

Production and Optimization of Citric Acid by *Aspergillus Niger* Isolated from Rotted Onion Bulb

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Abstract: Citric acid is one of the most valuable organic acid and is generally used in different industries. Apart from its consumption as a food additive, citric acid is also considered a vital component of various pharmaceuticals, synthetic detergents, cosmetics, and many other valuable products. It is predominantly produced by *Aspergillus niger*. The study was carried out to explore the potential of *Aspergillus niger* isolated from rotted onion bulb for citric acid production. The isolate was screened for citric acid production in Czapekdox agar incorporated with 1% bromocresol green indicator and was subjected to citric acid production in rice extract media. The effect of various physical and chemical parameters such as incubation time, temperature, pH, effect of different carbon sources and effect of different concentrations of methanol on Citric acid production by the isolate from onion was analyzed. The maximum amount of citric acid production was recorded at incubation time of 96hrs (1.15 gm/l), temperature-25°C(1.06 gm/l), pH-8.0(1.98 gm/l), carbon source Dextrose (1.21 gm/l) and methanol concentration [1% (v/v)].

Keywords: *Aspergillus niger*, Citric acid, Czapek dox agar

1. INTRODUCTION

Citric acid (C₆H₈O₇, 2-hydroxy-1,2,3-propane tricarboxylic acid), is the most adaptable organic acid and the common metabolite of plants and animals [1]. Citric acid derived its name from the Latin word citrus, and it was first isolated from lemon juice in 1784 by a Swedish chemist Carl Scheele [2]. The molecular weight of citric acid is 210.14 Da. It is used as a natural preservative in food materials and also used to add a sour taste to foods and drinks. The low toxicity and higher solubility characteristic of citric acid make it suitable for use in food, pharmaceutical and detergent industries [7]. Many bacteria, as well as fungi, produce citric acid but *Aspergillus niger* is a more preferable microorganism due to ease in handling, genetic stability, ability to utilize a variety of substrate and produce a large amount of citric acid. *Aspergillus niger* is a ubiquitous fungi and alone credit with the production of over 1 million tonnes of citric acid normally [2]. Though many chemical methods were developed for citric acid production, better successes were achieved by using microbial fermentations and nowadays this method has become an ultimate choice for the commercial production over chemical synthesis.

At an industrial level, the production of citric acid can be carried out using three different ways: by submerged fermentation, surface fermentation, and solid - state fermentation or Koji process. Citric acid production is mostly performed by submerged fermentation using *Aspergillus niger*. Approximately 80% of the world's citric acid is done by using submerged fermentation [7].

Nowadays the increasing demand for citric acid in industries, it has become more important to search for the cheap raw material sources from agricultural wastes for its production. Common agricultural residues such as sugarcane molasses, potato residue, banana peel, wheat bran, corncobs have been exploited as a raw material for the citric acid production. By using the agricultural waste for citric acid production, the industrial sector has been encouraged to waste management, reduction in the expenses and high yield [5].

Due to the rising demand for citric acid, there is need to investigate different aspects of fermentation and effects of various environmental parameters on citric acid productivity and yields to meet the ever-increasing demand for the commercially important metabolite. From 2008 the citric acid demand was found to be increasing by 5% yearly [1].

Citric acid production can be improved by optimizing the fermentation parameters such as initial substrate concentration, initial pH, nutrient concentration, additives, incubation period, fermentation temperature, etc. The optimal conditions vary depending on the species and substrates [1].

Recently major production of citric acid was conducted using microbial fermentation as it was economical and easy to handle. Citric acid has multiple uses in food and beverage industries as a flavor enhancer and antioxidant agent. It also has other industrial uses such as pharmaceuticals, cosmetics, and various chemical industries.

2. MATERIALS AND METHODS

2.1 Sample collection

The fungus was isolated from the rotted onion bulb.

