Optimization of Citric Acid Production Using *Aspergillus niger* Isolated from the Leaf Litter Soil of Sathuragiri Hills

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Abstract Soil samples were collected from the different locations in Sathuragiri Hills (Western Ghats, Tamilnadu). The Total Fungal Counts in the soil samples of the different locations were analyzed. The percentage frequencies of occurrence of fungal isolates are identified. The fungal isolates were identified based on the morphological and cultural characteristics they were identified as *Cladosporium* sp, *Aspergillus niger*, *Penicillium* sp, *Alternaria* sp, *Curvularia* sp, *Helminthosporium* sp and *Aspergillus flavus*. Among the isolates maximum amount of citric acid levels was produced by *Aspergillus niger* (2.2 ± 0.001 g/l). The effect of different pH, temperature, carbon source, nitrogen source, incubation time and agricultural residues on citric acid production by *Aspergillus niger* was analysed. The maximum amount of citric acid production was recorded in pH - 6.0 (2.32 ± 0.09 g/l), temperature - 30°C (2.85 ± 0.06 g/l), carbon source - Glucose (6.08 ± 0.10 g/l). Nitrogen source - ammonium chloride (10.22 ± 0.05 g/l), incubation time - 72 hours (13.56 ± 0.04 g/l) and agricultural residue - corn ears (17.2 ± 0.14 g/l).

Keywords Optimization, Citric Acid, Fungi, Leaf Litter, *Aspergillus niger*, Pharmaceutical

1. Introduction

Citric acid ($\text{C}_6\text{H}_8\text{O}_7$, 2-hydroxy-1,2,3-propanetricarboxylic acid), a natural constituent and common metabolite of plants and animals, is the most versatile and widely used organic acid in the field of food (70%) and pharmaceuticals (12%). It has got several other applications in various other fields. Currently, the global production of citric acid is carried out by fermentation [1]. There is constant increase (3.5-4%) each year in its consumption, showing the need of finding new alternatives for its manufacture [2].

Citric acid is the most produced organic acid measured in tonnage. The main reason for constant increase is the large number of applications that can be found for citric acid,

mainly in the food and pharmaceutical industries. Traditional processes, such as the submerged fermentation using the fungus *Aspergillus niger*, dominate the global production. However, different techniques of production are continuously being studied showing new perspectives for the production of citric acid. In this context, solid-state fermentation appears where agro-industrial residues can be used as substrate-supports to the filamentous fungi *Aspergillus niger*. Significant optimization of all citric acid processes can be observed with genetic amelioration of producer strains, which nowadays is the powerful tool of the citric acid market [3]. Since the Second World War, the production of citric acid has increased rapidly, reaching about 1.7 million tons per annum in 2008 with 5% predicted annual increase in the rate of production in order to meet the growing needs of the global market [4].

Citric acid is commercially produced by large scale fermentation mostly using selected fungal or yeast strains in aerobe bioreactors. 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 this commercially important metabolite. Different techniques for the hyper production of citric acid are continuously being studied for the past few decades. However, there is still a gap between demand and supply. Hence, there is obvious need to achieve industrially sustainable bio-production of citric acid. To meet the rising demand for citric acid in many applications in food and biomedicines, there is need for continual search for more efficient strains from our environment. Although genetic manipulations by classical mutagenesis techniques and rDNA technology are frequently exploited for overproduction of citric acid by microorganisms, there are problems with genetic stability of the strains and safety issues associated with the use of genetically modified organisms. Citric acid production can be improved by optimizing the fermentation parameters such as initial substrate concentration, initial pH, nutrient concentration, additives, stirrer speed, incubation period, fermentation temperature, air and O_2/N_2 supply. The optimal conditions vary depending on the species and substrates [5].

Citric acid is considered as one of the important organic acids that has a wide commercialization potential. Recently, major production of citric acid was conducted via microbial fermentation, as it was economical and easy to handle. Citric acid has multiple uses, particularly in food and beverage industries as a flavor enhancer and antioxidant agent. It also has other industrial uses, such as in pharmaceutical, cosmetic, and various chemical industries [6].

