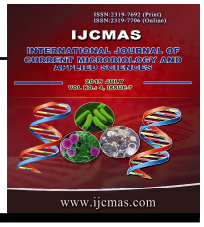
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Original Research Article

Fermentation of the fruit pulp of *Dialium guineense* (Velvet tamarind) for Citric Acid Production Using Naturally Occurring Fungi

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Keywords

Citric acid, Solid state fermentation, Fruit pulp, Czapek-dox agar, Substrate

A B S T R A C T

The aim of this research was to produce citric acid from the fruit pulp of *Dialium guineense* using naturally occurring fungi in a solid state fermentation. The fruit pulp was de-capped from the seed and dried at 60^{o}C in an oven. Natural fermentation was carried out for seven days at 28^{0}C ± 2. The fungi were isolated and identified as *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Penicillium citrinum, Mucor hiemalis, Mucor racemosus, Rhizopus stolonifer, Alternaria tenuis Syncephalastrum racemosus, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. They were screened for citric acid production using standard methods. Yellow coloration confirmed citric acid production after five days of incubation. Organisms that were strongly positive for citric acid production include *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Penicillium citrinum, Mucor racemosus,* and *Alternaria tenuris*. They were used to ferment the fruit pulp of *D. guineense* in a solid state fermentation by inoculating 2ml spore suspension of $2x10^{5}$ spores/ml of each of the isolates on separate Erlenmeyer flasks of 250ml for seven days at 28 ± 2 ^{o}C. Quantitative production of citric acid was determined using standard methods. *Aspergillus niger* had the highest citric acid production of 14.07mg/g while *Aspergillus flavus* had the lowest citric acid production of 6.34mg/g. The result of this investigation reveals that some of the naturally occurring fungal isolates from the natural fermentation of the fruit pulp of *D. guineense* are capable of utilizing the substrate for the production of citric acid and could be employed for the production of citric acid on large scale.

Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word *citrus*. Citric acid was discovered in 1784 by Karl Wilhelm Scheele and the first raw material from which it was produced was lemons during the 19th century. The first discovery of citric acid was in 1893 by Wehmer as a fungal by product (Soccol, *et al.*, 2006). The production of citric acid in high concentrations from disaccharides was reported by James Currie in 1917

(Vandenberghe *et al*., 1999). Ever since then many researchers have employed various raw materials for the production of citric acid. It is a weak organic acid commonly found in citrus fruits (Makut and Ade-Ibijola, 2012). It is a primary metabolic product formed in the tricarboxylic acid (or Krebs) cycle (Max *et al*., 2010). It is used extensively in the food, pharmaceutical, cosmetics, agricultural, dairy, biochemical industries among others (Dhillon, *et al*., 2011; Makut and Ade-Ibijola, 2012). Citric acid is one of the most common products which have a never ending demand in the global market. It plays a pivotal role of an acidulant in food and beverage industries. Citric acid fermentation is one of the primitive fermentations but still its production is going on increasing with passage of time (Pandey *et al*., 2013). Citric acid is an important commercial product. In the present day mostly citric acid is produced by fungal fermentation. Chemical synthesis of citric acid is possible but it is no cheaper than fungal fermentation (Dhandayuthapan, 2009). Though bacteria such as *Arthrobacter paraffinens, Bacillus licheniformis and Corynebacterium sp* have been employed for citric acid production, fungi is still the most commonly used microorganism for citric acid production. Fungal species that have been employed include *Aspergillus niger, A. aculeatus, A. carbonarius, A. awamori, A. foetidus, A. phoenics, P. janthinellum* and yeasts such as *Candida tropicalis, C. oleophila, C. guilliermondii, Yarrowa lipolytica and Saccharomycopsis lipolytica* among others (Vandenberghe *et al.*, 1999; Pandey *et al.*, 2001*;* Papagianni, 2007). *Dialium guineense* (Velvet tamarind) is a woody plant that occurs in the rain forest region of West Africa. It grows in dense savannah forests up to 30m high with dark green glossy leaves (Ewedje and Tandjiekpon, 2011). The young leaves are sometimes chewed for its tangy taste. Various parts of the plant are of economic and medicinal use. The fruits and leaves are used for food and fodder, the wood and roots are utilized for timber and charcoal. Also, roots, leaves and fruit bark are employed for medicinal purposes while the young stems and branches can be used as fishing tools (Ewedje and Tandjiekpon, 2011). The fruit pulp of *Dialium* guineense has a good nutritional content as reported by Borokini, (2006) with 8.64% crude protein, 4.36% crude fat, 2.74% crude fiber, 26% moisture, 2.04% ash and 55.58% carbohydrate. Due to its high carbohydrate content, the fruit pulp can be employed for citric acid production using microorganisms that would naturally ferment it most especially, fungi. The present investigation is focused on isolating naturally occurring fungi from the fruit pulp of *Dialium guineense* through solid state fermentation and screening the fungal isolates for their potential for citric acid production.

