Citric Acid Production from U-V Mutated Estuarine Aspergillus niger

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Abstract: The aim of this study was to improve the citric acid production by U-V mutated strain of Aspergillus niger and optimize the production. Citric acid is the most important organic acid produced in tonnage by fermentation. It is widely used in the food, beverage, pharmaceutical and cosmetic industries and range of other industries, from textiles to electroplating. Potato dextrose agar medium was used for isolation of Aspergillus niger from Vellar estuary Tamilnadu India. To improve the citric acid production strain improvement technique, U-V mutation was carried out at different time interval. Optimization was carried by using production media with various parameters such as pH, temperature, carbon source, nitrogen source and incubation period. Citric acid production was expressed as g/l. Among the mutated strains Aspergillus niger which was exposed to U-V for 4 hours was selected as the most potential. Maximum production was observed at pH 5, 32°C, sucrose as carbon source, ammonium nitrate as nitrogen source 1% of molasses as the best cheaper source and 7 days incubation as optimum period.

Key words: U-V Mutation • Aspergillus niger • Optimization • Cheaper source

INTRODUCTION

Citric acid is present in all citric fruits and it was first crystallized from lemon juice in the form of calcium citrate. It is the most important organic acid produced in tonnage by fermentation. worldwide demand for citric acid is increasing due to the increasing demand for industrialized foods [1]. It is widely used in the food, beverage, pharmaceutical and cosmetic industries and applications in a range of other industries, from textiles to electroplating. Citric acid production can be done by solid-state fermentation or with submerged fermentation [2]. It is used to flavour the drinks, jams and jellies, candies, water ice and wines [3]. It is produced commercially from the fermentation of bulk hydrated materials and by-product of sugar production by A. niger [4]. The mutant strains might show several fold increase in citrate production as compared to wild-type cultures [5,6].

Recently a wide range of citric acid production has been reported in response to different levels of nutrient supplementation [7,8]. The problem in the production of citric acid from yeasts is the simultaneous formation of iso citrate. The main advantages of using Aspergillus niger are its easy of handling, its ability to ferment a variety of cheap raw materials and high yields [7].

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is an intermediate in the TCA cycle and its accumulation is strongly influenced by the balance of nutrients. The limitation or starvation of nitrogen, phosphorus or other trace elements during the fermentation resulted in the limited growth of A. niger and to the enhancement of citric acid production [9]. In addition to the basal nutrients, to improve citric acid production, stimulators such as organic solvents, phytate and lipids can be applied [10]. Citric acid production is also known to be affected by inoculum density and fermentation time. Up to a specific limit, metabolite production generally increases with inoculum density [11]. The production of citric acid using cheap carbon source from agro-industrial by-products provides considerable combined benefit of waste material management as well as decrease of production cost [12,13]. Various waste materials have been evaluated for citric acid production, including sugar cane bagasse, grape pomace, apple pomace, wheat bran, coffee husk, kiwifruit peel, kumara, taro and cassava waste [14-20]. Citric acid production can be done by solid-state fermentation or with submerged fermentation [2]. To date, most industrial processes are carried out with submerged fermentation [12]. According to Ali et al. [21] about 700 thousand tons of citric acid is produced every year by
submerged fermentation. There are many microorganisms, including fungi, yeasts and bacteria, that can produce citric acid by fermentation. Aspergillus niger is one of the most well-known citric acid producers [22]. Apart from a number of parameters generally accepted as influencing biotechnological processes, temperature, humidity, pH, amount of inoculum, addition of nutrients, etc. also other parameters were found to be critical to the course of the solid state fermentation. Aspergillus niger is the main microbial strain used for citric acid industrial production [1,7].

The techniques of U.V- gamma ray-induced or chemical (NTG) mutagenesis have long been accepted as routine methods to improve the yield of citric acid by A. niger [22]. Mutations are abrupt and so are the hereditary modifications in the genetic material. The organisms containing the DNA are not static molecules and their bases are frequently exposed to natural or artificial agents that can cause modifications in their structure or in chemical composition [23, 24].

The main objective of present study was to improve the citric acid production by using U-V muted strain of Aspergillus niger compared with wild strain and optimized the media for citric acid production. It was hoped that information from this study might help to that U-V mutation is one of the useful method for strain improvement and also increase the production of citric acid.

