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Isolation and Screening of Citric Acid Producing Aspergillus Spp and Optimisation of Citric Acid Production by Aspergillus Niger S-6

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Abstract: *The present study was aimed to isolate and screen the ability of citric acid producing Aspergillus spp from soil. Citric acid is the most important organic acid, used as a natural preservative and conservative. It is also used to add an acidic or sour taste to foods and soft drinks. In this study, fungal samples were isolated from soil samples, using serial dilution agar plating method and the isolates were identified based on their microscopic and morphological characteristics. From the isolates, the dominant fungal species, Aspergillus niger and Aspergillus fumigatus were sub-cultured using Potato Dextrose Agar (PDA) medium and the fungal species were used for citric acid production. The spores were mixed in the fermentative medium for 72 hours at 30±1° C. After incubation, the citric acid content was estimated by titrimetric method. Among the two species, higher citric acid production was shown by Aspergillus niger S-6. Then it was undertaken for optimisation under various pH, temperature, Carbon and Nitrogen sources. In this assay, the maximum production of citric acid was observed in Aspergillus niger S-6 at pH 6.0, temperature 30±1° C, Carbon source Glucose and Nitrogen source Ammonium sulphate.*

Keywords*: Aspergillus niger, Aspergillus fumigatus, citric acid.*

I. Introduction

Citric acid (2-hydroxy-1, 2, 3-tricarboxylic acid) is an organic acid naturally present in all aerobic organisms, as it is produced during the process of cellular respiration. It is a ubiquitous intermediate product of metabolism and its traces are virtually present in all plants and animals[1]. This organic acid has found multiple uses in food, beverage, pharmaceutical, chemical, cosmetic and other industries. Citric acid is used to impart a characteristic tart taste to foods and beverages. It is used in industries for acidulation, anti-oxidation, emulsification, preservation, flavour enhancement and as plasticizer and synergistic agent[2]. This acid is biodegradable and palatable, highly soluble and low/non-toxic[3]. It is adjudged to be GRAS (Generally Recognised As Safe). Hence, it is one of the most important organic acids produced by fermentation and is the most exploited biotechnological/biochemical product[4]. It has an annual industrial production of 1.6 million tons[5] with an annual growth demand/consumption rate of 3.5-4%[6]. The food industry utilises about 70% of the total citric acid produced globally while about 12% id used in pharmaceuticals and the remaining 18% in other industries[7,8]. The natural supply of citric acid is very limited as compared to its growing industrial demand. Its ever increasing demand can only be satisfied by biotechnological processes[9].

Microbial fermentation is the most common method for large scale production of citric acid. Many micro-organisms such as bacteria, fungi and yeasts can produce citric acid. A large number of these micro-organisms have been used for citric acid production, but only a few of them can produce the acid on an industrial scale[10-14]. The micro-organisms which have been used for citric acid production by various workers include bacteria such as Arthrobacter paraffinens, Bacillus licheniformis and Corynebacterium spp, fungi such as Aspergillus niger, A. fumigatus, A. aculeatus, A.carbonarius, A. awamori, A. foetidus, Penicillium jenthinellum and yeasts such as Candida oleophila, C. tropicalis, C. citroformans, Torula spp, Torulopsis spp, Sacharomyces spp, etc. Among these, Aspergillus niger is the most commonly used strain for commercial production of citric acid as it produces more acid per unit time. A. niger remains the organism of choice for this purpose as it is easy to handle, it can ferment a variety of raw materials and it gives higher yields as compared to other micro-organisms used for the purpose. Different methods of fermentation have been employed by different workers for producing citric acid using specific substrates and microbial strains. In any technique used in citric acid production, good yield depends on various factors such as the microbial strain used, substrate used, fermentation conditions employed like temperature, pH, etc. Factors such as carbon source and nitrogen source used in the fermentation process also influence the yield of the acid in the medium. Different types of fermentation methods are employed for citric acid production on mass scale, which include solid-state fermentation, submerged fermentation and surface fermentation. The most common method however, is the submerged fermentation method. This study was carried out to isolate and screen the ability of citric acid producing Aspergillus spp and study the effect of various fermentation parameters on citric acid production.

