Screening of Citric Acid Producing Fungi from the Leaf Litter Soil of Sathuragiri Hills

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Abstract Soil samples were collected from ten different locations of Sathuragiri Hills (Western Ghats, Tamilnadu). Percentage frequency of occurrence of fungal isolates was calculated. Cultural characteristics of fungal isolates on Sabouraud's dextrose agar were observed. Fungal isolates were identified based on morphology, cultural characteristics and 18S rRNA sequencing. Citric acid production ability of fungal isolates was screened. Among the isolates *Aspergillus niger* found to produce the highest quantity of citric acid (2.5 ± 0.01 g/L) and it is selected for further work.

Keywords Screening, Citric Acid, Fungi, Leaf Litter, Pharmaceutical

in which it occurs (*i.e.* lemons, limes, figs, oranges, pears and goose-berries). Hence, citric acid is used to impart a pleasant tart flavours to foods and beverages. It is used in the industries to achieve acidulation, antioxidation, emulsification, preservation, flavour enhancement, and as plasticizer and synergistic agent [7]. The wide applicability of citric acid in industries is attributed to its low/non-toxicity, high solubility; biodegradability and palatability [8].

It is a product adjudged to be GRAS (Generally Recognized as Safe). The food industry consumes about 70% of total citric acid produced and pharmaceutical industries consume about 12% and the remaining 18% are consumed by other industries [6]. The demand for citric acid is increasing faster than its production and hence, more economical processes are required [9].

The supply of natural citric acid is very limited and the demand can only be satisfied by biotechnological processes [10]. Many microorganisms such as fungi, bacteria and yeast can produce citric acid. A large number of these microorganisms have been employed for citric acid production, but only a few of them can produce citric acid in industrial scale [7]. It is reported that *Aspergillus niger* is almost exclusively used for industrial scale production of citric acid. This is due to its high citric acid productivity at low pH, without secretion of toxic metabolites, ease of handling and ability to ferment a variety of cheap raw materials. Citric acid is commercially produced by large scale fermentation mostly using selected fungal or yeast strains in aerobe bioreactors [10].

A large number of microorganisms including fungi and bacteria such as *Arthrobacter paraffinens*, *Bacillus licheniformis* and *Corynebacterium* sp., *Aspergillus niger*, *A. aculeatus*, *A. carbonarius*, *A. awamori*, *A. foetidus*, *A. fonscaeus*, *A. phoenicis* and *Penicillium janthinellum*; and yeasts such as *Candida tropicalis*, *C. oleophila*, *C. guilliermondii*, *C. citroformans*, *Hansenula anamola* and *Yarrowia lipolytica* have been employed for citric acid production [11]. Most of them, however, are not able to produce commercially acceptable yields due to the fact that citric acid is a metabolite of energy metabolism and its

1. Introduction

Citric acid ($C_6H_8O_7$, 2-hydroxy-1,2,3-propane tricarboxylic 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 estimated to be around 736000 tones/year [1] and the entire production is carried out by fermentation. 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 an intermediate of tricarboxylic acid (TCA) cycle which is obtained when carbohydrates are oxidized to carbon dioxide. It has three carboxylic acid functional groups with three pKa values at pH 3.1, 4.7 and 6.4. It is a ubiquitous intermediate product of metabolism and its traces are found virtually in all plants and animals [3].

Citric acid is known as the most important organic acid produced in tonnage by fermentation and is the most exploited biochemical/biotechnological product [4]. It has an annual production of 1.6 million tons [5] with annual growth demand /consumption rate of 3.5-4.0% [6].

Citric acid is responsible for the tart taste of various fruits

accumulation rises in appreciable amounts only under conditions of drastic imbalances. Among the mentioned strains, the fungus *A. niger* has remained the organism of choice for commercial production because it produces more citric acid per time unit. The problem in the production of citric acid for yeasts is the simultaneous formation of isocitric acid. The main advantages of using *A. niger* are its ease of handling, its ability to ferment a variety of cheap raw materials, and high yields. Industrial strains which produce commercial citric acid are not freely available and only a few can be obtained from international culture collections.