2.2 Isolation and identification of the fungus

The spore suspension was prepared and 0.1ml of this suspension was streaked on Sabouraud's agar plates. The plates were incubated for 96hrs at 37° C. After incubation, the growth was observed and the isolated fungus was identified using morphological characteristics and the growth was determined microscopically to confirm its purity by lactophenol cotton blue staining.

2.3 Screening of isolates

Czapek dox agar medium containing Bromocresol green as an indicator using the plate method was used for the screening of citric acid producers. The surface of the medium was point inoculated with the spores of individual fungal isolate and incubated at 37°C for 3-5 days. The colonies showing yellow color zones around them were selected as citric acid producers.

2.4 Inoculum preparation

The inoculum was prepared by adding 10ml distilled water containing the 2 drops of 0.1% Tween 80 to the 5 days old culture. A sterile wire loop was used to remove the clusters of spores under the sterilized condition and then mixed by shaking thoroughly to prepare a homogenized spore suspension.

2.4 Fermentation using rice extract media

Rice(100gm) was boiled in 250 ml of distilled water for 3-5 min and the rice extract solution was separated from the rice using sieve cloth. The rice extract media (200 ml) was prepared using Rice extract(70 ml), KH_2PO_4 (0.24gm), MgSO_4 (0.32gm), distilled water (130ml) and pH 7.0 and autoclaved at 121°C for 15 min. After cooling the flasks were inoculated with 1ml of fungal spore suspension, following incubation at 25°C for 4 days on shaking water bath.

2.6 Determination of citric acid

Citric acid was determined titrimetrically using 0.1N NaOH and phenolphthalein indicator. In this procedure, 20 ml of fermented broth was taken into flask. 2-3 drops of phenolphthalein indicator were added and titrated against 0.1N NaOH. The endpoint was noted when the fermented broth changes from colorless to pink color, and then it was calculated using the formula

1] Normality of citric acid=

Normality of NaOH × NaOH volume ÷ volume of sample

2] Citric acid concentration in %=

Citric acid normality × 100 × 96 ÷ Volume of sample

2] Citric acid concentration in gm/l =

Titre × 100 × 10 × 0.0064 ÷ Volume of sample

The citric acid equivalent factor is 0.0064g/l citric acid. Total citric acid was reported in gm/l.

2.7 Qualitative analysis for citric acid by TLC

Qualitative detection of citric acid in the filtrate obtained after fermentation was done by thin layer chromatography (TLC) for confirming the production of citric acid by the fermentation process. A precoated silica gel plate of 0.25mm thickness was used to detect the production of citric acid. For the development of chromatogram, methanol and ammonium hydroxide (4:1 v/v) was used as a mobile phase. The TLC plate was observed under U. V. Transilluminator to elucidate the presence of citric acid. The retention factors (Rf) value of citric acid was calculated by the following formula :

Distance travelled by solute

Rf value =

Distance travelled by solvent

2.7 Recovery process

After completion of the fermentation process, the fermented broth was filtered for the separation of pellet form of fungal culture. Lime (CaOH_2) was added to the fermentation broth to allow the precipitation of citric acid in the form of calcium citrate. After that, the precipitate was treated with dilute sulphuric acid (H_2SO_4) to obtain the precipitated solution and then filtered. The precipitated solution containing citric acid was purified using carbon granules. The solution obtained was then evaporated for getting purified citric acid in crystal form.

2.8 Optimization of citric acid production

The physical and chemical parameters were selected for the optimization of citric acid production: incubation time, temperature, pH, the effect of different carbon sources and effect of different concentrations of methanol. Each variable was optimized by varying only a single parameter at a time i.e. incubation time (24 hrs, 48 hrs, 72 hrs, 96 hrs, and 120 hrs), reaction temperature (20°C, 25°C, 27°C, 30°C and 37°C), reaction pH (5,6,7,8 and 9), different carbon sources (dextrose, lactose, fructose and mannitol) and different concentrations of methanol (1%, 2%, 3%, 4% and 5%).