In this context, the isolation and identification of a potential citric acid producing fungal strain from the Western Ghats of Tamilnadu was attempted in the present investigation considering the fact that novel and potential citric acid producing strain can be isolated from biodiversity niche. *Aspergillus niger* was found to be very effective comparatively with the existing microbial strains. Microorganisms in Western Ghats of Tamil Nadu form a potential source for exploring novel microbial products, due to their unique natural habitat, distinct physiological characteristics, metabolic patterns and nutrient utilization.

2. Materials and Methods

Citric acid producing fungal strain (*Aspergillus niger*) was isolated from the Western Ghats of Tamilnadu, India. It was used for the optimization process [7]. All the experiments were carried out in triplicates.

2.1. Effect of pH

The pH of the culture broth was determined using a digital pH meter. The pH meter was allowed to warm up for about 30 minutes before use. The electrode was removed from distilled water in the storage beaker and dried. The electrode was then placed in a beaker containing a buffer solution of pH 7 and calibrated to the same figure. The electrode was removed and after rinsing in distilled water was placed in the culture broth to be tested. Care was taken not to allow the electrode have any contact with the glass. The pH was digitally readout from the pH meter and recorded [8].

The effect of optimum pH for citric acid production by *Aspergillus niger* was determined by culturing the fungi individually in the production media with different pH. The experiments were carried out individually at various pH such as 3, 4, 5 and 6. The citric acid determination was carried out after 72 hours of incubation at 30°C.

2.2. Effect of Temperature

The effect of optimum temperature for citric acid production by *Aspergillus niger* was determined by culturing the fungi individually in the production media with different temperature. The experiments were carried out individually at various temperatures such as 20, 30, 40, 50 and 60. The citric acid determination was carried out after 72 hours of incubation at 30°C.

2.3. Effect of Carbon Source

To identify suitable carbon source for the citric acid production by *Aspergillus niger* the following carbon sources were tested. The production medium containing glucose, act as carbon source. This glucose was replaced by sucrose, fructose, dextrose, lactose, trehalose, maltose, galactose, starch, mannose, raffinose, carboxyl methyl cellulose, arabinose and xylose. These carbon sources were tested individually at the concentration of 1% with dry substrate in the optimized production medium. The citric acid determination was carried out after 72 hours of incubation at 30°C.

2.4. Effect of Nitrogen Source

To identify suitable nitrogen source for the citric acid production by *Aspergillus niger* the following nitrogen source was tested. The production medium containing ammonium chloride, act as nitrogen source. This ammonium chloride was replaced by ammonium nitrate, peptone, yeast extract, glycine, ammonium sulphate, ammonium molybdate, gelatin, urea, casein, sodium nitrite and potassium nitrate. These nitrogen sources were tested individually at the concentration of 0.5% with dry substrate in the optimized production medium. The citric acid determination was carried out after 72 hours of incubation at 30°C.

2.5. Effect of Incubation Time

The effect of incubation time on citric acid production by *Aspergillus niger* was determined by culturing the fungi individually in the production media. The experiments were carried out individually at various incubation times. They were 24, 48, 72, 96 and 120 hours.

2.6. Effect of Agricultural Residues

The citric acid production by *Aspergillus niger* was optimized individually by supplementing different agricultural residues. For this, 18 different agricultural residues were tested individually at the concentration of 5% with dry powdered substrate in the optimized production medium. The different agricultural residues used for the citric acid production were groundnut pod, rice bran, cotton gin waste, doll husk, paper, black gram pod, paddy straw, cotton seeds, sugar cane bagasse, green gram husk, corn stalk, black gram husk, coir pith, green gram pod, banana pseudo stem, coir fiber, corn ears and wheat bran.

2.7. Citric Acid Production Media

In Hygiene Fermentor (3 Liters volume capacity), 50g of agricultural substrate (corn ears) and 1000 ml of production medium containing (g/l); glucose-10g; NH_4Cl -5g; KH_2PO_4 -2g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -1g; $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ -0.02g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -1g and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -1g in distilled

water-1000ml. The *Aspergillus niger* was inoculated and incubated for 72 hours. At the end of the fermentation it was centrifuged at 10,000 rpm for 5 minutes and the supernatant was used for further analytical work.

