

Full Length Research Paper

Citric acid producing fungi found in the soil environment of Keffi metropolis, Nasarawa state, Nigeria

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Abstract

This investigation was carried out to determine the species of citric acid producing fungi found in the soil environment of Keffi metropolis. Soil samples were obtained from ten (10) different sites. Ten different fungal species were isolated from the ten different sites and these were *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Cladosporium herbarum*, *Rhizopus stolonifer*, *Alternaria alternata*, *Curvularia lunata*, *Absida corymbifera*, *Penicillium* sp and *Trichoderma viride*. The results further showed that *Absida corymbifera*, *Penicillium* sp, *Trichoderma viride*, *Aspergillus flavus* and *Aspergillus niger* produced citric acid at varying levels of concentrations. *Aspergillus niger* produced the highest amount of $8.4\mu\text{g ml}^{-1}$ followed by *Trichoderma viride* which produced $5.1\mu\text{g ml}^{-1}$, while *Aspergillus flavus*, *Penicillium* sp and *Absida corymbifera* produced $3.2\mu\text{g ml}^{-1}$, $2.0\mu\text{g ml}^{-1}$ and $1.4\mu\text{g ml}^{-1}$, respectively. The results of this investigation revealed that strains of citric acid producing fungi are present in the soil environment of Keffi metropolis, Nasarawa state, Nigeria, and these could be harnessed locally for large scale industrial production of citric acid.

Keywords: Screening, citric acid, fungi, Keffi, Nigeria.

INTRODUCTION

Fungi are diverse group of either single-celled or multicellular organisms that obtain food by direct absorption of nutrients. Enzymes, secreted by fungi dissolved the food which thereafter absorbed through thin cell walls, and distributed by simple streaming of the protoplasm (Cooke and Whipps, 1993). Together with bacteria, fungi are primary decomposers present in natural habitat, found everywhere. They are responsible for the decay and decomposition of all organic matter. Some are parasitic on living matter and cause serious plant and animal diseases (Alexopoulos *et al.*, 1996).

Many species are free-living saprophytes which utilise dead organic materials as sources of nutrients and are responsible for the biodegradation of organic materials in the environment, particularly plant materials such as leaf litter and other plant debris. These fungi play a vital role in recycling essential elements, particularly carbon. Fungi are very effective and efficient biodegraders because of the wide range of extra cellular enzymes they produce,

which are capable of degrading complex polymers (Magnuson and Linda, 2007)

Fungi are vital for their ecosystem functions while some are used in the processing and flavouring of foods, and others are used in the production of antibiotics and organic acids. The characteristic wall components are chitin (beta-1, 4-linked homopolymers of N-acetylglucosamine in microcrystalline state) and glucans primarily alpha-glucans (alpha-1,3- and alpha-1,6-linkages) (Griffin, 1994).

Some species of fungi are used for the commercial production of different kinds of organic acids such as citric acid, gluconic acid and Gallic acid. Yet others are useful in the manufacture of inks and dyes. Similarly, synthetic resins are known to be manufactured from fumaric acid formed by black bread mould. Commercially usable oils have been obtained from species of several genera of fungi (Bomstein and Johnson, 1992).

Citric acid, first isolated from lemon juice, is a 6-carbon containing tricarboxylic acid. It is a weak organic acid, naturally found in citrus fruits. It is a natural preservative and also used to add an acidic (sour) taste to foods and soft drinks. It is an important intermediate in the citric acid

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cycle and therefore occurs in the metabolism of almost all living things. It also serves as an environmentally benign cleaning agent and acts as an antioxidant (Abe *et al.*, 1992; Lopez-Garcia, 2002).

Citrate salts of various metals are used to deliver those minerals in a biologically available form in many dietary supplements. The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals (Talaro, 2005) and to increase the solubility of brown heroin (Zhang and Röhr, 2002).

Although, citric acid exists in a variety of fruits and vegetables, it is most concentrated in lemons and limes, where it can comprise as much as 8% of the dry weight of the fruit. At room temperature, citric acid is a white crystalline powder. It can exist either in an anhydrous (water-free) form, or as a monohydrate that contains one water molecule for every molecule of citric acid (Talaro, 2005).

