



FERMENTATION CONDITIONS AND PROCESS OPTIMIZATION OF CITRIC ACID PRODUCTION BY YEASTS

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ABSTRACT

Article History

Received: 3 October 2018

Revised: 8 November 2018

Accepted: 11 December 2018

Published: 31 December 2018

Keywords

Citric acid

Yeast

Yield

Parameters

Recrystallized.

The aim of this study was to isolate and screen citric acid producing yeasts using low cost substrates. Thirty three yeast isolates were obtained from pineapple, plantain and sugar cane waste and identified as; *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida tropicalis*, *Pichia guilliermondii*, *Debaromyces* sp., *Candida parapsilosis*, *Candida rugosa*, and *Candida krusei*. *Candida tropicalis* had zone of clearance of 49 ± 2.1 mm in diameter, *Pichia guilliermondii* had 40 ± 1.2 mm. *Saccharomyces cerevisiae* produced citric acid with glucose with 105.0 mg/l. *C. tropicalis* yielded 132.2 mg/l with sodium nitrate. *S. cerevisiae* and *C. tropicalis* produced citric acid at pH 6 with 23.70mg/l and 23.80mg/l. *P. guilliermondii* at pH 4 produced 23.00mg/l. The temperature of 30°C favoured *S. cerevisiae* and *C. tropicalis* yielding 40.80mg/l and 39.80 mg/l. After extraction, the yield of the citric acid was 4.231g, 3g of which was recrystallized to yield 2.16g of pure citric acid resulting into 72% recovery. The result indicated that pineapple wastes, plantain wastes and sugarcane cane are potential sources of yeasts that can be used for the production of citric acid.

Contribution/Originality: This study contributes in the existing literature by providing basic information for other researchers regarding the isolation and the screening of yeasts for the production of citric acid using low cost substrates, as well as to study the effect of various fermentation parameters on citric acid production.

1. INTRODUCTION

Citric acid (2-hydroxy-1, 2, 3-propanetricarboxylic acid) is an intermediate and important commercial product of metabolism and its traces are found in all plants and animals tissues (Kamzolova *et al.*, 2008). Citric acid is a commercially valuable organic acid, widely used in food, pharmaceutical and beverage industries. It has several applications in cosmetic industries as an acidulant flavour enhancer, preservative, antioxidant and emulsifier and chelation agent (Roehr, 1998).

Citric acid (C₆H₈O₇) is a natural component and common metabolite of plants and animals. It is the most versatile and widely used organic acid in foods, beverages, detergents and pharmaceuticals. Citric acid is accepted as GRAS (Generally Recognized as Safe) and approved by the Joint FAO/WHO Expert Committee on Food Additives (Dhillon *et al.*, 2011). In its pure form, this citric acid is colourless, soluble in water and solid at room temperature. Due to its functionality and environmental acceptability, it is used in numerous industrial and research applications for chelation, buffering, pH adjustment, and also as a source of energy for controlled bacterial metabolism (Yoo *et al.*, 2004).

The annual production of citric acid is estimated to be about 1.4 to 1.5 million tons per year and its demand is estimated to be growing at a rate of about 3.5 to 4.0% annually (Soccol *et al.*, 2006; Lazar *et al.*, 2011; Show *et al.*, 2015). Of the total amount of citric acid produced annually, about 70% is utilized by the food industry; about 12% is utilized by pharmaceutical industries as flavouring, anticoagulant and preservative while the remaining 18% is utilized by other industries such as cosmetics, detergent, textile, oil recovery and paper (Soccol and Vandenberghe, 2003; Silva *et al.*, 2011). Citric acid is also used in the detergent industry as a phosphate substitute, because of less eutrophic effect, and in the cement industry to slow down the process of hardening (Max *et al.*, 2010). Citric acid is responsible for the tart taste of various fruits in which it occurs (*i.e.* lemons, limes, figs, oranges, pineapples, pears and goose-berries) (Francis, 2000). Hence, citric acid is used to impart a pleasant tart flavours to foods and beverages. It is also used in the industries to achieve acidulation, antioxidation, emulsification, preservation, flavour enhancement, and as plasticizer and synergistic agent (Soccol *et al.*, 2006).

Citric acid production by fermentation is the most economical and widely used way of obtaining this product. More than 90 % of the citric acid produced in the world is obtained by fermentation. Citric acid can be produced using three types of microbial fermentation. These are surface fermentation, submerged fermentation and solid-state fermentation (Papagianni *et al.*, 2005). The use of citric acid in industries is attributed to its low/non-toxicity, high solubility; biodegradability and palatability (Ali *et al.*, 2002).

Citric acid is known as the most important organic acid produced in tonnage by fermentation and is the most exploited biochemical product (Soccol *et al.*, 2006; Lotfy *et al.*, 2007). All developed countries follow a conventional procedure involving the use of *Aspergillus niger* (producer) and molasses (substrate) for citric acid production. Use of yeasts such as *Y. lipolytica* however has several advantages over the use of *Aspergillus* spp, since yeast strains show lower sensitivity to low dissolved oxygen concentrations and heavy metals, and give higher product yields.

