol, single-cell protein, mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, among other products (Soccol and Vandenberghe 2003).

Sugarcane bagasse is a lignocellulosic agro-residue generated in high amount by the sugar and alcohol industry in Brazil. This can be achieved by chemical or enzymatic hydrolysis, preceded by appropriate pretreatments that enhance the efficiency of hydrolysis. The aim of the pre-treatment is to separate lignin and break the structure of lignocellulose, and it is one of the most expensive steps in the process of converting biomass to fermentable sugars (Binod et al. 2012). The aim of this review is to describe different pretreatment strategies to promote delignification of the sugarcane bagasse by thermo-chemical and biological processes.

Citric acid is a weak organic acid found in citrus fruits. It is a good, natural preservative and is also used to add an acidic (sour) taste to foods and soft drinks and other food products. Utilization of citric acid includes flavor enhancement, bacterial inhabitant, pH adjustment and as an anti oxidant. *Aspergillus niger* is most commonly used for citric acid production. (Usami, S. 1977 usami s. 1978 & Steinbock, F.A 1991). This is because of the fact that this organism has capacity to utilize varieties of substrates due to its well-developed enzymatic system. (Alexopolous, C.J., 1962). *A niger* is normally a haploid fungus producing white septate hypha which is profusely branched. It produces black mass of conidia, which are found in chain arising from the secondary sterigmata. Citric acid is mainly produced by a fungus *Aspergilus niger* by utilizing starchy and sugar substrates. (Kristiansen, B. and C.G. Sinclair, 1978).

The present investigation was therefore, undertaken with a view to determine the feasibility of using raw and cheap materials such as molasses for citric acid production and optimization under fermentation condition on these substrates. The main raw materials used in citric acid production are carbohydrates such as sucrose and molasses.

Citric acid is considered as one of the important organic acids that has a wide commercialization potential. It has been used by many researchers and in many studies, mainly in solid-state fermentation (SSF), for its ability to live and grow in an environment similar to its natural habitat (Bari et al., 2009; Karthikeyan and Sivakumar, 2010; Dhillon et al., 2011). In the last 3 decades, SSF has gained great interest from researchers and industries as an alternative technique to the traditional submerged fermentation (SmF). The unique characteristics of SSF, using solid materials, stimulated researchers to use waste such as agro-residual and agro-industrial wastes as an alternative to raw materials for the production of citric acid. Karthikeyan and Sivakumar (2010) used banana peel, and Khosravi-Darani and Zoghi (2008) used bagasse. The remarkable demand for citric acid stimulated researchers and industries to look for and modify an economic production process by reducing the cost of the raw materials and the additives.

Besides fungi, it is known that several yeasts produce citric acid from *n*-alkanes and carbohydrates. (Mattey, M. (1999). Especially species belonging to the genera *Candida*, *Hansenula, Pichia, Debaromyces, Torula, Torulopsis,Kloekera, Saccharomyces, Zygosaccharomyces* and *Yarro wia*. During the '60s and '70s oil was cheap, and citric acid was produced industrially from this source by *Candida* sp., including *C. tropicalis, C. catenula, C. guilliermondii* and *C. intermediate*. Papagianni, M. (2007). Although many microorganisms can be employed to produce citric acid, *A. niger* is still the main industrial producer. Despite a long and successful history of producing citric acid, there is not unanimous explanation of the biochemical basis of the process. Kristiansen, B.; Sinclair, C.G. (1978). To address the lack of cycle intermediates consequent to the metabolic dysfunction responsible for the accumulation of citric acid, pyruvic acid produced from glucose is not only decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex, but it is also partially carboxylated to oxaloacetic acid during the idiophase. Kubicek et al (1979).

II. MATERIALS & METHOD

Materials required: H2SO4, HNO3, HCL, Potato dextrose agar (PDA),

Sub culturing of *A. niger*

Potato dextrose agar slants were prepared. *A. niger* from stock culture was streaked / sub cultured on to potato dextrose slant. These potato dextrose agar slants were incubated at room temperature for 5-6 days.