MATERIALS AND METHODS

Study Area: This study was conducted in Faculty of marine science, Annamalai University, Parangipettai, Tamilnadu, India. Samples were collected from Vellar Estury in Tamil Nadu, India. The river Vellar flowing on the southeast coast of India originates in the Shervaryan Hills of Salem District, Tamilnadu, India. After meandering through a distance of 480 kms, it forms the estuarine system at Parangipettai, before it joins the Bay of Bengal. The Vellar estuary is always open with the Bay of Bengal and it said to be a “true estuary” as there is no complete closure of the mouth.

Isolation of Strain: Soil sample were collected from Vellar Estury in Tamil Nadu, India. Samples were serially diluted and inoculated in to a Potato Dextrose agar plate (Hi-media) and incubated for 5 days at 37°C.

Strain Improvement by U-V: Aspergillus niger strains were exposed to u-v light for 2hr, 4hr, 6hr, 8hr. They were analyzed for the production of citric acid. They named as A1, A2, A3, A4 respectively. Unexposed wild strain was named as A.

Citric Acid Production Medium: Strains were screened for citric acid production in liquid culture which contained sucrose (g/l) 120g, NaNO3 -5g, KH2PO4 - 2g, MgSO4.7H2O -1g, CuSO4.7H2O-0.02g, FeSO4.7H2O - 1g, ZnSO4.7H2O-1g, 50% Seawater-1000ml. The pH of medium was adjusted to 6.5 [25].

Selection of Strain: A, A1, A2, A3, A4 Strains were inoculated in a production broth and incubated at 37°C for 5 days then analyzed for the citric acid production.

Citric Acid Determination: Citric acid (CA) was determined titrimetrically [26] by using 0.1N NaOH and phenolphthalin as indicator and calculated as % according to the following formula:

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\%\text{CA} = \frac{\text{Normality} \times \text{volume of NaOH} \times \text{Equiv. wt. of CA}}{\text{Weight of sample} \times 10}
\]

Effect of Cabon Source: Production medium with the following composition was used with different carbon sources such as 1%, sucrose, fructose, glucose, dextrose, lactose. NANO3 -5g, KH2PO4 - 0.2g, MgSO4.7H2O -0.1g, CuSO4.7H2O-0.02g, FeSO4.7H2O - 0.1g, ZnSO4.7H2O-0.1g, at pH-6.5, 50% Seawater-100 ml of broth was prepared in a Erlenmeyer shaker flask(250ml) and inoculated 1ml of A2 spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days at 37°C.

Effect of Nitrogen Source: Medium with different nitrogen sources such as 2g of ammonium nitrate, peptone, yeast extract, used as nitrogen source with production media at pH-6.5 100 ml was taken in a Erlenmeyer shaker flask(250ml) and inoculated 1ml of A2 spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days at 37°C.

Effect of P: Production medium with different pH-4, 5, 6, 7, 8, 9 was used. About100 ml of production media was taken in a Erlenmeyer shaker flask (250ml) and inoculated 1ml of strain A2 spore suspension. Flasks were incubated in a rotary shaker at 150 rpm for 5 days at 37°C.
Effect of Temperature: About 100 ml of Production broth was prepared in an Erlenmyer shaker flask (250 ml) and sterilized. Then inoculated 1 ml of A2 strain spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days. Then analyzed for citric acid production with different temperature ranging from 28°C, 30°C, 32°C, 35°C.

Effect of Incubation Time: About 100 ml of Production broth was prepared in an Erlenmyer shaker flask (250 ml) and sterilized. Then inoculated 1 ml of A2 strain spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days at 37°C. Then analyzed for citric acid production with different incubation time ranging from 2 to 7 days.

Effect of Cheaper Source: Media with the following composition such as 1% Molasses, Ricebran, Wheatbran, Coconut cake as the sole carbon source with production media at pH 6.5 50% Seawater 100 ml was taken in an Erlenmyer shaker flask (250 ml) and inoculated 1 ml A2 spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days at 37°C.

Mass Scale Culture: 1000 ml of optimized production media which contain carbon source from cheaper source with optimized nitrogen source with optimized pH level at optimum temperature and incubation time fermentation was carried out in a rotary shaker at 150 rpm.