Citric acid can be produced by microorganisms and related yeast species. Presently most citric acid is being produced using *Aspergillus niger* because of its high citric productivity but there is dearth of information on the use of yeast for the production of citric acid. The constant increase in citric acid consumption, compared to its natural supply and some problems in traditional production process (molasses treatment and environmental issues) leads to the need of exploring the use of alternative microorganisms like yeasts species. Use of yeasts such as *Y. lipolytica* however has several advantages over the use of *Aspergillus* spp, since yeast strains show lower sensitivity to low dissolved oxygen concentrations and heavy metals, and give higher product yields.

The aim of this study was to isolate and screen citric acid producing yeasts using low cost substrates, as well as to study the effect of various fermentation parameters on citric acid production.

2. MATERIALS AND METHODS

2.1. Sample Collection

Samples of pineapple wastes, plantain wastes and sugarcane were collected in a sterile polythene bag from Agbowo and Bodija market in Ibadan, Oyo State, Nigeria. The samples were transported to the laboratory immediately for analysis.

2.2. Sample Preparation

The samples were rinsed with sterile distilled water and then kept in a sterile container from which samples were analysed daily. Old samples were discarded after some days and replaced

2.3. Isolation of Citric Acid Producing Yeasts

Isolation was done by using pour plate method. One gram of the plantain and pineapple wastes and sugarcane waste was dispensed in 9mls of sterile distilled water and 1ml of the sugarcane juice was dispensed in 9mls of sterile distilled water. One ml from each sample was plated out using Potato Dextrose Agar (PDA) and then incubated at

room temperature for 48-72hrs at 25 °C. The culture was maintained on Potatoe Dextrose Agar (PDA) plate at 4°C for further studies.

2.4. Characterization and Identification of Yeast Isolates

Different identification system or techniques were used in the identification of the isolates using standard morphological and physiological test and identification keys described by Kurtzman *et al.* (2003) and Barnett (2007) which includes: macroscopic identification, microscopic evaluation, sugar fermentation etc.

2.5. Screening of Isolates for Citric Acid Production in a Solid Medium.

The basal medium for screening of citric acid was modified as described by Finogenova *et al.* (2008). The yeast isolates were grown on a medium of the following composition (g/L): (NH₄)₂SO₄ 1.0; KH₂PO₄ 1.0; K₂HPO₄ . 3H₂O 0.16; MgSO₄ . 7H₂O 0.70; NaCl 0.50; Ca(NO₃) . 4H₂O 0.40; bromocresol green 0.40; glucose 20.0; agar 20.0 and 1 L of distilled water. The yeast growth, which showed yellow colour zones around them, was selected as the citric acid producers.

2.6. Fermentation Medium and Conditions

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. The production medium used contained (g/l): Glucose 30, (NH₄)₂SO₄ 0.5, yeast extract (Fluka, nitrogen content: 7% (w/w) 0.5, KH₂PO₄ 7, Na₂HPO₄ 2.5, MgSO₄.7H₂O 1.5, CaCl₂ 0.15, FeCl₃.6H₂O 0.15, ZnSO₄.7H₂O 0.02, MnSO₄.H₂O 0.06 (Papa Nikolaou *et al.*, 2002) and were cultivated at 30°C in a rotary shaker at 180 rpm speed for 7 days.

2.7. Measurement of Citric Acid:

The concentration of citric acid in the fermentation medium was estimated titrimetrically (AOAC, 1995) as reported by different researchers (Imandi *et al.*, 2008). The readings were taken at regular intervals.

2.8. Citric Acid Assay

After fermentation process, the fermentation medium was centrifuged at 4000rpm for 15minutes. Titration was used to determine the amount of citric acid in the filtrate. 0.1 M NaOH was titrated against 10 mL of the filtrate and 2 - 3 drops of phenolphthalein was used as an indicator. Once the endpoint has been reached, the volume of NaOH used (titre) on the burette was read and the titration was completed and the final calculations can be done using citric acid, 1ml 0.1M NaOH is equivalent to 0.0064 g citric acid (Emeka *et al.*, 2012) the amount of citric acid can be expressed in terms of g/l by citric acid factor (0.0064) formula. The titration was performed in duplicates and the average titre was determined and used for the calculation using the equation below:

$$\text{g/l acid} = \frac{\text{Titre} \times 0.0064 \times 100 \times 10}{10\text{ml of analyte (Sample)}} = \text{Titre} \times 0.64$$

2.9. Optimization of Fermentation Medium:

The next phase of the study conducted was optimization of the culture conditions taking 4 different parameters i.e. pH, Carbon sources and Nitrogen sources, for citric acid production as it showed the maximum levels of acid production so far. The citric acid production was estimated after 7 days of incubation of the inoculated medium.