Materials and Methods

Collection and procession of fruits

The *Dialium guineense* fruits were collected from Ketu market in Lagos. They were taken to the herbarium section of Plant Biology Department, University of Ilorin and identified as *Dialium guineense* with voucher No. UIH1064. The Velvet tamarind fruits were processed by dehulling the shells manually. The fruit pulp were separated from the seeds and then dried in an oven at 60^{o}C for 24 hrs. 250g of the fruit pulp was put in plastic fermentors after mixing thoroughly with 250ml sterile distilled water using a sterile rod to obtain a uniform mash and then covered with clean muslin cloth for fermentation to commence for 168 hours at ambient temperature (Kayode and Sani, 2008).

Isolation and maintenance of the microorganisms

The fungi were isolated on potato dextrose agar from the fermenting substrate by serial dilution method; they were then incubated for 5 days at (28 ± 2)°C. Sub culturing was done until pure cultures were obtained. Each isolate was maintained on PDA slants at 4°C. The characterization and identification were done as described by A.O.A.C, (2000) and Fawole and Oso (2004).

Screening microorganisms for citric acid production

The 11 naturally occurring fungal isolates were screened qualitatively for citric acid production by plate method on Czapek-dox agar containing Bromocresol green as indicator. The spore suspension of the isolates were put in the agar wells of 5mm in diameter of the medium plates and allowed to grow for 5 days. Yellow zones indicated citric acid production.

Mono culture fermentation

The fruit pulp of *Dialium guineense* was fermented for seven days by solid state fermentation using naturally occurring fungal isolates. A spore inoculum of each of the fungal isolate that showed yellow zones after the screening was prepared by washing the culture with sterile distilled water and the number of spores was counted to be 2 x 10^5spores/ml (Hang and Woodhams, 1984; Kuforiji *et al.*, 2010). This was used to inoculate sterilized pre-weighed sample (fruit pulp of *D. guineense*) of twenty milligrams in Erlenmeyer flasks (250ml) of distilled water in a solid state fermentation. Fermentation was allowed to go on for 7days and samples were withdrawn for analysis on a daily basis (Sani *et al.*, 1992). One gram of the fermenting fruit pulp was dissolved in 100 millilitres of distilled water. It was filtered and used for further analysis for citric acid assay and titratable acidity.

Determination of pH

The pH of the fruit pulp was measured using a Crison micro pH meter (Model 2000). Five grams of the dried fruit pulp of the fruit was weighed into a 100 ml beaker; 45 ml of distilled water (pH 7.0) was added and allowed to stand for 30 minutes with occasional stirring with a glass rod. The suspension was allowed to stand for a period of thirty minutes before the pH was measured (AOAC, 2000).

Determination of Titratable Acidity

Sodium Hydroxide solution (0.01M) was titrated against 10 ml of the fruit pulp filtrate using two drops of phenolphthalein as indicator. The filtrate was obtained by dissolving 1 gram of the dried fruit pulp in 100 ml of distilled water. It was then filtered using filter paper. The point at which the color changes from colourless to pink was taken as the end point, AOAC, (2000). The amount of acid was measured in milligram/per hundred grammes (mg/100g) as shown by the formula below:

$$\text{Titratable acidity} = \frac{0.01x\ 0.064\ x\ T\ x\ 1000}{Ft\ x\ S}$$

Where 0.01= Molarity of NaOH used

0.064 = conversion factor for citric acid

T = titre value, Ft = quantity of filtrate used; S= quantity of sample weighed

10 = dilution factor and 1000 = conversion factor from mg to 100g

Assay for citric acid

This was done according to the method of Marier and Boulet (1958), Pandey *et al.* (2013). 1ml of the diluted culture filtrate

along with 1.30ml of pyridine was added in the test tube and swirled briskly. Then 5.70ml of acetic anhydride was also added to the test tube. The test tube was then placed in a water bath at 32 ^{o}C for 30 minutes. The absorbance then was measured on a spectrophotometer (420nm) and the citric acid contents of the sample were estimated by calculating the quantity of citric acid from the citric acid curve. Samples were drawn at 24hours intervals over a period of 7days and they were assayed for citric acid. Blanks of acids without substrate were used with all acid assays.