In this context, the isolation and identification of a potential citric acid producing fungal strain from the Western Ghats of Tamilnadu will be attempted in the present investigation considering the fact that novel and potential citric acid producing strain can be isolated from biodiversity niche. This species may be 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

2.1. Sampling Area

The leaf litter soil samples were collected out in Sathuragiri Hills (Western Ghats, Tamilnadu) is about 15

Kms from Srivilliputhur. The town is situated on latitude 9.727288300000001000" North and longitude 77.618638299999930000" East. The town is about 4500 feet above the sea level.

Sample collection

Soil samples were collected from ten different locations in leaf litter degrading soil environment of Sathuragiri Hills (Western Ghats, Tamilnadu). Using a sterilized spatula, 250g of soil sample was collected from each location into 300g-capacity plastic container which was previously washed and rinsed with 70% alcohol. The samples from the ten different sites were conveyed to the Microbiology Laboratory within same day, where they were analyzed.

2.2. Isolation and Identification of Fungal Isolates

Serial dilutions of soil samples from each site were prepared and inoculated in duplicates into Sabouraud's Dextrose Agar (SDA) plates by spread plate methods. The plates were incubated at 30°C for 3 days and observations were made daily to determine the presence of filamentous fungi. Pure cultures of isolates were obtained by repeated sub-culture on SDA [12].

The fungal isolates were identified based on the basis of their cultural and morphological characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically. The isolates were identified with reference to the work of Domsch *et al.* [13].

Figure 1. Map shows the sampling site - (Sathuragiri Hills, Western Ghats, Tamilnadu)

2.3. Determination of Percentage Frequency of Occurrence of Isolates

The percentage frequency of occurrence for each species of fungus was determined by the method of using the formula: $A/B \times 100$; where, A=Number of plates in which species appear and B=Total number of plates incubated for each sites [14].

2.4. Citric Acid Production Medium

Strains were screened for citric acid production in liquid culture which contained sucrose (g/l) 120g; NaNO_3 -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 pH of medium was adjusted to 6 [15].

2.5. Selection of Strain for Citric Acid Production

Based on the colony morphology the fungal isolates were designated as AS1, AS2, AS3, AS4, AS5, AS6 and AS7. These strains were inoculated individually in the production broth and incubated at 37°C for 3 days and then they were analyzed for the citric acid production.

2.6. Isolation and Identification of Fungal Strains

The isolated colonies AS1, AS2, AS3, AS4, AS5, AS6 and AS7 were examined by lactophenol cotton blue technique and identified according to their morphological characteristics [16].

2.7. Screening of the Fungal Cultures

All the seven cultures were screened qualitatively for the production of citric acid as described by Ali, [17]. Czapek-Dox agar medium (20 mL) was poured into individual sterile Petri plates and allowed to cool at room temperature. The plates were incubated at 30°C for 3–5 days. The plates were observed after incubation for yellow zones due to citric acid formation. Strains of *Aspergillus niger* with the widest yellow zone were used for further studies.

2.8. Determination of Citric Acid Production by Fungal Isolates

Czapek-Dox broth (50 mL) was prepared in seven conical flasks and sterilized. They were allowed to cool at room temperature. The conical flasks were inoculated with seven different fungal isolates and incubated at 30°C for 3–5 days. The broth was centrifuged at 5000rpm for 10 minutes and the supernatant was taken for citric acid estimation. Citric acid was determined titrimetrically [18] by using 0.1 NaOH and

phenolphthalein as indicator and calculated as % according to the formula:

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

3. Results

The results of the soil samples of the different locations in Sathuragiri Hills (Western Ghats, Tamilnadu) are presented here. While Table 1 shows the results of the Total Fungal Counts in the soil samples of the different locations. The percentage frequencies of occurrence of fungal isolates are presented in Table 2, while the results of the cultural characteristics of isolates are presented in Table 3. The results of the citric acid levels produced by the fungal isolates are presented in Table 4.

Sri Santhana Mariamman, Thavasipaarai and Periyapasukidai had high fungal counts of 2.4×10^4 , 2.2×10^4 and 2.1×10^4 respectively. Also, **Thaniparai entrance and** Nadu kali had counts of 1.9×10^4 , 1.8×10^4 respectively. Also, by this Pacharisi-Paarai and Pala-AdiKaruppar Swamy had a similar count's of 1.6×10^4 respectively. Whereas, Chinnapasu-kidai sampling area had the least amount of fungal count of about 1.2×10^4 (Table 1).