2.9.1. pH of the Medium

The pH of the fermentative medium was another criteria that was worked upon. A range of pH starting from 4, 5, 6, 7 and 8.0 were selected for citric acid production by the yeast isolates.

2.9.2. Carbon Sources

Five Carbon sources were chosen and tested for their suitability as substrates for citric acid production, which were; Glucose, Sucrose, Glycerol, Maltose and Fructose.

2.9.3. Nitrogen Sources

Five different Nitrogen sources were used and their suitability was checked for citric acid production. The sources selected were: Ammonium phosphate, Yeast extract, Sodium nitrate, Peptone, and Urea.

2.9.4. Temperature

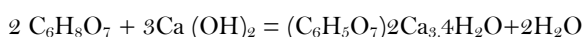
Different temperature ranges were selected i.e. 30°C, 35°C, 40° and the fermentative media were allowed to produce citric acid under these temperature ranges. The amount of citric acid produced at different temperatures by the yeasts was estimated gravimetrically, using pyridine-acetic anhydride method as reported by [Marier and Boulet \(1958\)](#) and [Ikram-ul et al. \(2004\)](#) One ml of the culture filtrate along with 1.3ml of pyridine was added in the test tube and shaken then 5.70ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath for 30min. The optical density was measured on a spectrophotometric (450 nm) and citric acid contents of the sample were estimated with reference (run parallel, replacing 2.0 ml of the culture filtrate with distilled water) to the standard.

2.9.5. Agitation

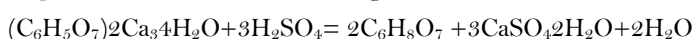
The effect of agitation on the citric acid production by incubating a set up containing the fermentation medium and the yeasts in an incubator (static) while another set up was incubated in a rotary shaker at 180rpm for 7 days (agitated).

2.10. Recovery of Citric Acid Process

After the completion of the fermentation process, the incubated broth (300mls) was filtered for the separation of pellet form of yeast culture and the fermentation broth, which acts as the source of citric acid. Lime (Ca (OH)₂) was added to the fermentation broth to allow precipitation of citric acid in the form of calcium citrate. The precipitate was recovered (by centrifuging the suspension at 3000 rpm for 10 minutes) and dried at room temperature.



Again, the precipitate was treated with concentrated sulphuric acid (H₂SO₄) to precipitate insoluble calcium sulphate, and then filtered, leaving citric acid in solution.



The resultant supernatant (presumed to be citric acid in solution) was decanted and was kept in the refrigerator for 2 hours. The crystals were formed and washed with small amount water twice, dried at room temperature and weighed prior to the estimation of melting temperature ([Jinglan et al., 2009](#)).

2.10.1. Assessment of the Purity of the Crystals

A melting point apparatus was used to assess the purity of the product obtained. The temperature at which the product crystals melted was compared with the melting temperature of pure, anhydrous citric acid crystals (153°C; melting point of monohydrate approx. 75°C).

2.10.2. The Recrystallization Procedure

The purpose of recrystallizing the recovered citric acid was to remove any by-product and impurities that might be attached to the crystals. Most impurities are believed that the impurities would be soluble in the solvent, hence filtered out of the mixture.

To 3g of citric acid, 5ml of hot distilled water to totally dissolve the recovered citric acid crystals and 45ml of hot acetone, place on ice cubes where it will not be disturbed for about 10 minutes to allow the crystals to collect at the bottom of the beaker. It was then filtered and dried at room temperature.

2.10.3. Melting Point Determination

A melting point capillary was used to pick small amount of the citric acid until the crystals fall to the bottom of the capillary. The capillary was placed in the melting point apparatus (also melting- temperature apparatus) and the crystals was watched from the lens to determine the approximate range. The melting temperature range was determined from the first appearance of liquid in the capillary tube to the sample's complete liquefaction. The melting points and the appearances of the sample with a standard (Laboratory citric acid) (Downham and Collins, 2000).

2.11. Data Analysis

All the data were analyzed using statistical analyses.

3. RESULT

3.1. Isolates Obtained From the Samples

The total number of isolates obtained from pineapple waste (PP) was eight, that of plantain waste (PL) was six, while the isolates obtained from sugarcane juice (SC) was nineteen. In the course of this study, the total number of yeast isolates obtained were 33, belonging to six (6) Genera and eight (8) species.

3.2. Distribution of Isolates Obtained From the Samples

Table 4.3 shows the distribution of yeast isolates obtained from the samples, *Saccharomyces cerevisiae* was found to occur throughout the sampling and also has the highest total count of 13. Also, most of the yeast isolates obtained were isolated from sugarcane juice (*Saccharomyces cerevisiae*, *Candida parapsilosis*, *Candida tropicalis*, *Candida rugosa*, *Pichia guilliermondii*, *Debaromyces sp.*, and *Candida krusei*), while the least were obtained from pineapple waste (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida parapsilosis*).