Effect of pH and Titratable Acidity on the fermenting substrate

The pH and titratable acidity of the fermenting substrate (fruit pulp of *D.guineense*) was monitored during the period of fermentation by the naturally occurring fungi. The total titratable acidity was determined by 0.1N NaOH. The samples were taken in two replicate readings.

Effect of incubation period on citric acid production

The citric acid production by the fungal isolates that were used for the fermentation was studied on a daily basis for 7days.

Results and Discussion

11 fungal isolates were obtained during the natural fermentation of the fruit pulp of *D.guineense*. The fungal isolates were characterized and identified after obtaining pure culture of isolates by repeated sub culturing. The basic examinations carried out for the fungal characterization on the pure cultures were macroscopic and microscopic examinations. The isolates were identified on the basis of their cultural and morphological characteristics in accordance with Harrow (1968); Samson and Van Reen-Hoekstra (1988); Cheesbrough (2000). These include *Mucor racemosus, Mucor hiemalis Alternaria tenuis, Schizosaccharomyces pombe, Saccharomyces cerevisiae*, *Syncephalastrum racemosus, Rhizopus stolonifer*, *Aspergillus flavus, Penicillium chrysogenum, Aspergillus niger* and *Penicillium citrinum*. All the 11 fungal isolates were screened by agar plate method (Table I) and the zones of yellow colouration as a result of citric acid production was measured in mm as shown in Table II. The isolates that were strongly positive after the screening for citric acid production were used for further studies. The zone of yellow clearance was the highest in the plate seeded with *Aspergillus niger* with 4.45cm while it was the lowest in the plate seeded with *Mucor racemosus* with 2.0 mm. There was a significance difference between these fungal isolates at $p<0.05$. Some of the fungal isolates that were positive after screening which had the ability to produce citric acid has been reported by many researchers to accumulate citric acid. *Penicillium* spp, *Aspergillus* spp and *Mucor* spp have been reported by Vandenberghe, *et al.* (1999); Sinha *et al.* (2001); Papagianni (2007); Berovic and Legisa (2007); Max *et al.* (2010) among others. The result also agrees with that of Makut and Ade-Ibijola (2012) that reported that some species of fungi other than *A. niger* can produce citric.

Effects of pH and titratable acidity

The pH and titratable acidity of the fermenting medium was monitored during the period of mixed fermentation. It was observed that the pH decreased considerably as the total titratable acidity increased (Figure I). The pH of the medium is of utmost importance especially in the production of acid. It is paramount to ensure that the substrate to be used is capable of

producing acid by the fungi that ferments it. This is evident in the low pH of the fermenting fruit pulp of *D. guineense* which was used as the substrate for fermentation to produce citric acid. The pH and titratable acidity of the fermenting substrate determines its suitability for the production of citric acid because a low pH and high titratable acidity indicates the accumulation of acid. This is similar to the findings of Kayode and Sani (2008) who reported that the gradual decrease in the pH of substrate with a concurrent increase in titratable acidity may be as a result of the fast growth of the various fungi in the fermenting substrate and the resultant production of some metabolic substances, such as organic acids since the fermentation was basically aerobic.

Table.1 Screening of Fungal Isolates for Citric Acid Production

S/N	CODES	REACTION
1	*Mucor racemosus*	++
2	*Mucor hiemalis*	_
3	*Alternaria tenuis*	++
4	*Schizosaccharomyces pombe*	_
5	*Saccharomyces cerevisiae*	+
6	*Syncephalastrum racemosus*	+
7	*Rhizopus stolonifer*	_
8	*Aspergillus flavus*	++
9	*Penicillium chrysogenum*	++
10	*Aspergillus niger*	++
11	*Penicillium citrinum*	++