II. Materials And Methods

2.1 Isolation of Aspergillus spp: Soil samples from sugarcane growing areas of Boko, Kamrup District (Rural), Assam, India during 2013-14. The soil samples were taken by means of sterilised spatulas and collected sterile sealed polythene bags. The samples were brought to the Research laboratory, Department of Botany, Cotton College, Guwahati, Assam, India and stored at room temperature for microbiological study. 1 gram of soil sample was taken in a 250 ml conical flask containing 100 ml of sterile distilled water and shaken well for 20 minutes. From the stock, various dilutions were prepared from 10^{-2}, 10^{-4} and 10^{-6} using sterile distilled water. 1 ml of the serially diluted sample was poured in the sterile petridishes containing Potato Dextrose Agar (PDA) medium. Streptomycin was added in the molten PDA medium in the petridishes and were incubated at 30±1° C for 3-5 days for the isolation of Aspergillus spp. After incubation, different fungal colonies grow on the PDA medium. Preliminary identification of the fungi was done based on their morphological characteristics, with the help of "A Manual of Soil Fungi"[15]. A total of 10 different Aspergillus isolates were obtained, 6 belonging to Aspergillus niger and 2 belonging to Aspergillus fumigatus. The dominant isolates of Aspergillus niger and Aspergillus fumigatus were then sub-cultured in PDA medium. These isolates have been tentatively named as Aspergillus niger S 1 to 6 (Aspergillus niger S-1, S-2, S-3, S-4, S-5 and S-6) and Aspergillus fumigatus S-7 and S-8. The isolated Aspergillus spp were purified by restreaking on the medium and the pure cultures were maintained on the PDA slants and stored at 5°C in the refrigerator.

2.2 Basal Screening Fermentation Medium: The isolate Aspergillus spp were screened quantitatively for the production of citric acid in liquid culture medium containing soluble starch – 10 gm/L; $(NH_4)_2SO_4$ – 2.2 gm/L; K_2HPO_4 – 1 gm/L; $MgSO_4.7H_2O$ – 0.05 gm/L; $CaCl_2$ – 0.05 gm/L. The basal screening media was autoclaved at 121°C for 15 minutes. The basal medium was inoculated with 1ml of the spore suspension of the concentration 2 x 10^8 spores/ml prepared from 5 days old cultures of the isolated Aspergillus strains. A total of 8 Aspergillus strains were screened for citric acid production. Based on the screening results for citric acid yield, only Aspergillus niger S-6 was selected for further study.

2.3 Fermentation Condition: Fermentation was carried out in the laboratory in 250 ml Erlenmeyer flasks as small scale laboratory fermentor containing 100 ml of fermentation medium in each flask. Aspergillus strains were inoculated separately to the fermentation medium at an inoculum concentration of 1 ml containing the spore suspension of the concentration 2 x 10^8 spores/ml. Experiments were carried out at 30±1°C in the incubator.

2.4 Measurement of Citric acid: The concentration of citric acid in the fermentation medium was estimated titrimetrically (AOAC, 1995)[16] as reported by different investigators[17,18]. The readings were taken at regular intervals of 4 hours from the time of inoculation of the medium with the fungal spores.
The amount of citric acid produced by the different isolates of Aspergillus spp are shown in Table 3.1.

2.5 Optimisation of fermentation medium: The next phase of the study conducted was optimisation of the culture conditions taking four different parameters i.e. Temperature, pH, Carbon sources and Nitrogen sources, for citric acid production, using Aspergillus niger S – 6, as it showed the maximum levels of acid production so far. The citric acid production was estimated after 60 hours of incubation of the inoculated medium.

2.5.1 Temperature: Different temperature ranges were selected i.e. 25°C, 30°C, 35°C, 40°C and 45°C and the fermentative media were allowed to produce citric acid under these temperature ranges. The amount of citric acid produced at different temperatures by Aspergillus niger S-6 are shown in Table 3.2.

2.5.2 pH: The pH of the fermentative medium was another criteria that was worked upon. A range of pH starting from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were selected for citric acid production by A niger S-6. The results are shown in Table 3.3.