3.3. Diameter of Zones of Inhibition and Percentage of Occurrence of the Yeast Isolates

Table 4.4 shows the diameter of zone of clearance (in mm) from screening of the yeast isolates for citric acid production. All the yeast isolates showed significant zones of clearance from the screening. SC9 (identified to be *Candida tropicalis*) gave the highest zone of clearance with 49 ± 2.1 mm, while the lowest zone of clearance was obtained from PL5 (identified as *Saccharomyces cerevisiae*) with 09 ± 2.3 mm.

Fig. 4.1 represents the percentage of occurrence of yeast isolates obtained from the samples (Pineapple waste, plantain wastes and sugarcane juice). *S. cerevisiae* had the highest occurrence of 39.4% and *C. rugosa* had the lowest with 3.03%.

3.4. Optimization of the Fermentation Medium

The effect of different carbon sources on citric acid yield by the yeast isolates was represented in fig. 4.2, where *S. cerevisiae*, *P. guilliermondii* and *C. tropicalis* produced the highest citric acid with glucose and gave the lowest yield with fructose and glycerol respectively.

In fig. 4.3, the effect of different nitrogen sources on the yield of citric acid was represented. Sodium nitrate was observed to favour the highest yield of citric acid by the yeast isolates (*S. cerevisiae*, *P. guilliermondii*, and *C. tropicalis*). Ammonium phosphate gave the lowest yield of citric acid for *S. cerevisiae* and *P. guilliermondii* while *C. tropicalis* gave the lowest yield with Urea.

The effect of different pH on the yield of citric acid was also done and this was represented in fig. 4.4. *S. cerevisiae* produced highest at pH 7 and lowest pH 6, *P. guilliermondii* gave the highest yield at pH 4 and lowest at pH 6, while *C. tropicalis* gave the highest yield of citric acid at pH 6 and lowest at pH 5.

Fig. 4.5 represents the effect of agitation on the yield of citric acid by the yeast isolates. All the yeast isolates produced more when agitated, compared to when static fermentation was done.

In fig. 4.6, the effect of different temperatures on citric acid yield was represented. *S. cerevisiae* and *C. tropicalis* produced highest at 30°C, while *P. guilliermondii* produced highest at 35°C. Meanwhile, it was observed that at 40°C, the yeast isolates had the lowest yield.

The effect of different incubation period on the yield of citric acid was represented in fig. 4.7. *P. guilliermondii* and *C. tropicalis* produced highest on day 6, while *S. cerevisiae* gave the highest yield on the 5th day after which the yield declined.

Table-1. Distribution of Yeast Isolates Obtained from the Samples

Most Probable Organism	Total Number (N)	Pineapple Wastes	Plantain Wastes	Sugarcane Juice
<i>Saccharomyces cerevisiae</i>	13	+	+	+
<i>Schizosaccharomyces pombe</i>	2	+	+	-
<i>Candida parapsilosis</i>	5	+	+	+
<i>Candida tropicalis</i>	5	-	-	+
<i>Candida rugosa</i>	1	-	-	+
<i>Pichia guilliermondii</i>	3	-	+	+
<i>Debaromyces sp.</i>	2	-	-	+
<i>Candida krusei</i>	2	-	-	+
Total	33			

Table-2. Yeast Isolates and Their Zones of Clearance from Screening For Citric Acid Production

No.	Yeast Isolates	Diameter of their Zones of Clearance (mm)
1	PP1	34± 0.5
2	PP2	27±0.3
3	PP3	45±1.7
4	PP4	18±1.3
5	PP5	25±0.5
6	PP6	34±0.5
7	PP7	37±0.8
8	PP8	38±0.9
9	PL1	36±0.7
10	PL2	40±1.1
11	PL3	29±0.06
12	PL4	30±0.05
13	PL5	09±2.3
14	PL6	15±1.6
15	SC1	21±0.9
16	SC2	40±1.2
17	SC3	12±2.0
18	SC4	35±0.6
19	SC5	29±0.06
20	SC6	33±0.4
21	SC7	22±0.8
22	SC8	22±0.8
23	SC9	49±2.1
24	SC10	33±0.8
25	SC11	37±1.6
26	SC12	21±0.9
27	SC13	39±1.0
28	SC14	30±0.05
29	SC15	29±0.06
30	SC16	20±1.1
31	SC17	29±0.06
32	SC18	30±0.05
33	SC19	27±0.3