Codes: + = positive, ++ = strongly positive, - negative

Table.2 Zones of Citric Acid Production of Fungal Isolates on Czapek-dox Agar

S/N	FUNGAL ISOLATES	ZONES OF CITRIC ACID PRODUCTION (mm)
1	*Mucor racemosus*	2.0 ± 0.00^{a}
2	*Alternaria tenuis*	3.75 ± 0.05^{b}
3	*Aspergillus flavus*	4.3 ± 0.30^{bc}
4	*Penicillium chrysogenum*	4.1 ± 0.10^{bc}
5	*Aspergillus niger*	4.45 ± 0.05^{c}
6	*Penicillium citrinum*	3.75 ± 0.25^{b}

Values represented in the table are means of two replicate readings and standard error of means of zones of yellow colouration on Czapek-dox agar plates of fungal isolates. Values within the column having different superscripts are significantly different at $p<0.05$

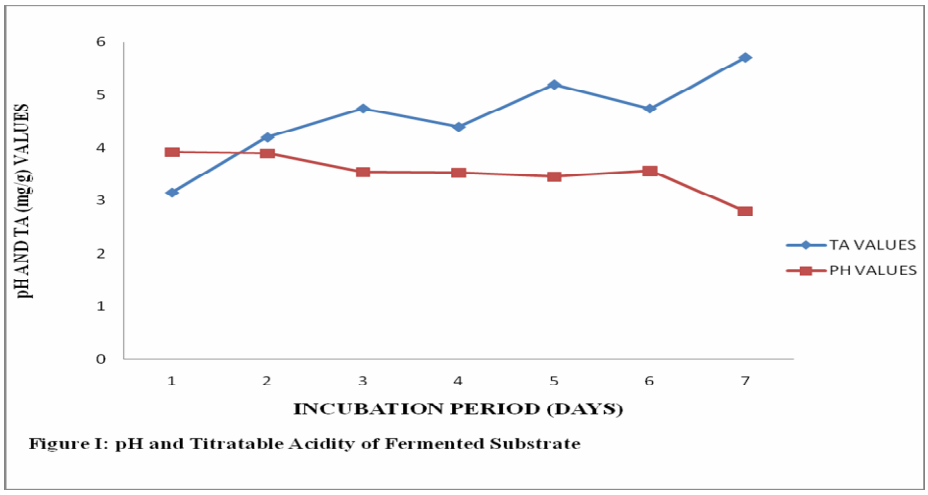
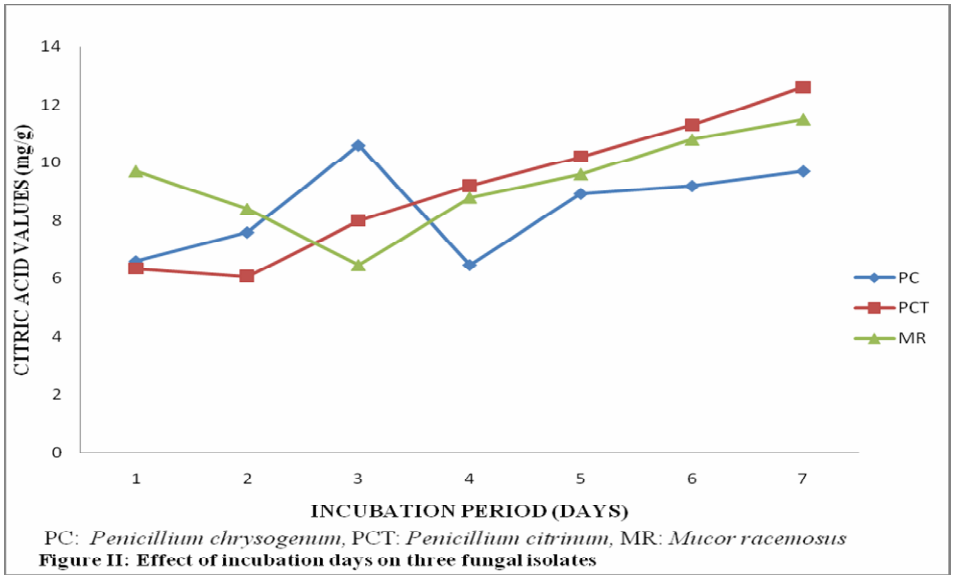
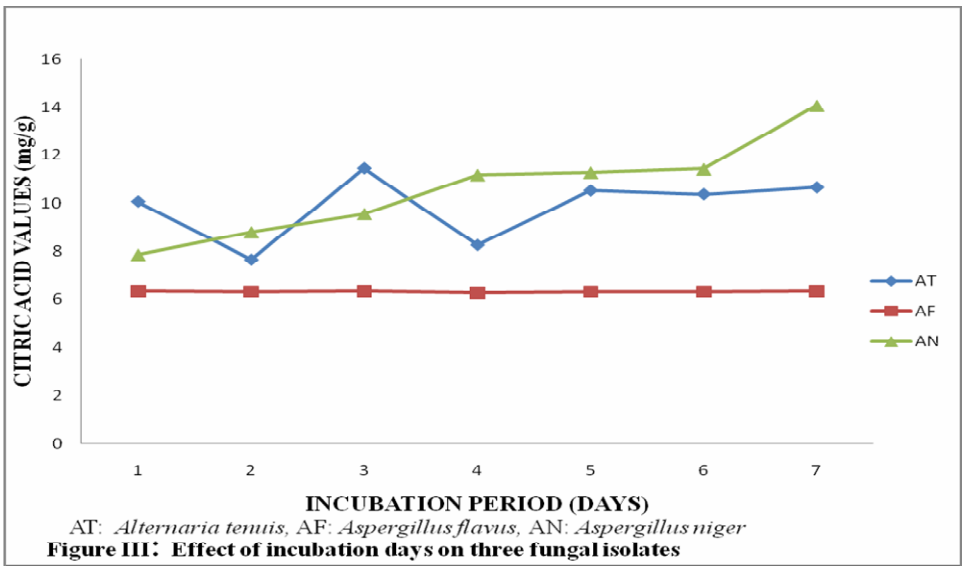


