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PRODUCTION OF CITRIC ACID FROM THE ETHIOPIAN SUGAR CANE MOLASSES USING ISOLATED, 16SRDNA SEQUENCED *ASPERGILLUS NIGER*[1]

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ABSTRACT

Citric acid (CA) is a weak organic acid found naturally in the citrus fruits with a wide range of applications, including, preservation, flavor enhancement, bacterial inhibition, pH regulation and as an antioxidant. The main goal of this work was to produce citric acid from Ethiopian sugar cane molasses using an isolated fungal species via submerged fermentation process. The organism was identified as Aspergillus niger by 16S rDNA sequencing. In this study, the sugar cane molasses after dilution, is pretreated with 35 mL of 1N H_2SO_4 per liter, boiled, cooled, neutralized with CaO and clarified prior to fermentation. The effect of pH (5, 7 and 9) and the fermentation time is recorded during the process and the yield of citric acid by the fungal species is determined.

Keywords: *Aspergillus niger, 16S rDNA, Cane Molasses, Citric Acid, Submerged Fermentation*

1. INTRODUCTION

Sugar cane molasses is a black, viscous liquid by-product of refining sugarcane into sugar. It is used as a substrate for the production of ethanol and citric acid due to its high reducing sugar content which is about 62%. This content may vary based on the amount of sugar, method of extraction and age of plant (cane). Citric acid (CA) has more health and economic benefits [1]. They exist in two forms, monohydrous and anhydrous, which differs in their degree of hydration [2].Conventional citric acid production is carried out in three ways, from the citrus fruit extracts, chemical synthesis and by using carbohydrate source material such as sugar cane molasses, and other fruit peels.

Of all the methods, fermentation is the most economical method for producing citric acid and is a predominant way of producing of citric acid as it accounts for about 90% of world production. Though all the three types of fermentation processes have been widely employed, including the submerged, surface fermentation and solid state fermentation [3,7], in the present study submerged

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fermentation was used to produce citric acid due to the liquid nature of the substrate, the sugar cane molasses. Submerged fermentation technology has been reported by several workers as an attractive process to produce citric acid due to its advantage of low cost, short period and high yield. There are many sugar manufacturing industries in the country, which provide cheap and ample cane molasses making the production of citric acid reliable and consistent. The most important aspect during the citric acid production is the clarification of the raw molasses that comes from the sugar milling factories and its pretreatment with sulfuric acid to form complex compounds of trace elements in the molasses and thereby removing them[4,8].*Aspergillus niger* is one of the most common species used to utilize starchy and sugar substrates like molasses and converted into different products . It is the most promising microorganism used in production of citric acid compared to other microorganisms due to its ability of utilizing starchy and sugar substrates [5]. The production of citric acid using submerged fermentation depends strongly on an appropriate strain and on operational conditions such as aeration, type and concentration of carbon source, nitrogen and phosphate limitation, pH, concentration of trace elements and morphology of the fungal species.

2. EXPERIMENTAL SECTION

2.1. Microorganism-Inoculation and culture conditions

The fungus used in this study was obtained from the Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia. The isolates were initially grown on Potato Dextrose Agar at 328 K for 24 h. The fungus was further sub-cultured on the Potato Dextrose Agar at regular intervals and incubated at 313 K. For further propagation, the minimal medium composition employed in mg/L was, 0.223 NH_4NO_3, 0.1 K_2HPO_4, and 0.023 $MgSO_4.7H_2O$. Cane molasses was employed as the chief carbon source. All the submerged fermentations were carried out using the isolated fungus. The strain was maintained on PDA slants at 30°C for 6 d. The strain was prepared as conidial suspensions by washing slant cultures with 5 mL sterilized water. Spore suspension was counted at 25×10^6 spores/mL by Haemacytometer. All the trials were carried out in 250 mL Erlenmeyer flasks containing 100mL of molasses, at pH between 5 and 9[6]. The flasks were sterilized prior to the inoculation with three milliliters of prepared spores and incubated at a temperature of 28°C on a shaker incubator at100 rpm for 11 days in succession.

2.2. Feedstock-Molasses pretreatment

Cane molasses contains the following contents: water, 20%, sugar, 62%, and non-sugar, 10%, and inorganic salts (ash contents), 8%. It is a blackish homogenous liquid with high viscosity[20]. Ash contents include ions such as Mg, Mn, Al, Fe and Zn in variable ratios. Sugar content was diluted to 24% with deionized water. This was followed by the addition of 35 mL of 1N H_2SO_4 per liter, boiling for half an hour, cooling and neutralizing using the lime-water (CaO). The contents were then left to stand overnight for clarification [7]. The clear supernatant liquid was further diluted to 12-24 % sugar

using deionized water.