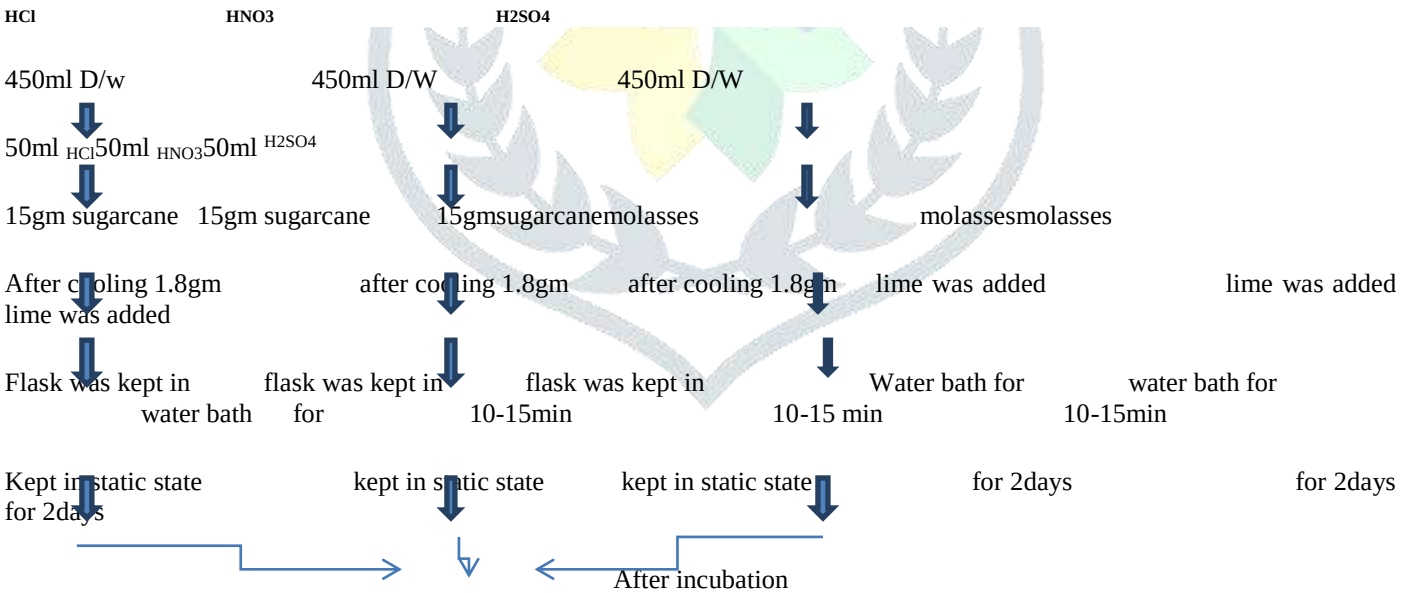
Composition of cane molasses

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.

Molasses clarification

Three flasks containing different chemicals like H2SO4, HNO3, HCL respectively. Cane molasses were obtained from market. The substrate and chemicals were added follows:-





Two layers were formed, the upper shiny black & lower yellowish brown due to the presence of trace metals. The clear supernatants were diluted to desired sugar level.

Preparation of conidial inoculum

Sterile distilled water was added to the *A. niger* slant. Inoculating needle was used to break the conidia. The tube was shacked and mixture of conidial suspension was obtained. The conidial count in 1.0ml of inoculum was calculated to be 1.2×10^6 conidia.

Fermentation technique

50ml of clarified cane molasses medium was added into individual 250ml cotton plugged conical flasks. The conidial suspension was added in each flask. These flasks were kept in a rotary shaker for 7 days.

Assay methods

The culture filtrate of 2ml was taken in a test tube. 2ml Benedict's solution was added to it and was heated till the color to bluish green. Similarly steps were followed for HNO3 and HCl. The color change obtained was turbid yellow color for both.

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.

% Total acid =

1000 x sample of Volume acid of weight equivalent x alkali of normality x Titre × 100

H_2SO_4 = {(78.8×0.1×24) ÷ 50×1000}× 100

= 378240

HNO_3 = {(10.2 × 0.1 ×24) ÷ 50 × 1000 } × 100

= 48960

HCl = {(4.5 × 0.1 × 24) } ÷ 50 × 1000 } × 100

= 21600

- **Citric acid**

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). The % citric acid (w/v) was determined.