Key: PP = Pineapple waste PL = Plantain waste SC = Sugarcane juice

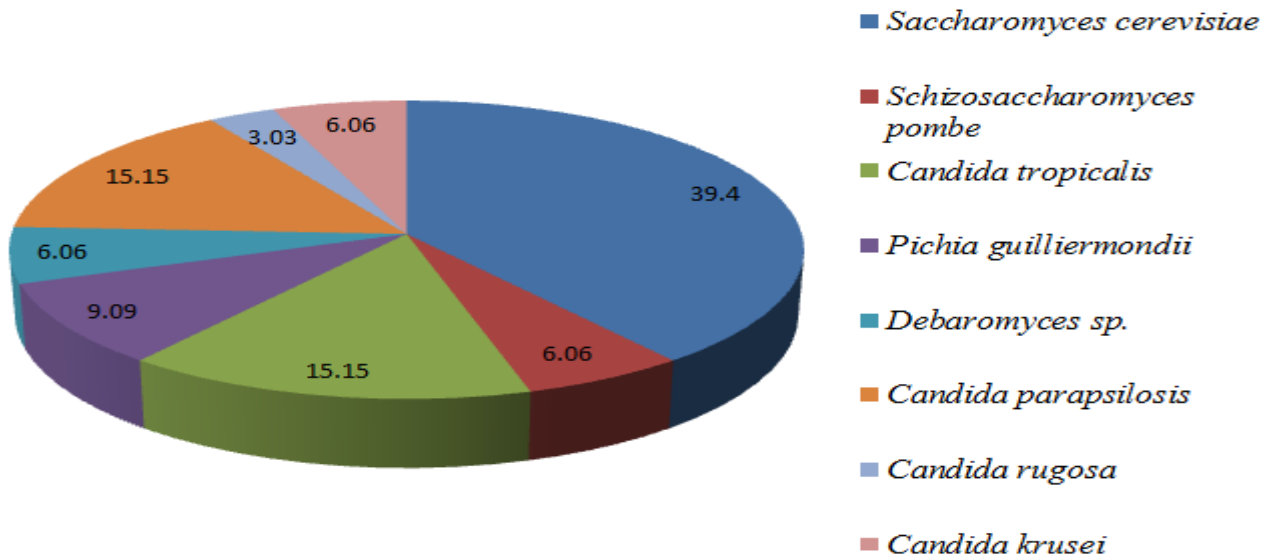


Figure-1. Percentage of occurrence of yeast isolates obtained from Pineapple waste, Plantain waste and Sugarcane juice.

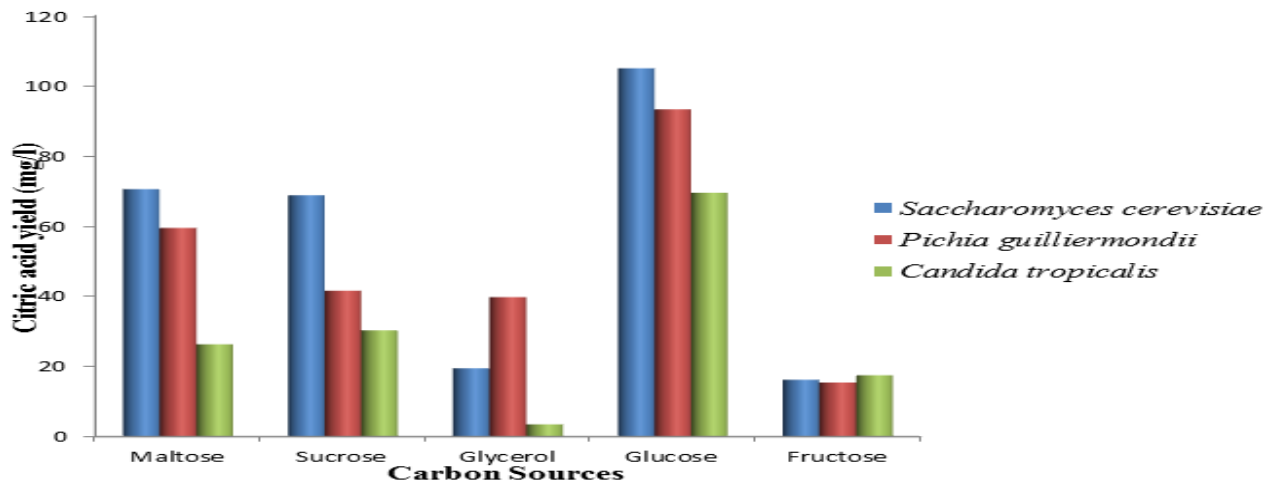


Figure-2. Effect of different carbon sources on citric acid yield by the yeast isolates