In microbial technique, *Aspergillus niger* are mostly used for the production of citric acid. Cultures of *Aspergillus Niger* are inoculated on sucrose containing medium to produce citric acid. The mould, *Aspergillus niger*, is used to ferment a carbohydrate source such as molasses. Many of the industrial applications of Citric acid are based not only upon its acidic properties, but also upon its powerful sequestering action with various transient metals, such as iron, copper, nickel, cobalt, chromium and manganese (Kirimura *et al.*, 2000).

Approximately 70% of citric acid produced is used in the food and beverage industry for various purposes, 12% in pharmaceuticals and about 18% in other industrial uses. The estimated world production of citric acid was reported as 350.000 tons/year in 1986. It was however recently reported that the world market requirement for citric acid is around 500.000 tons/year (Kubicek *et al.*, 1980).

Although, *A. niger* has primarily been used in the citric acid production process, other strains of fungi apart from *A. niger*, various kinds of yeast and some bacteria are known to accumulate citric acid in various kinds of media. The reason for choosing *A. niger* over other potential citrate producing organism include the availability of raw materials (molasses) used as substrate and high consistent yields. The source of sugar in this technique is corn steep liquor, molasses, hydrolyzed corn starch or other inexpensive sugary solutions (Torres, 1994). In the biotechnology and pharmaceutical industries, citric acid is preferentially used in place of nitric acid because nitric acid is considered hazardous to dispose after usage (Zhang and Röhr, 2002).

Citric acid can be added to ice cream to keep fat globules, and can replace fresh lemon juice in recipes. Citric acid is used along with sodium bicarbonate in a wide range of effervescent formulae, both for ingestion (as powders and tablets) and for personal care (such as bath salts, bath bombs, and cleaning of grease). Citric

acid is commonly employed in wine production as a substitute or enhancer during fermentation in preference to other acids, such as tartaric acid, because of its high stability, pleasant flavour and ability to impart brilliance to the finish wine (Kubicek *et al.*, 1980; Röhr *et al.*, 1993).

When applied to hair, citric acid opens up the outer layer, also known as the cuticle. While the cuticle is open, it allows for a deeper penetration into the hair shaft. It can be used in shampoo to wash out wax and coloring from the hair. Citric acid is also used as a stop bath in photography. The developer is normally alkaline, so a mild acid will neutralize it, increasing the effectiveness of the stop bath when compared to plain water (Zhang and Röhr, 2002).

The goal of this work is to obtain high citric-acid producing fungi in the local soil habitat of Keffi city in Nasarawa State of Nigeria.

MATERIALS AND METHODS

Study Area

The research was carried out in Keffi Metropolis, the headquarters of Keffi Local Government Area of Nasarawa State, Nigeria. Keffi is about 58Km from Abuja (the Federal Capital Territory) and 128Km from Lafia, the Nasarawa State Capital. The town is situated on latitude 8° 5" North and longitude 7° 50" East. The town is about 850 meters above the sea level (Awka *et al.*, 2007).

Sample Collection

Soil samples were collected from ten (10) different locations in Keffi town, and these locations were Government Reserve Area (GRA), Dadinkowa, Angwan Lambu, High Court, Nasarawa State University Main Campus, Angwan waje, Angwan Tiv, Kofar Hausa, BCG Area and Total Round-About area.

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 30 minutes, where they were analyzed.

Determination of Physico-Chemical Properties of the Soil Samples

Soil Types

The soil samples from each sites was sieved and evaluated by the unified analysis technique so as to determine the soil type (Whitbread *et al.*, 1996, Pettijohn,

2006).

Soil pH

The pH of the soil samples were determined using digital pH meter according to standard methods of Association of Official Analytical Chemists (AOAC,1990).

Soil Temperature

The soil temperature was determined by the use of a field thermometer. At each site, the thermometer was inserted into the soil up to the depth of 5 cm and then allowed to stay for 10minutes, after which the temperature reading was obtained. The temperature values of three consecutive readings were recorded and the mean was determined for each site (Dix and Webster, 1995).