3. RESULTS

3.1 Identification of Isolate

In the present study, the isolate was isolated from the rotted onion bulb. The isolate was purified and examined microscopically to show its purity and characterized by its cultural characteristics. The isolated fungus was identified by observing morphological characteristics and lactophenol cotton blue staining and confirmed as *Aspergillus niger*. After screening on Czapek dox agar

medium containing Bromocresol green as an indicator yellow color zone was observed around the colonies of isolate and were determined as citric acid producers.

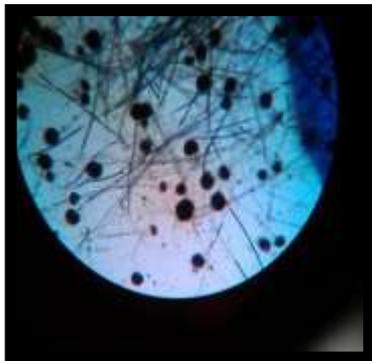


Fig-1: Lactophenol cotton blue staining



Fig-2: Primary screening on Czapek dox medium containing bromocresol green

3.2 Production of citric acid

Citric acid production was carried out using rice extract media. The results showed that rice extract media proved to be a better substrate for citric acid production and hence was selected for the further stages of the experiment. The citric acid produced using rice extract media was 1.47 gm/l.



Fig No- 3: Fermentation of citric acid

3.3 Determination of citric acid concentration using Titration method

This is one of the most widely used methods to confirm the production of citric acid. 20 ml of fermentation broth was taken from the fermentation medium and 2 to 3 drops of phenolphthalein indicator was added and it was titrated against 0.1 N NaOH solution. The maximum production of citric acid in rice extract media was determined (11.04%).

3.4 Qualitative analysis of citric acid by TLC

The citric acid produced was confirmed by performing Thin layer chromatography (TLC). The retention factors (Rf) value was calculated based on the mobility and size of citric acid molecules. The Rf value of citric acid produced by *Aspergillus niger* was 0.74



Fig - 4 Thin Layer Chromatography

3.5 Recovery of citric acid

Citric acid was recovered from fermentation broth using the precipitation method. The obtained citric acid was purified by using activated charcoal.

3.6 Optimization of Citric acid production using *Aspergillus niger* isolated from rotted Onion.

The various physical and chemical parameters were examined for citric acid production. The effect of different kinds of incubation time was tested on citric acid production. The maximum amount of citric acid production by *A.niger* was observed in 96 hrs of incubation time (1.3 gm/l). It was noted that as the incubation time was further increased the production of citric acid was decreased [Chart-1]. Among the various temperatures examined, the maximum production of citric acid was obtained at 25°C (1.06 gm/l) and the further increase in the temperature lowered the citric acid production [Chart-2]. The effect of pH on citric acid production by *A.niger* showed maximum production at pH 8 (1.98 gm/l) and the increase in pH of medium reduced the production of acid [Chart-3]. About 1.21 gm/l of citric

acid was produced by using dextrose as a carbon source, so it can be said as dextrose is the best-suited carbon source for citric acid production [Chart-4]. The effect of different concentrations of methanol was examined on the production of citric acid. The maximum citric acid was recorded in 1 % (v/v) methanol concentration and the further increase in concentrations of methanol decreased the production of citric acid[Chart-5].

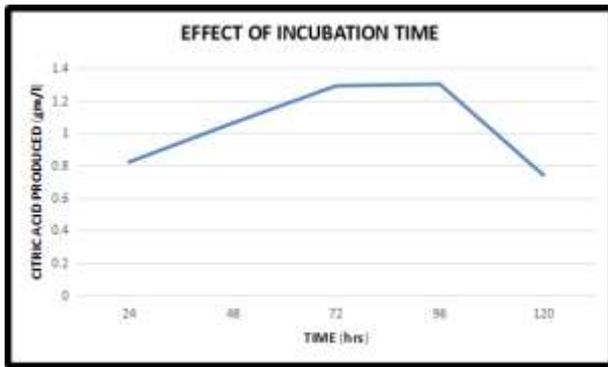


Chart-1: Effect of incubation time on citric acid production.