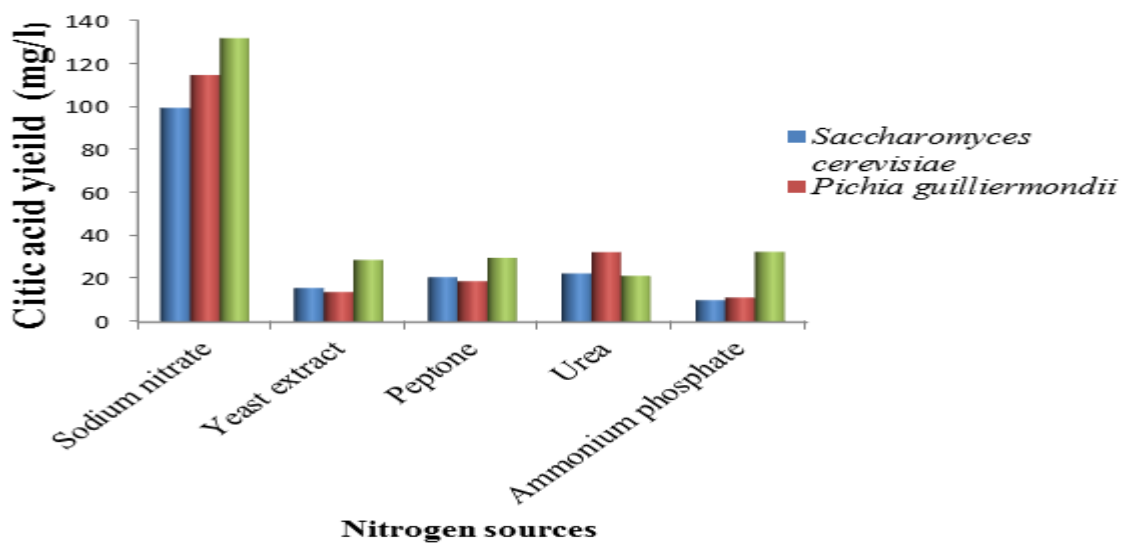


Figure-3. Effect of different Nitrogen sources on citric acid yield by the yeast isolates