2.5.3 Carbon Sources: Three Carbon sources are chosen and tested for their suitability as substrates for citric acid production, among starch, sucrose and maltose. Different concentrations of solutions containing starch, sucrose and maltose are used to find out the suitable concentration at which the citric acid production is the optimum. The results obtained, are shown in Table 3.4.

2.5.4 Nitrogen Sources: Five different Nitrogen sources have been used and their suitability was checked for citric acid production. The sources selected were: Ammonium sulphate, Ammonium nitrate, Sodium nitrate,

Yeast extract and Peptone. The citric acid production shown by these 5 Nitrogen sources under different concentrations, are shown in Table 3.5.

III. Tables

Table 3.1: Amount of citric acid produced by the different isolates of Aspergillus spp recorded at regular intervals of 4 hours over a span of 72 hours:-

No. of Hours	Name of the different isolates of Aspergillus spp obtained and the corresponding amount of citric acid (gm/100 ml of solution) produced by them.							
	A niger S-1	A niger S-2	A niger S-3	A niger S-4	A niger S-5	A niger S-6	A fumigatus S-7	A fumigatus S-8
4	0.026	0.034	0.031	0.072	0.030	0.08	0.000	0.050
8	0.030	0.036	0.032	0.075	0.031	0.10	0.000	0.050
12	0.031	0.036	0.032	0.078	0.031	0.11	0.009	0.050
16	0.031	0.037	0.035	0.080	0.033	0.12	0.012	0.040
20	0.033	0.039	0.037	0.081	0.034	0.12	0.012	0.050
24	0.034	0.043	0.039	0.083	0.037	0.13	0.014	0.070
28	0.036	0.044	0.041	0.083	0.038	0.14	0.016	0.050
32	0.036	0.049	0.042	0.086	0.041	0.15	0.019	0.040
36	0.038	0.052	0.044	0.089	0.043	0.15	0.020	0.050
40	0.041	0.053	0.045	0.090	0.047	0.17	0.020	0.030
44	0.043	0.055	0.045	0.097	0.048	0.19	0.022	0.040
48	0.045	0.059	0.052	0.099	0.048	0.23	0.025	0.050
52	0.046	0.064	0.055	0.14	0.056	0.33	0.026	0.040
56	0.049	0.068	0.056	0.17	0.058	0.35	0.029	0.050
60	0.050	0.068	0.063	0.23	0.061	0.39	0.030	0.060
64	0.050	0.068	0.063	0.24	0.065	0.39	0.030	0.060
68	0.050	0.068	0.063	0.24	0.067	0.39	0.030	0.060
72	0.050	0.065	0.060	0.23	0.066	0.39	0.030	0.050

Table 3.2: Citric acid production shown by A niger S-6 at different temperatures (pH ~6.0):

Sl No.	Temperature (°C)	Citric acid Production (gm/100 ml)
1	25	0.31
2	30	0.38
3	35	0.33
4	40	0.32
5	45	0.31

Table 3.3: Citric acid production shown by A niger S-6 at different pH ranges (Temperature 30°C):

Sl No.	pH of the fermentative medium	Citric acid Production (gm/100 ml)
1	5.0	0.22
2	5.5	0.28
3	6.0	0.33
4	6.5	0.38
5	7.0	0.30
6	7.5	0.20
7	8.0	0.12

Table 3.4: Citric acid production by A niger S-8 on different Carbon sources (under temperature 30°C and pH ~6.0):

Carbon Source	Concentration (% solution)	Citric acid production (gm/100 ml)
Starch	10	0.32
	20	0.38
	30	0.35
Sucrose	10	0.32
	20	0.34
	30	0.30
Maltose	10	0.30
	20	0.32
	30	0.22

Table 3.5: Citric acid production shown by A niger S-6 under the 5 different Nitrogen sources used (under temperature 30°C and pH ~6.0)

Nitrogen Source	Concentration (% solution)	Citric acid production (gm/100 ml)
$(NH_4)_2SO_4$	10	0.26
	20	0.38
	30	0.25
$NaNO_3$	10	0.08
	20	0.08

	30	0.05
NH_4NO_3	10	0.07
	20	0.05
	30	0.03
Yeast Extract	10	0.00
	20	0.04
	30	0.02
Peptone	10	0.00
	20	0.03
	30	0.03

IV. Results And Discussion

4.1 Screening of stock cultures of Aspergillus spp: 8 cultures of Aspergillus spp were screened for citric acid production on the above mentioned fermented medium at 30±1°C for 72 hours. Of these cultures, Aspergillus niger S-6 produced higher citric acid (0.33 gm/100 ml). Out of these 8 cultures, only Aspergillus niger S-6 was used for optimisation studies.