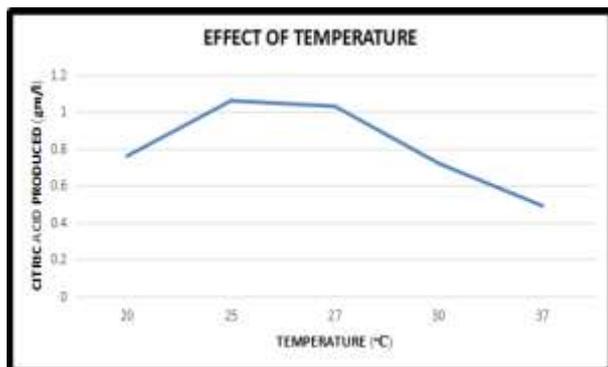


Chart-2: Effect of temperature on citric acid production

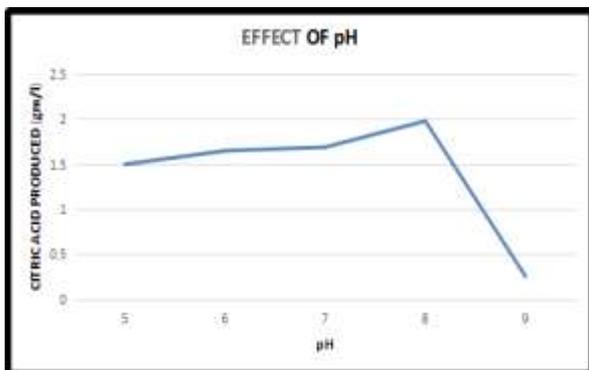


Chart-3: Effect of pH on citric acid production

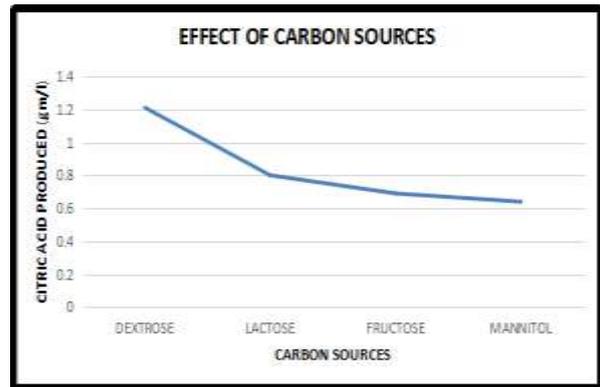


Chart-4: Effect of various carbon sources on citric acid production

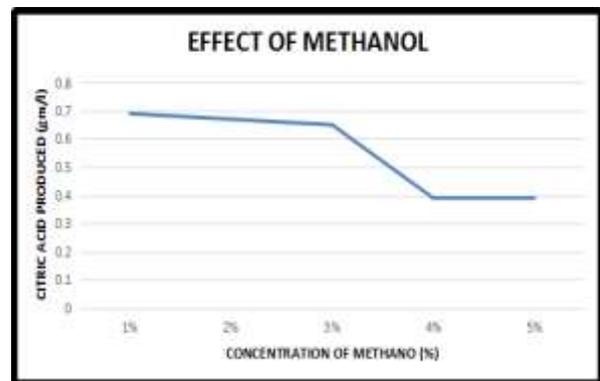


Chart-5: Effect of methanol concentration on citric acid production

4. CONCLUSION

Citric acid is the most commercially used organic acid. Although citric acid can be produced chemically from the juice of citrus fruits, fungal fermentation to produce citric acid has gained more importance than chemical synthesis because the latter is a much more expensive endeavor. In niger, we reported that it is a simple and cost-effective approach. The produced citric acid was estimated by titration method and detected by performing Thin Layer Chromatography (TLC) which showed Rf value 0.74. We also studied the optimization parameters such as incubation time, temperature, pH, different carbon sources and effect of methanol. The maximum production of citric acid was observed at incubation time 96hrs, temperature 25° C, carbon source dextrose, 1 % methanol (v/v) and pH 8.

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