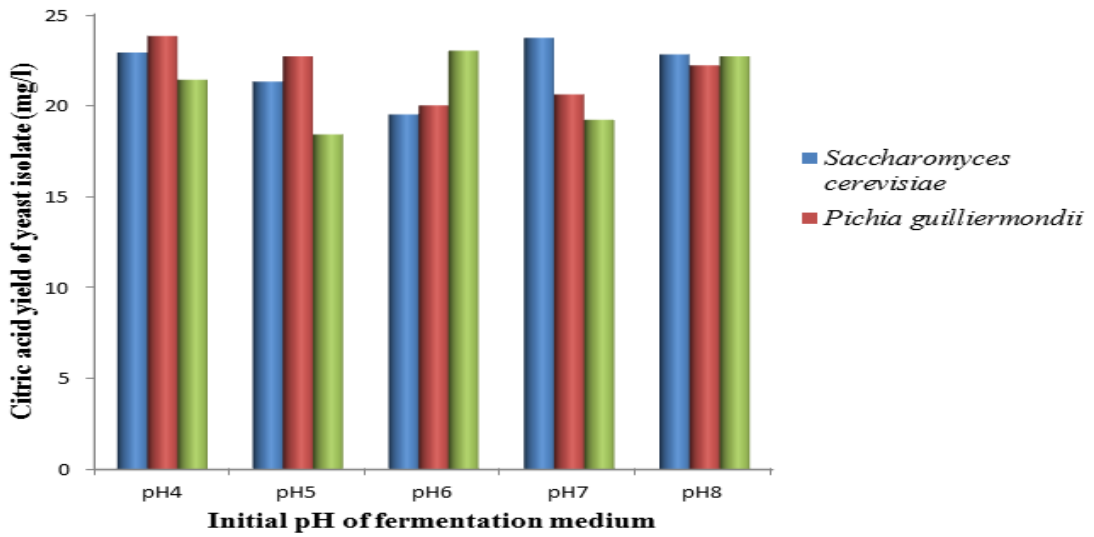


Figure-4. Effect of different pH on citric acid yield by the yeast isolates

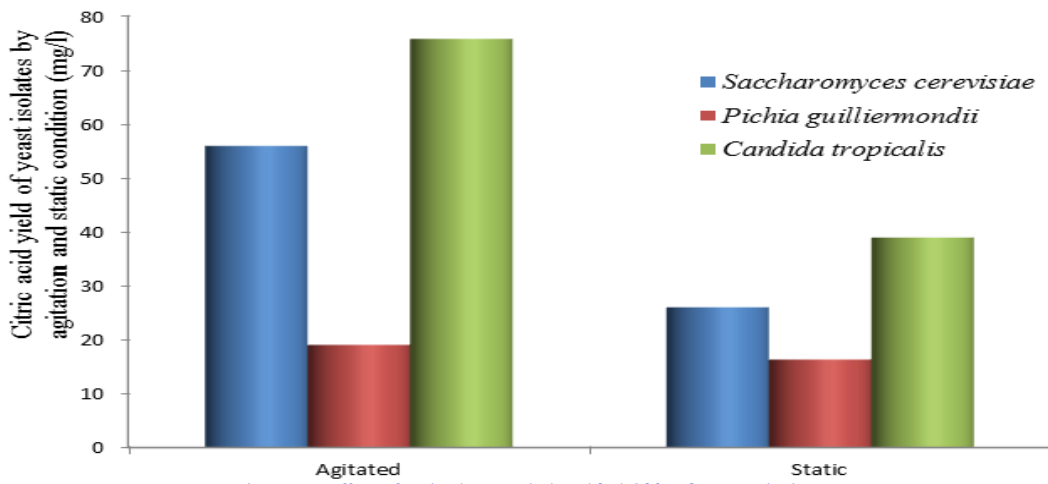


Figure-5. Effect of Agitation on citric acid yield by the yeast isolates

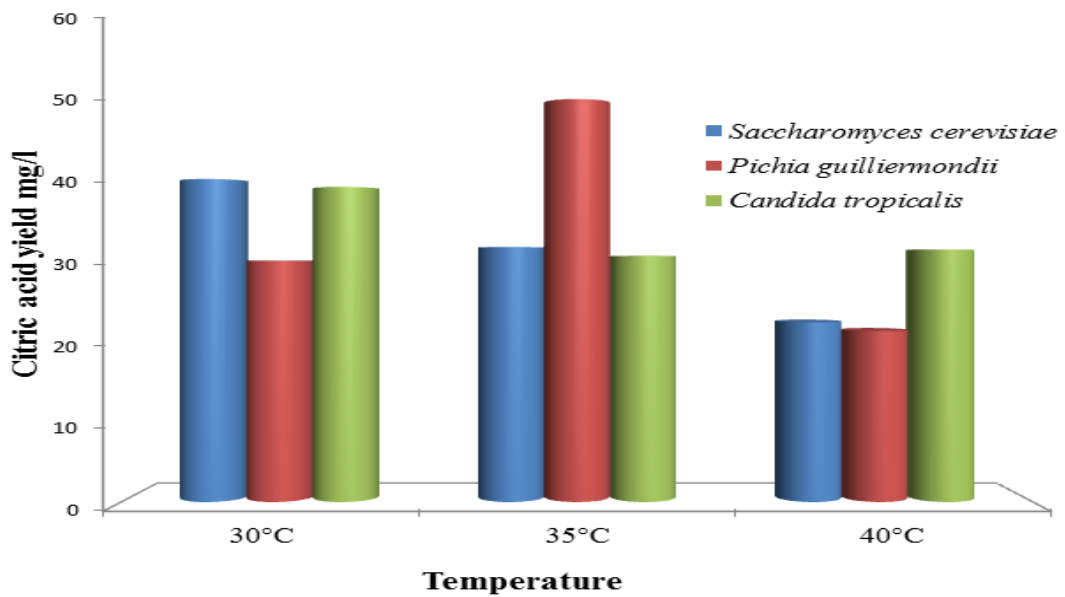


Figure-6. Effect of different temperature on citric acid yield by the yeast isolates

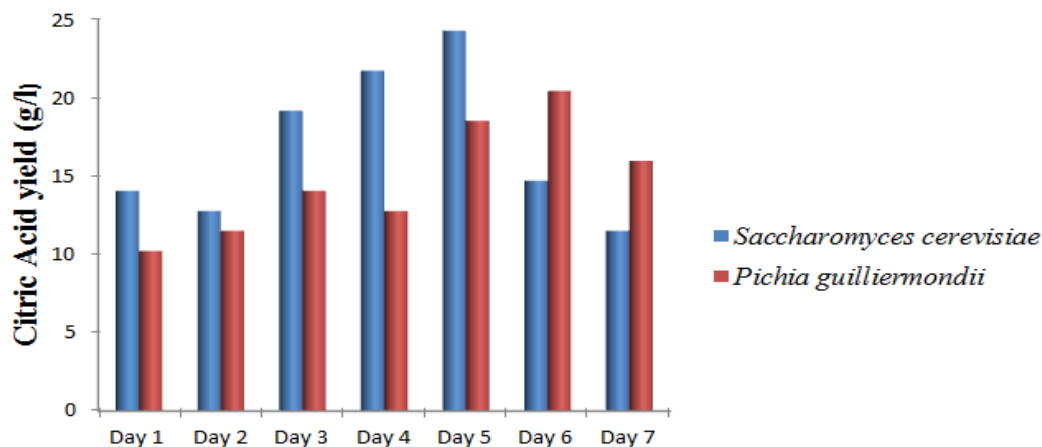


Figure-7. Effect of different incubation period on citric acid yield by the yeast isolates

Table-3. Melting point range of the Citric acid crystals.

	Melting point range of crystals
Before recrystallization	140 - 141°C
After recrystallization	151 - 152°C

4. DISCUSSION

In the course of the study, a total number of 33 yeasts were isolated and identified. They include; *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida tropicalis*, *Pichia guilliermondii*, *Debaromyces* sp., *Candida parapsilosis*, *Candida rugosa*, and *Candida krusei* as shown in Table 4.1.

4.3 shows the distribution and total number of each isolate with *Saccharomyces cerevisiae* having the highest number of 13, followed by *C. parapsilosis* and *C. tropicalis* representing 5 isolates each, *Pichia guilliermondii*, with 3, *Debaromyces* sp., *C. krusei* and *Schizosaccharomyces pombe* being 2 each, while the lowest number was recorded with *C. rugosa* which was 1.