RESULTS AND DISCUSSION

Citric Acid Production by Potential Strain: The present study revealed that hyper production of citric acid was observed in mutated strain compared to wild type strain. Among the mutated strain A2 (Fig. 1) which is exposed to U-V for 4 hours is the most potential and it has produced 295.2 g/l citric acid. It was already found [21] that 31.1 g/l citric acid was produced in parental strain whereas 50.0 g/l was produced by U-V mutated strain. This hyper production may be due to the gamma ray is a packet of electromagnetic energy (photon) emitted by the nucleus of some radionuclides following radioactive decay. After gamma-irradiation and the breaking of the DNA double-strands, the cell can repair the damaged genetic material in the limit of its capability and genetic improvement may occur [27]. The most frequently used method is induction by U-V irradiation. Although the U-V rays do not have much energy and, consequently, they do not induce ionization directly, the unicellular organisms are potent mutagenic agents, since U-V rays are absorbed by purine and pyrimidines, making them reactive and inducing mutation [28,24]. Therefore, the mutations induced by U-V can randomly provide a strain with a higher capacity of citric acid production when compared to the control strains citric acid production.

Effect of Carbon Sources on Citric Acid Production: About 319.0 g/l of citric acid was produced by using sucrose as carbon source (Fig. 2). Sucrose is the most effective carbon source followed by fructose, dextrose, glucose, lactose. It was proved [29] that the increase in sucrose concentration had a positive effect on citric acid production. It was suggested that the mycelial growth and the strain have an extracellular invertase linked to mycelium and this breaks the sucrose producing energy exactly at the level in which the increase in citric acid production was observed.

Effect of Nitrogen Sources on Citric Acid Production: 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 the basic part of cell proteins. 479.0 g/l of citric acid was produced by using ammonium nitrate as nitrogen source followed by yeast extract, peptone (Fig. 3). About 16.9% of increased production was got when ammonium nitrate was used as nitrogen source [30].

Effect of Better Source: Media with the following composition such as 1% Molasses, Ricebran, Wheatbran, Coconut cake as the sole carbon source with production media at pH 6.5 50% Seawater 100 ml was taken in an Erlenmyer shaker flask (250 ml) and inoculated 1 ml A2 spore suspension. Flask was incubated in a rotary shaker at 150 rpm for 5 days at 37°C.

Effect of pH on Citric Acid Production: The maintenance of a favorable pH is very essential factor for the successful production of citric acid. Fig. 4 shows the citric acid production was 467 g/l at pH 5. pH 5 was the optimum followed by 4.6, 7.8, 9. At pH 5 472.4 g/l of citric acid production was obtained [31]. Decrease in pH caused reduction in citric acid production. It might be due to that at low pH, the ferrocyanide ions were more toxic for the growth of mycelium [32].

Effect of Temperature on Citric Acid Production: The temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid. About 30°C temperature was found to be the best for citric acid production in present study (Fig. 5). According to Steel et al. [33] incubation temperature should be in the range of 28 to 32°C, while Gerhardt et al. [34] found that 30°C was the optimum temperature for citric acid production. It was also
Corroborated that at temperatures between 24 and 31°C, temperature of 30-31°C was optimum [35, 36]. Temperatures lower than 27°C slowed down growth and production substantially. When the temperature of medium was low, the enzyme activity was also low, giving no impact on the citric acid production. However when the temperature of medium was increased above 30°C, the biosynthesis of citric acid was decreased. It might be due to the accumulation of by-products such as oxalic acid [37, 38].

**Effect of Incubation Time on Citric Acid Production:**
In the present observation 7th day was the optimum incubation time for the production (Fig. 6). In stationary culture, the production starts after a lag phase of approximately after 2 to 3 days and reaches maximum at the stationary phase [37].

**Effect of Cheaper Sources on Citric Acid Production:**
In order to minimize cost of the production cheaper sources were used for the production of citric acid.
When 1% of molasses was used as cheaper source compared to coconut cake, wheat bran, rice powder, rice bran production was 333 g/l (Fig. 7). When using 15% molasses was used, a maximum concentration of 120 g/L citric acid was obtained [39]. The maximum 191 mg/ml of citric acid production was observed when molasses were used as substrate [40]. In mass scale culture, citric acid production was carried out using optimized parameter by using molasses as carbon source. About 523 g/l of citric acid production was observed. The result concluded that Mutated strain of \textit{A. niger} showed highly potential citric acid producing marine fungi compared to wild strain by using molasses as carbon source.

**CONCLUSION**

This study proved that citric acid production was increased with \textit{u-v} mutated strain of \textit{Aspergillus niger} compared with wild type strain of \textit{Aspergillus niger}. This study also concluded that molasses as the best cheaper source for the citric acid production. It is also easily available source and very economic. This cheaper source can be useful in industry scale fermentation of citric acid production.

**REFERENCES**