2.3. Process variables for citric acid production

Citric acid production by fermentation can be divided into three phases, which includes preparation and inoculation of the raw material, fermentation, and recovery of the final product. After the pretreatment of the molasses, nutritive salts (like ammonium nitrate) are added and it is diluted with distilled water to make a solution [8]. This solution is sterilized and after cooling down to 30°C, it is transferred to a sterilized submerged fermenter and inoculated with the spore suspension of *Aspergillus niger*. pH of the substrate (solution) was adjusted to 5.5-5.9, and the temperature 28°C, the most suitable for the germination of the conidial aggregation. This was followed by the addition of nutrients and microorganism to the bioreactor. Then the parameters such as pH and temperature were adjusted to an initial value of 2.5-3.0 and 28°C respectively. The fermenter is then aerated, when a thin film of mycelium is created in 24 h (germinating period). This was followed by the production period, when the mycelium gets stronger, and the temperature of the fermented solution rises[9]. During this phase, the exothermic citric acid production is initiated by the environment.

3. RESULTS AND DISCUSSIONS

3.1. Microscopic observation and molecular testing

The isolate was identified based on the colony morphology, microscopic observation and molecular identification [12]. The fungus was identified as *Aspergillus niger* based on the production of clear carbon black /brown spores from the biseriate phialides. The colonies were fast-growing, whitish to blackish or brownish, and usually thick [13]. The morphology of the isolate was examined on potato dextrose agar (PDA in the dark. Colonies on PDA were fast growing with sporangiophores measuring 2.3 to 3.6 mm long and 1.2 to 2.9 mm wide after 3 days. DNA was extracted from the hyphae of a 36 h culture on PDA slants and suspended in UltraPURE distilled water in 2 ml Eppendorf tubes, each containing one sterile 4.5-mm steel shot pellet.

3.2. Fungal strain identification

For the nucleotide sequence analysis, fungal genomic DNA was purified using the Fungi Genomic DNA Isolation Kit (MTK 08) (Modern Science Co., Nasik). The fungal primer pairs annealing at the 50 and 30 end of the 18S rRNA, 50-GTAACCCGTT-GAACCCCATT-30 and 50-CCATCCAATCGGTAGTAGCG-30, respective-ly, were used for amplification. The PCR was run for 35 cycles in a DNA thermal cycler (Thermal Cycler Applied Biosystems 2720, USA). Amplified PCR products were then analyzed in a 1% (w/v) agarose gel and purified. Purified products were cloned and subsequently sequenced using an automated DNA sequencer (ABI 3130 Genetic Analyzer, USA). The 16S rDNA sequence obtained was compared with the sequence obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI)[12]. The phylogenetic analysis of the

strain, using its nucleotide sequence data showed that this strain had the highest homology of 98% and 99% with the *Aspergillus niger* strains, *Aspergillus niger* SH-2 and *Aspergillus niger* ATCC 10864, respectively. Based on the evolution distance and partial sequencing, this strain isolated was identified as *Aspergillus niger*.

3.3. Citric acid production

After the citric acid production by the submerged fermentation, the fungal mycelium is separated from the solution, washed to prevent loss of considerable quantity of acid contained by the mycelium. After the hydrolysis, the liquid fraction of the hydrolysate samples were analyzed for their reducing sugar content was determined using the phenol-sulfuric acid method. The absorption values of the samples were recorded at 490 nm on a spectrophotometer [3]. The acid solution is then separated from the mycelium and is reconditioned through filtering to remove the by-products such as oxalic acid. The calcium citrate is precipitated using lime and then the resulting solution is filtered. The resulting solution is decolorized batch-wise by the addition of activated carbon. The percentage of citric acid produced by *Aspergillus niger* is then determined by the titrimetric method[4]. After the decolorization, the solution is concentrated by evaporation and crystallized at a temperature of 22-26°C to form a white monohydrate citric acid powder as the final product.