4.2 Effect of incubation temperature on Citric acid production: Temperature is a critical physical factor for citric acid production. The effect of temperature on citric acid production was shown in Table 3.2. In this study, maximum citric acid production was obtained at 30±1°C (0.38 gm/100ml). At a temperature of 25°C, lower concentration of citric acid (0.31 gm/100ml) was produced. This may be due to low enzyme activity which makes no impact on citric acid production. When the temperature of the fermentation medium was increased above 10°, the biosynthesis of citric acid decreased (35°C – 0.33 gm/100ml, 40°C – 0.32 gm/100ml, 45°C – 0.31 gm/100ml). It might be due to the accumulation of different by-products such as oxalic acid. Similar results were also obtained by various workers by using 30°C as the cultivation temperature and obtained higher production of citric acid.[19, 20]

4.3 Effect of pH on citric acid production: The pH of the medium is an important physical factor that affects the performance of submerged fermentation. In this study, the optimum initial pH of the medium for citric acid production is shown in the Table 3.3. A range of initial pH 5 to 8 was examined to investigate the effect on citric acid production. There was a significant increase in citric acid production uptil pH 6.0. Further increase to pH 8.0 was associated with a decrease in citric acid yield. A higher initial pH leads to the accumulation of oxalic acid. The nature of the substrate and production technique also influences the pH techniques.[21] Hence, the initial pH must be well defined and optimized depending on the type of micro-organisms, different substrates and the production techniques.

4.4 Effect of different Carbon sources for Citric acid production: The effect of different carbon sources viz. soluble starch, sucrose and maltose concentration were estimated by changing the carbon source while keeping all the other factors unchanged (TABLE 3.4). The starch concentration (20%) is the optimum for citric acid production as compared to the other carbon sources. It was observed that maximum quantity of citric acid was obtained in starch during this investigation (0.38gm/100ml). Citric acid accumulation is strongly influenced by the type of concentration of Carbon sources.[22, 23] The nature of the carbohydrates which are taken up by the micro-organisms has been found to be most essential for the good production of citric acid. Among the easily metabolised carbohydrates, starch is the favourable carbon source followed by sucrose and maltose.[24] Based on the results, several raw materials can be employed for citric acid production. Moreover, there are some other factors that should be taken into account such as the cost of raw materials and the need of pre-treatment at the time of choosing the type of substrate.

4.5 Effect of different Nitrogen sources on citric acid production: Nitrogen constituent has a profound effect on citric acid production. In this study, five different nitrogen sources i.e. $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3, Yeast Extract and Peptone were used to check the suitability of each as a source of nitrogen in the fermentation medium for citric acid production. As shown in the Table 3.5, the maximum production of citric acid was seen in the medium containing $(NH_4)_2SO_4$, while the other nitrogen sources did not yield any satisfactory levels of citric acid in the fermentation medium. The maximum amount of citric acid production (0.38 g/100 ml) is clearly influenced by the concentration and nature of nitrogen sources. Physiologically, ammonium salts are preferred such as ammonium nitrate, ammonium sulphate, sodium nitrate, yeast extract and peptone. Nitrogen sources are necessary to maintain the pH of the fermentation medium. High nitrogen concentrations increase fungal growth and sugar concentration, but decrease the citric acid production.[25]

V. Conclusion

The present study concluded that, to isolate and to screen the citric acid production, Aspergillus strains from sugarcane growing areas to explore their citric acid production potential and to optimise the cultural conditions for possible future production and application. The factors such as different carbon sources, nitrogen sources, pH, temperature, etc need to be considered in the cultivation of Aspergillus niger S-6 since it affects citric acid production. Substrate requirement as well as biomass and product yields are some of the basic parameters that need to be considered in determining the feasibility of the fermentation process.

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