Isolation and identification of fungal isolates

Serial dilutions of soil samples from each sites were prepared and inoculated in duplicates unto Potato Dextrose Agar (PDA) and the Malt Extract Agar (MEA) plates by both spread plate and pour plate methods. The plates were incubated at 30°C for 7days, and observations were made daily to determine the presence of filamentous fungi (Nester *et al.*, 1998; Wainright *et al.*, 1993). Pure cultures of isolates were obtained by repeated sub-culture on PDA and MEA.

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.*(1980).

Determination of percentage frequency of occurrence of isolates

The percentage frequency of occurrence for each species of fungus was determined by the method of Sampo *et al.*(1997) 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.

Determination of citric acid production by fungal isolates

The fungal isolates were inoculated into Nutrient Broth and incubated at 30°C for 7 days (Magnuson and Linda, 2007; Sikander *et al.*, 2001). The medium (Nutrient Broth)

was centrifuged after incubation period, and 9ml of the supernatant was obtained and precipitated with 5ml of Calcium Hydroxide ($\text{Ca}(\text{OH})_2$). A characteristic milky color change indicated the presence of Citric Acid. The mixture was then treated with 3ml of sulphuric acid to precipitate Calcium Sulphate (Roehr *et al.*, 1996). The solution mixture was then filtered and citric acid was purified by ion exchange chromatography (Magnuson and Linda, 2007; Sikander *et al.*, 2001). The pure citric acid was then measured by the recommended pyridine – acetic anhydride method (Marrier and Boulet, 1958).

RESULTS

The results of the physico-chemical properties of soil samples of the different locations in Keffi Metropolis are presented in Table 1, while Table 2 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 3, while the results of the cultural characteristics of isolates are presented in Table 4. The results of the citric acid levels produced by the fungal isolates are presented in Table 5.

DISCUSSION

The soils of Dadin Kowa,Angwan Lambu,Main Campus,Angwan Wuje,Kofar Hausa,B.C.G. and Total Round About are sandy, while those of and G.R.A. and Angwan Tiv are loamy and that of High Court is clay. Soil samples from Angwan Lambu, High Court, B.C.G and Total Round About had relatively high pH values which ranged from 7.2 and 8.2, while soil samples from Agwan Wuje and Kofar Hausa had low pH values which ranged from 5.7 to 5.9. The temperature of the soil at all the sites during the time of this investigation (rainy season) ranged between 25°C and 26°C.

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 .

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%.

Aspergillus fumigatus, *Cladosporium herbarum*,

Table 1. Physico-Chemical Properties of Soil Samples of the Different Locations in Keffi Metropolis

Sites	Soil Types	pH	Temperature ($^{\circ}$ C)
A	Sandy	6.9 \pm 0.4	26 \pm 0.5
B	Loamy	6.7 \pm 0.1	25 \pm 0.5
C	Sandy	8.2 \pm 0.2	26 \pm 0.5
D	Clay	7.2 \pm 0.3	25 \pm 0.5
E	Sandy	6.2 \pm 0.6	26 \pm 0.5
F	Sandy	5.9 \pm 0.9	26 \pm 0.5
G	Loamy	6.0 \pm 0.8	25 \pm 0.5
H	Sandy	5.7 \pm 1.1	25 \pm 0.5
I	Sandy	8.0 \pm 1.1	26 \pm 0.5
J	Sandy	7.8 \pm 0.9	25 \pm 0.5

KEY:

A=Dadinkowa
 B=G.R.A
 C=Angwan Lambu
 D=High Court
 E=Main Campus
 F=Angwan Wuje
 G=Angwan Tiv
 H=Kofar Hausa
 I=B.C.G
 J=Total Round About

Table 2. Total Fungal Count (TFC/g) of Soil Samples from Different Location of Keffi Metropolis

Sites	TFC/g
A	2.1 X 10 ⁵ \pm 0.4
B	1.3 X 10 ⁵ \pm 0.3
C	1.5 X 10 ⁵ \pm 0.1
D	2.5 X 10 ⁵ \pm 0.8
E	1.8 X 10 ⁵ \pm 0.1
F	1.0 X 10 ⁵ \pm 0.6
G	2.2 X 10 ⁵ \pm 0.5
H	1.5 X 10 ⁵ \pm 0.1
I	1.2 X 10 ⁵ \pm 0.4
J	1.8 X 10 ⁵ \pm 0.1