% Citric acid = citric acid ×100

sugar used

- Readings O.D. at 450nm

Citric acid = 0.3, HCl= 0.5, HNO_3 = 0.13, H_2SO_4 = 0.12

RESULTS AND DISCUSSION:

Fig. 1 flask containing sugarcane molasses & lime

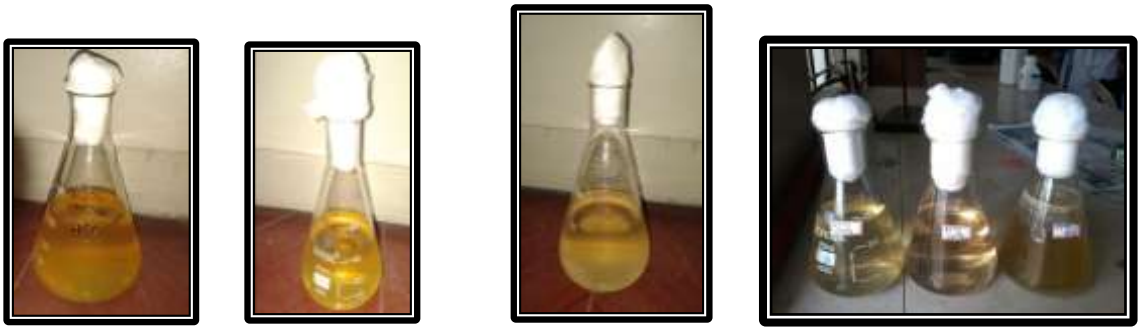


Fig. 2 flask after incubation for 2 days. Fig 3 change in flask after keeping in rotary shaker for 7 days

Pre-treatment of cane molasses with HCL: The effect of pre-treatment of cane molasses with HCl (i.e 1.0 N) for citric acid production by *A. niger* strain NG-4 was also carried out . Maximum amount of citric acid (21.6g/l) was produced when 1.0 N HCl pre-treated cane molasses was used as a substrate.

Pre-treatment of cane molasses with HNO3: The effect of pretreatment of cane molasses with HNO3 (1.0 N) for citric acid production by *A. niger* strain NG-4. HNO3 was added in the production medium during the time of molasses clarification. The maximum amount of citric acid (48.96 g/l) was produced when 1.0 N HNO3 pretreated cane molasses was used as a substrate.

Pre-treatment of cane molasses with H2SO4: The effect of pre-treatment of cane molasses with H2SO4 (1.0 N) for citric acid production by *A. niger* strain NG-4 was undertaken. The amount of citric acid (37.82g/l) was produced when 1.0 N H2SO4 pre-treated cane molasses was used as a substrate.

Fig 4- results found after performing Benedict's test

Fig 5- After titration method

Fig 6- Result compared on addition of pyridine & acetic acid with citric acid

The present study investigates the production of citric acid by *A. niger* with the acidic pre-treatment of sugarcane molasses. The project was done using sugarcane molasses as a substrate for the production of citric acid it has been carried out using different chemicals H2SO4, HCl, HNO3.

Similar work carried out a project in which a minimum citric acid production (15.48g/kg) was obtained in banana waste while supplementation with nitrogen sources led to an increase in citric acid secretion. Among the supplements, peptone gave a maximum citric acid yield (30.8g/kg) followed by ammonium phosphate (42.32g/kg). Nitrogen had been reported to be an important factor in fermentation processes due to an increase in C/N ratio. Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also basic part of cell proteins. In this report it was found that fermentation media for citric acid biosynthesis should consist of substrates necessary for the growth of microorganism primarily the carbon, nitrogen and phosphorus sources.

A different method was done using different substrates and the results from sesamum oil cake and rice chaff having different particle sizes have fermented with the strains of the *A. niger* ATCC 9142, ETGP12 and ETGP18. The yield of the citric acid by *A. niger* ATCC 9142 increased as the particle size of both the substrates increased upto 4 mm, thereafter a decrease in the yield of citric acid was observed. The maximum citric acid yields 94.8 and 102.3 g/kg were observed at particle size 4 mm and the least yield was observed at the particle size 2 mm from sesamum oil cake and rice chaff wastes. The maximum yields of citric acid obtained were 97.5 and 102.4g/kg from sesamum oil cake and rice chaff respectively. The similar set of findings were made from *A. niger* ETGP18

In the present study the citric acid production by the acidic pre-treatment method using *A. niger* was obtained in varied amount like HCl = 21.60g/l , HNO3 = 48.96g/l , H2SO4 = 37.82g/l. larger amount of citric acid was produced in case of HNO3 when compared to H2SO4 & HCl. Though a total good amount of citric acid was produced using sugarcane molasses.

CONCLUSION

Thus it can be concluded, many factors need to be considered by citric acid producers to obtain the economically favorable 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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