3.4. Analysis of total reducing sugar content

In this study, the amount of reducing sugar in the molasses was investigated. The total sugar content of molasses samples was determined using phenol sulfuric acid method. During the phenol sulfuric acid method, the reducing sugar content in the samples is dehydrated due to the reaction with sulfuric acid and produced furfural derivatives[3]. Further reaction between furfural derivatives and phenol develops a detectible Yellow-Orange color. The concentrations of unknown sugar content of samples were determined from the standard curve of glucose. The resulting molasses substrate was subjected to fermatation by the *Aspergillus niger.*

3.5. Analysis of citric acid content

The fermented samples were subjected to the assay for citric acid by the titrimetric method using phenolphthalein as an indicator. The filtrate obtained is titrated against an alkali of known strength using phenolphthalein as indicator. The end point is the formation of pale pink color. The volume of alkali used for neutralization is used to find the normality and the percentage of acid in the sample. In this study, a solution of 2.1g citric acid per 100ml distilled water is prepared. And from the prepared solution, 10 mL of solution was pipetted in to a conical flask, with an addition of 2-3 drops of indicator, titration was carried out against 0.1N NaOH in the burette till a pale pink color was formed[4]. The titration was repeated till expected value was obtained. During the sampling, a solution was prepared using 5 mL of the sample from each test tubes of citric acid per 20 mL distilled water. From the prepared solution, 10 mL of solution was pipetted into a conical flask and 2-3 drops of

indicator were added and titrated following the procedure previously mentioned. The percentage of citric acid produced is then calculated.

$$\text{Normality of Citric acid} = \frac{\text{Normality of NaOH x Volume of NaOH}}{\text{Volume of Citric acid}}$$

$$\text{\% of Citric acid} = \frac{\text{Normality of citric acid x Equivalent wt of Citric acid} \times 100}{\text{Volume of Citric acid}}$$

3.6. Effect of pH on the citric acid production

In this study, a considerable amount of citric acid was produced using molasses as a carbon substrate by the submerged fermentation from the molasses containing about 62% of sugar. Stoichiometrically, by the conversion of 100 g sucrose with oxygen, 123g monohydrate or 112g anhydrous citric acid can be obtained. Biochemical conversion of sucrose into citric acid by the microorganism is shown below,

$$C_{12}H_{22}O_{11}+4.95O_2+0.133NH_4NO_3 \longrightarrow 1.56CH_{1.72}O_{0.55}N_{0.17}+3.54CO_2+5.32H_2O+1.15C_6H_8O_7$$

During the fermentation process, the pH of the medium was found to decrease initially at non regular intervals, from the initial pH of 7.0 to 4.32 on the eleventh day, indicating the acidity of the media and more evidently the citric acid stable pH (Table 1). Figure. 1 shows the effect of fermentation pH on the citric acid production.

Table 1. Data collected throughout the fermentation process

Day	Biomass weight (g)	Citric acid(%)	Initial pH	Final pH
3	3.42	24.00	7	6.40
5	9.60	28.88	7	5.90
7	1.98	38.40	7	4.60
9	5.29	67.20	7	4.52
9	1.64	26.88	5	4.78
9	4.33	39.55	9	5.09
11	4.42	30.72	7	4.32

The production of citric acid increased exponentially until the day 9, at which maximum yield was observed. But as the fermentation time exceeded the day 9, the yield decreased due to the possible

conversion of Citric acid into its byproducts. The production of citric acid was maximum at pH 7, implying the stability of the product at the favorable pH value of around 7[10].