Figure I: pH and Titratable Acidity of Fermented Substrate



PC: *Penicillium chrysogenum*, PCT: *Penicillium citrinum*, MR: *Mucor racemosus*

Figure II: Effect of incubation days on three fungal isolates



AT: *Alternaria tenuis*, AF: *Aspergillus flavus*, AN: *Aspergillus niger*

Figure III: Effect of incubation days on three fungal isolates

Effects of fermentation period on citric acid production

The optimal time of incubation for maximum citric acid production has been reported to vary both with the organism and fermentation conditions (Ali *et al*., 2002). The effect of fermentation period was carried out for each of the selected fungal isolates and it was observed that citric acid production increased gradually when the isolates were used for the fermentation, however citric acid production attained its maximum values for some of the isolates after 7days (168 hours) of inoculation except for *Penicillium chrysogenum* and *Alternaria tenuis* that had their optimum production after 3days (72hours) of inoculation (Figure II and III). This agrees with the findings of Rao and Reddy, (2013) when they produced citric acid from oat bran inoculated with *A. niger*. Increasing the fermentation period did not enhance an increment of citric acid production. This might be as a result of an inhibitory effect of high concentration of citric acid, depletion of sugar and decay in enzyme system responsible for citric acid biosynthesis (Nwoba *et al*., 2012). However, in contrast some researchers such as Arzumanov *et al*. (2000); Alvarez *et al*. (2007); Lofty *et al*. (2007); Nadeem *et al*. (2010); Maharani *et al*. (2014) observed that maximum citric acid concentration was obtained after 8 days (192 hours) of fermentation period.

Naturally occurring fungi growing on fermented *Dialium guineense* fruit pulp have high potential of producing citric acid which has great demand in the food and non food application industries. Although many microorganisms can be employed to produce citric acid, *Aspergillus niger* still remains the main industrial producer (Max *et al*.2010). From the findings of this research work, each of the fungal isolates produced citric acid optimally on different incubation days but *A. niger* had the highest production of 14.07mg/g on day 7 while *A. flavus* had the lowest citric acid production of 6.34mg/g.

The increase in the use of citric acid in various industries has led to a corresponding increase in demand. However, the high cost of chemically synthesizing the acids has led to the search for cheaper means of producing citric acids. *Dialium guineense* fruit pulp is a promising substrate for citric acid production using naturally occurring fungi. This can reduce drastically the wastage of the fruits in the wild and maximize fully the potentiality of the pulp for industrial uses. However there's the need for further investigation of high yielding strains of these fungal isolates that shows a considerable potential for production of citric acid.

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