2.8. Determination of Citric Acid Production

The broth was centrifuged at 10000rpm for 5 minutes and the supernatant was taken for citric acid estimation. Citric acid was determined titrimetrically [9] by using 0.1N NaOH and phenolphthalein as indicator and calculated as % according to the formula:

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

2.9. Statistical Analysis

All the works were done in triplicates. The results obtained in the present investigation were subject to statistical analysis like Mean ($\bar{x}$) and Standard deviation (SD) by Zar, (1984)[10]. Statistical analysis were carried out using Microsoft office excel package.

3. Results

Effect of pH

The effect of different pH on citric acid production by *Aspergillus niger* after 72 hours of incubation period at 30°C showed maximum amount of citric acid production in pH 6.0 (2.32 ± 0.09 g/l). Minimum citric acid production was recorded in pH 3.0 (0.90 ± 0.09 g/l) (Fig. 1).

Effect of Temperature

Among the various temperatures tested, maximum citric acid production by *Aspergillus niger* was recorded in 30°C (2.85 ± 0.06 g/l). On the other hand, minimum amount of citric acid production was recorded in 60°C (0.93 ± 0.05 g/l) (Fig. 2).

Effect of Carbon Sources

About 6.08 ± 0.10 g/l of citric acid was produced by using glucose as carbon source (Fig. 4). Glucose is the most effective carbon source followed by galactose, maltose, dextrose, fructose, sucrose, lactose and trehalose. It was proved that the increase in glucose concentration had a positive effect on citric acid production. It was suggested that the mycelial growth and the strain have an extracellular citric acid linked to mycelium and this breaks the glucose producing energy exactly at the level in which the increase in citric acid production was observed (Fig.3).

Effect of Nitrogen Sources

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. 10.22 ± 0.05 g/l of citric acid was produced by using ammonium chloride as nitrogen source followed by ammonium sulphate (9.13 ± 0.07 g/l). Minimum citric acid production was observed in urea (6.36 ± 0.04 g/l) (Fig.4).

Effect of Incubation Time

The effect of different kinds of incubation time was tested on citric acid production. Maximum amount of citric acid production by *Aspergillus niger* was observed in 72 hours incubation time (13.56 ± 0.04 g/l). Minimum amount of citric acid production was obtained in 96 hours of incubation time (8.56 ± 0.04 g/l) (Fig. 5).

Effect of Agricultural Residues

The effect of different agricultural residue on citric acid production by *Aspergillus niger* after 72 hours of incubation period at 30°C showed maximum amount of citric production in corn ears (17.2 ± 0.14 g/l) supplemented medium and next to that cotton seeds supplemented medium produced a maximum production of citric acid about 15.3 ± 0.13 g/l. Minimum amount of citric acid production was observed in wheat bran (10.5 ± 0.16 g/l) supplemented medium (Fig. 6).

Figure 1. Effect of pH on citric acid production

Figure 2. Effect of temperature on citric acid production

Figure 3. Effect of various carbon sources on citric acid production

Figure 4. Effect of various nitrogen sources on citric acid production

Figure 5. Effect of incubation time on citric acid production

Figure 6. Effect of agricultural residues on citric acid production

4. Discussion

Correspondingly, the effect of pH was observed that the concentration of citric acid increased with pH up to a maximum at a pH of 5.5 after which it declined. The results show that the best citric acid concentration of 1.94 g/dm³ was obtained at a pH of 5.5. The pH is important in two respects. Firstly, spore germination which is required for fermentation requires a pH of 5 and above to occur. Secondly, protons are released when ammonia is absorbed by germinating spores. This causes a release of hydrogen ions thus lowering the pH of the medium. The low pH has the effect of improving citric acid production and providing a close to sterile environment which reduces the risk of contamination [11].

Similar results were observed in a study which showed pH 6 was the optimum followed by 4, 6, 7, 8 and 9. At pH 5 472.4 g/l of citric acid production was obtained. 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 [12].