Table 1. Total Fungal Count (TFC/g) of Soil Samples from Different Location of Sathuragiri Hills (Western Ghats, Tamilnadu)

Sites	TFC/g
A	$1.9 \times 10^4 \pm 0.3$
B	$1.2 \times 10^4 \pm 0.2$
C	$1.4 \times 10^4 \pm 0.7$
D	$2.4 \times 10^4 \pm 0.4$
E	$1.6 \times 10^4 \pm 0.5$
F	$1.3 \times 10^4 \pm 0.6$
G	$2.1 \times 10^4 \pm 0.1$
H	$1.6 \times 10^4 \pm 0.2$
I	$1.8 \times 10^4 \pm 0.3$
J	$2.2 \times 10^4 \pm 0.5$

KEY:

A=Thaniparai entrance; B=Chinnapasu-kidai; C=Naaval Ootru; D=Sri Santhana Mariamman; E=Pacharisi-Paarai; F=Vana-Durgai Amman; G=Periyapasukidai; H=Pala-AdiKaruppar Swamy; I=Nadu kali; J=Thavasipaarai.

Aspergillus sp. had the highest percentage frequency of occurrence of 80%, followed by *Aspergillus flavus* which had 70% *Cladosporium* sp. & *Helminthosporium* sp had 40 & 30% frequency of occurrence respectively. While, *Curvularia* sp had a lowest frequency of occurrence about 10% (Table 2).

Table 2. Percentage frequency of occurrence of fungal isolates

Fungal Isolates	Sites										Occurrence percentage
	A	B	C	D	E	F	G	H	I	J	
<i>Aspergillus niger</i>	+	+	-	+	+	-	+	+	+	+	80
<i>Aspergillus flavus</i>	+	-	+	+	+	-	+	-	+	+	70
<i>Penicillium</i> sp	+	+	-	+	-	+	-	+	-	-	50
<i>Cladosporium</i> sp	+	-	-	+	+	-	-	+	-	-	40
<i>Helminthosporium</i> sp	+	-	-	-	+	+	-	-	-	-	30
<i>Alternaria</i> sp	-	-	+	-	-	-	+	-	-	-	20
<i>Curvularia</i> sp	-	-	-	-	-	-	+	-	-	-	10

Table 3. Cultural characteristics of fungal isolates on Sabouraud's dextrose agar

Isolates	Surface	Reverse
<i>Cladosporium</i> sp.	Black	Brownish
<i>Aspergillus niger</i>	Greenish-Black	Blackish-White
<i>Penicillium</i> sp.	Brownish	Whitish-Red
<i>Alternaria</i> sp.	Brownish	Greenish
<i>Curvularia</i> sp.	Whitish	Whitish
<i>Helminthosporium</i> sp.	Greenish-Black	Brownish
<i>Aspergillus flavus</i>	Greenish	Black

Aspergillus niger found to produce the highest quantity of citric acid (2.2 ± 0.001 g/L), followed by *Aspergillus flavus* (1.32 ± 0.01 g/L), *Penicillium* sp (1.28 ± 0.06 g/L), *Helminthosporium* sp. (1.22 ± 0.02 g/L). Least amount of citric acid was produced by *Cladosporium* sp. (0.92 ± 0.04 g/L). These findings have further confirmed the superior of *Aspergillus niger* as the industrial production species for the citric acid production (Table 4).

Table 4. Citric acid production by fungal isolates.