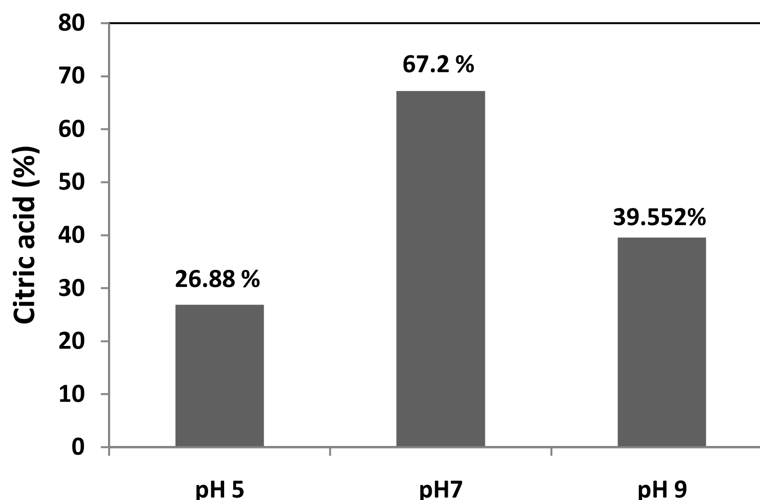

Fig.1. Effect of pH on the citric acid production

In the present batch-wise fermentation of citric acid, the production started after the lag phase of 1 day and reached maximum at the onset of stationary phase or late-exponential phase. Further, it was observed that there was no enhancement in the citric acid production during the increase in the incubation period. It may be due to the age of fungus used and depletion of sugar contents in the culture broth. The acid production was found to start at the initial stage of the idiophase (between 80-120 h) of fungal growth. At this stage, the pH was five. The yield gradually increased and reached to the maximum at the late idiophase (180-220 h). In this stage, the pH must be below 3 in order to suppress oxalic acid and gluconic acid formation. The acceleration of fermentation can be explained by the higher starting biomass concentration in fermenter and by the adaptation of biomass to very high osmotic pressures[11].

3.7. Effect of Temperature on Citric acid production

Temperature plays an important role in the citric acid production. Temperature between 25°C and 30°C is usually employed for culturing the *Aspergillus niger.* The optimum temperature for citric acid production is 28°C, but during the citric acid production, the temperature of the medium increases above 30°C and the biosynthesis of citric acid decreases. This may be due to high temperature, which causes the denaturation of enzyme-citrate synthase and accumulation of other by-products such as oxalic acid. In addition to this, enzyme catabolite repression, could be a possible inhibitor[8].

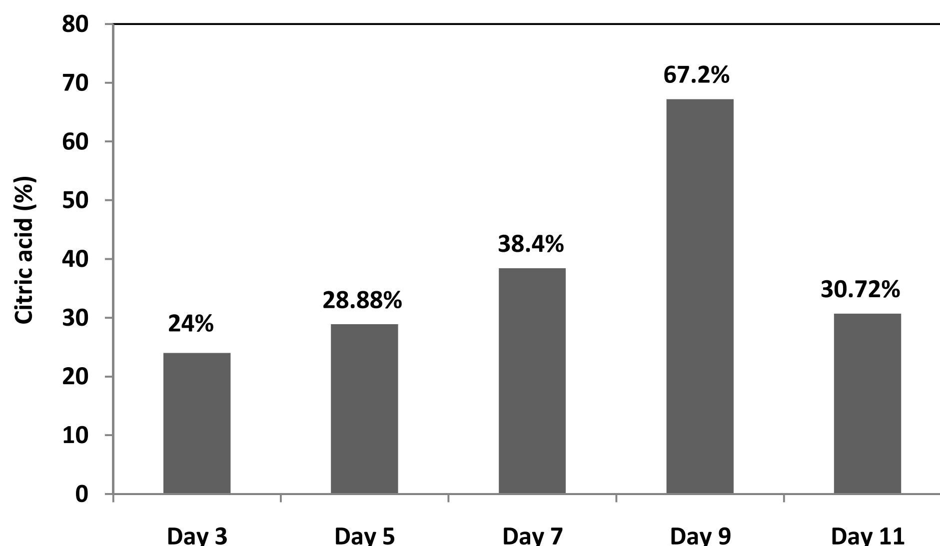

Fig.2. Effect of fermentation time on the production of citric acid

Nitrogen is another limiting factor in the citric acid production. Nitrogen is usually supplied in the form of ammonium nitrate, which is completely metabolized during fermentation periods. Citric acid starts to appear when the nitrogen concentration falls below a low limiting value[6,11]. Figure. 2 shows the effect of fermentation time on the citric acid production at a pH of 7. Earlier studies have reported that the factors affecting citric acid production by fermentation includes, the nutrient composition of the media, environmental conditions, deficiency of manganese, types and concentration of sugars, chelating effect of metal ions, ammonium nitrate and aeration[10]. The optimum time of incubation for maximum citric acid production varies with the organism and fermentation conditions used.

4. CONCLUSIONS

The study shows that the concentration and type of molasses, influences the yield of citric acid produced by *Aspergillus niger*. In the controlled production medium, the initial pH of 7 was found to decrease to 4.32 during fermentation confirming the production of citric acid. Sucrose in the molasses is the substrate responsible for the citric acid production in this medium. *Aspergillus niger* utilizes sucrose and produces 67.2% citric acid. Based on our study, the maximum amount of this citric acid was produced in 9 days at the pH 7.

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