KEY:

A=Dadinkowa
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Table 3. Percentage Frequency of Occurrence of Fungal Isolates

Fungal Isolates	Sites										Percentage occurrence
	A	B	C	D	E	F	G	H	I	J	
<i>Aspergillus niger</i>	+	-	+	+	-	+	-	+	-	+	60
<i>A. flavus</i>	-	+	-	+	-	+	+	-	-	-	40
<i>A. fumigatus</i>	-	+	-	-	-	-	+	-	-	-	20
<i>Absida corymbifera</i>	+	-	-	-	-	-	-	-	-	-	10
<i>Penicillium sp</i>	+	+	-	+	+	+	-	-	+	-	60
<i>Cladosporium herbarum</i>	-	+	+	-	-	-	+	-	+	-	40
<i>Trichoderma viride</i>	+	-	+	-	+	-	-	+	-	+	50
<i>Rhizopus stolonifer</i>	+	+	-	-	+	-	+	-	+	-	50
<i>Alternaria alternata</i>	-	-	+	-	-	+	-	+	+	-	40
<i>Curvularia lunata</i>	-	-	-	+	-	-	-	-	-	-	10

KEY: A=Dadinkowa; B=G.R.A; C=Angwan Lambu; D=High Court;
E=Main Campus; F=Angwan Wuje; G=Angwan Tiv; H=Kofar Hausa;
I=B.C.G; J=Total Round About

Table 4. Cultural Characteristics of Fungal Isolates on Malt Extract Agar (MEA)

Isolates	Surface	Reverse
<i>Aspergillus niger</i>	Black	Yellowish
<i>Aspergillus fumigatus</i>	Blue-Green	White
<i>Absida corymbifera</i>	Greyish	Whitish
<i>Penicillium sp</i>	Greenish	Yellowish
<i>Cladosporium herbarum</i>	Greenish-Black	Black
<i>Trichoderma viride</i>	Whitish-Green	Pale-Yellow
<i>Rhizopus stolonifer</i>	Greyish-White	Pale- White
<i>Alternaria alternata</i>	Greenish-Black	Brownish
<i>Curvularia lunata</i>	Pinkish-Grey	Brownish-Black
<i>Aspergillus flavus</i>	Greenish-Yellow	Reddish-Brown

Table 5. Citric Acid Production by Fungal Isolates

Fungal Isolates	Citric Acid Production	Amount of Citric Acid Produced (μgml^{-1})
<i>Aspergillus niger</i>	+	8.4 \pm 0.01
<i>Aspergillus fumigatus</i>	-	0.0 \pm 0.00
<i>Absida corymbifera</i>	+	1.4 \pm 0.02
<i>Penicillium sp.</i>	+	2.0 \pm 0.01
<i>Cladosporium herbarum</i>	-	0.0 \pm 0.00
<i>Trichoderma viride</i>	+	5.1 \pm 0.02
<i>Rhizopus stolonifer</i>	-	0.0 \pm 0.00
<i>Alternaria alternata</i>	-	0.0 \pm 0.00
<i>Curvularia lunata</i>	-	0.0 \pm 0.00
<i>Aspergillus flavus</i>	+	3. 2 \pm 0.00

Key:

+ =Positive

- =Negative

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 produced 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 (Rohr *et al.*, 1993; Kirimura *et al.*, 2000). The results also agree with that of Torres (1994) where it was reported that some species of fungi other than *Aspergillus niger* also do produce citric acid.

CONCLUSION

This investigation demonstrated that *Aspergillus niger*, *Trichoderma viride*, *Aspergillus flavus*, *Penicillium* sp. and *Absida corymbifera* do produce varying levels of citric acid. The results thus revealed that the soil environment of Keffi metropolis has species of fungi that are capable of producing reasonable amounts of citric acid. These fungal isolates could be useful for the industrial production of citric acid if the process is scaled up.

However, there is need for further survey of other locations in the area which may lead to development of high yielding strains of fungal isolates that exhibit potential for the production of citric acid. The assertion that certain species of fungi other than *A. niger* have ability to produce citric acid is to a large extent verified by this investigation.

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