Isolates Numbering	Fungal Isolates	Amount of Citric Acid (g/l)
AS1	<i>Cladosporium</i> sp.	0.92 ± 0.04
AS2	<i>Aspergillus niger</i>	2.20 ± 0.01
AS3	<i>Penicillium</i> sp.	1.28 ± 0.06
AS4	<i>Alternaria</i> sp.	1.12 ± 0.01
AS5	<i>Curvularia</i> sp.	1.14 ± 0.03
AS6	<i>Helminthosporium</i> sp.	1.22 ± 0.02
AS7	<i>Aspergillus flavus</i>	1.32 ± 0.01

Figure 2a. Lactophenol cotton blue mount of AS1 = *Cladosporium* sp. **Figure 2b.** AS1 = *Cladosporium* sp. on Sabouraud's Dextrose Agar (SDA) plate

Figure 3a. Lactophenol cotton blue mount of AS2 = *Aspergillus niger*

Figure 3b. AS2 = *Aspergillus niger* on Sabouraud's Dextrose Agar (SDA) plate

Figure 4a. Lactophenol cotton blue mount of AS3 = *Penicillium* sp

Figure 4b. AS3 = *Penicillium* sp on Sabouraud's Dextrose Agar (SDA) plate

Figure 5a. Lactophenol cotton blue mount of AS4 = *Alternaria* sp.

Figure 5b. AS4 = *Alternaria* sp. on Sabouraud's Dextrose Agar (SDA) plate

Figure 6a. Lactophenol cotton blue mount of AS5 = *Curvularia* sp

Figure 6b. AS5 = *Curvularia* sp. on Sabouraud's Dextrose Agar (SDA) plate

Figure 7a. Lactophenol cotton blue mount of AS6 = *Helminthosporium* sp.

Figure 7b. AS6= *Helminthosporium* sp. on Sabouraud's Dextrose Agar (SDA) plate

Figure 8a. Lactophenol cotton blue mount of AS7 = *Aspergillus flavus*

Figure 8b. AS7= *Aspergillus flavus* on Sabouraud's Dextrose Agar (SDA) plate

4. Discussion

Similarly, in a study carried out in Dadin Kowa, High Court and Angwan Tiv had high fungal counts of 2.1×10^5 , 2.5×10^5 and 2.2×10^5 respectively followed by those of the Main Campus and Total Round about which had counts of 1.8×10^5 , respectively. Also, Angwan Lambu and Kofar Hausa had counts of 1.5×10^5 while G.R.A and B.C.G had counts of 1.3×10^5 and 1.2×10^5 , respectively. Angwan Wuje had the lowest count of 1.0×10^5 [16].

Equally, *Aspergillus niger* and *Penicillium* sp had the highest percentage frequency of occurrence of 60%, followed by *Rhizopus stolonifer* and *Trichoderma viride* which had 50%. *Aspergillus flavus* and *Alternaria alternata* had 40% frequency of occurrence respectively. *Aspergillus fumigatus* had 20%, while *Absida corymbifera* and *Curvularia lunata* had the lowest frequency of occurrence of 10% [16].

Whereas in a study, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Rhizopus stolonifer*, *Alternaria alternata* and *Curvularia lunata* did not produce citric acid, while *Absida corymbifera*, *Penicillium* sp, *Trichoderma viride*, *Aspergillus flavus* and *Aspergillus niger* produced citric acid found to produce the highest quantity of citric acid ($8.4 \mu\text{g ml}^{-1}$), followed by *Trichoderma viride* ($5.1 \mu\text{g ml}^{-1}$), acid at varying concentrations. *Aspergillus niger* was *A. flavus* ($3.2 \mu\text{g ml}^{-1}$), *Penicillium* sp ($2.0 \mu\text{g ml}^{-1}$) and *Absida corymbifera* ($1.4 \mu\text{g ml}^{-1}$), respectively. These findings have further confirmed the superior of *Aspergillus niger* as the industrial species for the production of citric acid as reported by several workers [19, 20]. The results also agree with Torres, [21] where it was reported that some species of fungi other than *Aspergillus niger* also do produce citric acid.

5. Conclusions

Aspergillus niger found to produce the highest quantity of citric acid ($2.20 \pm 0.01 \text{ g/L}$), followed by *Aspergillus flavus* ($1.32 \pm 0.01 \text{ g/L}$), *Penicillium* sp. ($1.28 \pm 0.06 \text{ g/L}$), *Helminthosporium* sp. ($1.22 \pm 0.02 \text{ g/L}$). Least amount of citric acid was produced by *Cladosporium* sp. ($0.92 \pm 0.04 \text{ g/L}$). 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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