Similarly in a study the effect of temperature observed is such that there is an increase in the production of citric acid between 25°C and 30°C after which production decreases. The best citric acid concentration of 1.431 g/dm³ was obtained at a temperature of 30°C. This was the optimum temperature identified for citric acid production. The fermentation temperature is important in that when cells are grown under non ideal temperature conditions, they exhibit signs of adverse growth and metabolic production [13]. Nampoothiri *et al.* (2004) [14] reported that citric acid production could be affected by a slow germination of the fungi, slow metabolic activity, enzyme denaturation and reduced cell viability when *Aspergillus niger* cells are incubated under low or high temperatures. The optimum temperature obtained in this study is in agreement with the fact that filamentous fungi such as *Aspergillus niger* are mesophilic thus requiring optimal temperatures between 25°C and 35°C for growth [15, 16].

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 the present study. According to Steel *et al.* (1955) [17] incubation temperature should be in the range of 28 to 32°C, while Gerhardt *et al.* (1946) [18] found at 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 [19, 20]. 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 [21, 22].

It was proved that the increase in glucose concentration had a positive effect on citric acid production. It was

suggested that the mycelial growth and the strain have an extracellular citric acid linked to mycelium and this breaks the glucose producing energy exactly at the level in which the increase in citric acid production was observed. Equally, the proximate composition of cheese whey was used as the basic fermentation media. It was found to be 4.9% lactose, 1.0% crude protein, 0.5% ash, 0.2% fat, 6.4% total soluble solid (TSS) and 93.3% water. Citric acid production by *A. niger* from whey as a basic fermentation media, and with different concentrations of sucrose, glucose, fructose and galactose. Low amount of CA (2.43 g/l) was produced from whey alone. Adding different sugars to whey enhanced CA production with a maximum value of 106.5 g/l with 15% sucrose. Significantly lower values were obtained using same concentration of other sugars. The poor CA production from whey alone is believed to be at least partly due to the presence of galactose moiety of lactose in the whey [23].

It was believed that *A. niger* can readily utilize galactose its presence or that of its metabolic products causes inhibition of citric acid production and also reduce the rate of glucose utilization. These authors found that galactose interferes with the glucose repression of the key enzyme, 2-oxoglutarate dehydrogenase. There is a strong relationship between citric acid production and the activities of this enzyme and pyruvate dehydrogenase in cell free extracts [24].

Hossain *et al.* (1985) [25] explained that the nature of sugar source has a marked effect on citric acid production by *A. niger*. Sucrose is the traditional commercial substrate for CA production. Glucose, fructose and maltose have also been used as substrates for CA production [26]. Sucrose is of relatively low molecular weight and readily transported into microbial cells for hydrolysis by intracellular enzymes [27]. The result of the influence of different concentrations of each of riboflavin, TCP and methanol added to whey media containing 15% sucrose on citric acid and biomass production are presented. The highest citric acid values of 92.46-92.86 g/l were produced in the whey media containing 15% sucrose with or without 1% methanol, respectively. Much lower CA values were obtained with the addition of riboflavin and TCP throughout 16 days fermentation period. Higher methanol concentration (up to 5%) caused drastic decrease in CA production reaching its minimum (5.4 g/l) with the addition of 5%. CA values steadily increased with incubation time. Relatively higher biomass values (42-46 g/l) were found in the cultures containing riboflavin after 16 days. Lower values (31.1-38.9 g/l) were recorded in the cultures with the TCP. Biomass in the cultures containing methanol decreased from 37.4g/l with 1% methanol to 6.9g/l with 5% methanol.

Previous reports [24, 25] stated that the presence of methanol in the fermentation media may increase CA production by *A. niger*. The inductive effect of methanol for citric acid production may be due to reduction of the inhibitory effect of metal ions [28]. In the absence of methanol little or no citric acid was produced from galactose. The addition of Tricalcium phosphate (TCP) to date extract

induced CA production which probably chelates high levels of inhibitory metal ions like Mn, Fe, and Zn present in the date extract [29]. Adding TCP, riboflavin and methanol to whey may cause similar adverse effects by chelating certain metal ions like Cu^{+2} which is reported to be necessary component in the structure of the productive fungal pellets [30].

Citric acid, residual sugar, biomass and pH values from whey (with 15% sucrose) fermentation media by *A. niger* during 20 days are presented. A gradual increase was obtained in both citric acid values with fermentation time, reaching its maximum of 83.7 mg/l after 16 days, followed by a decrease to 42 g/l after 20 days. Biomass increase continues to the 20th day. These increases in CA and biomass values were accompanied with steady decrease in residual sugar from 173.8 g/l initially to a minimum of 35.6 g/l after 20 days, as well as in pH values from 3.0 initially to 1.5 after 16 days. In conclusion, we found that using whey alone as a natural fermentation medium for citric acid production was inferior to whey fortified with sugars. Maximum citric acid was produced from whey with 15% Sucrose [31].

About 319.0 g/l of citric acid was produced by using sucrose as carbon source. Sucrose is the most effective carbon source followed by fructose, dextrose, glucose, lactose. It was proved [32] 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 citric acid linked to mycelium and this breaks the sucrose producing energy exactly at the level in which the increase in citric acid production was observed [2].

Kristiansen and Sinclair, (1979) [33] used continuous culture and concluded that nitrogen limitation is necessary for citric acid production. Pellet formation in filamentous fungi has been discussed in many cases and among the factors considered to induce it, is the limitation of particular nutrients, including nitrogen. In a study, the highest values of kinetic parameters *i.e.*, $\text{Yp/s} = 0.908 \pm 0.05\text{a g/g}$, $\text{Qp} = 0.618 \pm 0.02\text{a g/l/h}$ and $\text{qs} = 0.036 \pm 0.01\text{b g/g cells/h}$ were observed at 0.2% NH_4NO_3 [34].

Similarly, 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. 479.0g/l of citric acid was produced by using ammonium nitrate as nitrogen source followed by yeast extract, peptone. About 16.9% of increased production was got when ammonium nitrate was used as nitrogen source [2].

Whereas, in an observation 7th day was the optimum incubation time for the production. In stationary culture, the production pH starts after a lag phase of approximately after 2 to 3 days and reaches maximum at the stationery phase [22].

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

333g/l. When using 15% molasses was used, a maximum concentration of 120 g/l citric acid was obtained [35]. The maximum 191mg/ml of citric acid production was observed when molasses used as substrate [36]. In mass scale culture citric acid production was carried out using optimized parameter by using molasses as carbon source. About 523g/l of citric acid production was observed. The result concluded that Mutated strain of *A. niger* showed highly potential citric acid producing marine fungi compared to wild strain by using molasses as carbon source [2].

5. Conclusions

Aspergillus niger found to produce the highest quantity of citric acid (2.20 ± 0.01 g/L). The maximum amount of citric acid production was recorded in pH - 6.0 (2.32 ± 0.09 g/l), temperature - 30°C (2.85 ± 0.06 g/l), carbon source - Glucose (6.08 ± 0.10 g/l). Nitrogen source - ammonium chloride ($10.22 \pm 0.05\text{g/l}$), incubation time - 72 hours (13.56 ± 0.04 g/l) and agricultural residue - corn ears (17.2 ± 0.14 g/l). These findings have further confirmed the superior of *Aspergillus niger* as the industrial production species for the citric acid production.

Even though there are many microbial sources available for citric acid production only a few are recognized for commercial production. Their vast diversity and specific range of action have attracted the attention of biotechnologists' worldwide. Presently, in India, little effort is being made to prepare citric acid using microorganisms. The best possible solution considering cost effectiveness may be the utilization of the indigenous and cheaper substances as like agricultural wastes and by products as substrate for the production of this valuable organic acid and then its further use in application like food and pharmaceutical industry. Therefore, the plan for future research investigations has been chalked out to produce citric acid using *Aspergillus niger*.

Conflict of Interest

There is no conflict in this study.

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