The samples employed in the course of this study were pineapple waste, plantain waste and sugarcane juice. The yeast isolates obtained from pineapple wastes (Table 4.3) has been reported by several researchers such as Chanprasartsuk *et al.* (2013) that isolated yeasts from the spontaneously fermentation of pineapple, and Santoshkumar and Patil (2006) who isolated wine yeast from pineapple fruits. Also the yeast species isolated from plantain wastes (Table 4.3) has been reported by Rao *et al.* (2008) who isolated and characterized ethanol-producing yeasts from fruits and tree barks. While the isolates from sugar cane juice (Table 4.3) correlates with the findings of Qureshi *et al.* (2007) that isolated Strains of *S. cerevisiae* from sugar cane based on their maltose utilization capacity. Ahmed *et al.* (2010) also conducted a study on the mycoflora associated with sugar cane juice isolated and Shehata (1960) where he isolated yeast from sugarcane juice to produce aguardente de cana where similar yeast were isolated and identified.

As shown in figure 4.1, *Saccharomyces cerevisiae* had the highest percentage of occurrence of 39.4%, followed by *C. tropicalis* and *C. rugosa* having 15.15% each, *P. guilliermondii* had 9.09% while *Schizosaccharomyces pombe*, *Debaromyces* sp. and *C. krusei* had 6.06%, and lastly *C. rugosa* had the least with 3.03%. This results were similar to the findings of Matapathi *et al.* (2010) who observed that *Saccharomyces* species dominates during fermentation more than any other yeast.

All the yeast isolates showed zones of clearance during the screening for citric acid production but with varying diameters. It was concluded that pineapple wastes, pineapple wastes and sugar cane juice is potentially a suitable source to select fermentative yeasts (Silva *et al.*, 2011). It was observed that even isolates that was identified as the same species gave different diameters of clearance which could be attributed to being different strains of the same species. The yeast isolates which was used for further studies were, SC9 (*Candida tropicalis*) which was isolated from

sugarcane juice gave the highest zone of clearance with 49 ± 2.1 mm followed by PP3 (*Saccharomyces cerevisiae*) which gave 45 ± 1.7 mm. Yeasts (*Saccharomyces* spp.) have been used as an alternative to filamentous fungi to produce citric acid (Burden and Eveleigh, 1990) and PL2 (*Pichia guilliermondii*) isolated from plantain waste gave 40 ± 1.1 mm, all in diameter. The result of the screening was as shown in Table 4.4. It was observed that SC2 also gave a clearance of 40 ± 1.2 but was not considered for further studies because it was also identified as *Candida tropicalis* (Table 4.2). This was in line with the previous work of Rymowicz *et al.* (2006) and Crolla and Kennedy (2001).

The carbon sources employed for the study were maltose, sucrose, glycerol, glucose and fructose. The three yeast isolates was observed to give the highest yield of citric acid utilizing glucose with *S. cerevisiae* yielding 105.0 mg/l, *P. guilliermondii* with 93.3 mg/l and *C. guilliermondii* with 69.5 mg/l. This is similar to a previous study performed with *C. lipolytica*, by Hamissa *et al.*, 1981, where it was reported that high citric acid yields were obtained when glucose was used as substrate. The least yield of citric acid for *S. cerevisiae* and *P. guilliermondii* was with fructose, yielding 16.2 mg/l and 15.40 mg/l respectively, while *C. tropicalis* gave the least yield with glycerol with 3.49 mg/l. This was represented in Fig. 4.2.

Fig. 4.3 shows the yield of citric acid with different nitrogen sources. The sources of nitrogen were sodium nitrate, yeast extract, peptone, urea, and Ammonium phosphate. It was observed that the yeast isolates employed in the course of this study (*S. cerevisiae*, *P. guilliermondii* and *C. tropicalis*.) yielded the highest with Sodium nitrate with 99.70 mg/l, 115.0 mg/l and 132.2 mg/l respectively. These results agree with the report of Socol *et al.* (2006) that inorganic sources of nitrogen have the potential of increasing the yield of citric acid. However, *S. cerevisiae* and *P. guilliermondii* yielded the least citric acid with 10.20 mg/l and 11.60 mg/l respectively when they utilized Ammonium phosphate, but *C. tropicalis* produced the least citric acid utilizing Urea with 21.60 mg/l.

In Fig. 4.4, It was observed that at pH 6, *S. cerevisiae*, produced the highest citric acid with 23.70 mg/l and the least yield was at pH 7, while *P. guilliermondii* produced highest at pH 4 with 23.80 mg/l. *C. tropicalis* also produced highest at pH 6, yielding 23.00 mg/l. It has been reported When working with yeasts, initial pH values above 5 should be used since citric acid production is adversely affected below pH 5 (Mattey, 1992).

The effect of agitation on the production of citric acid by the yeast isolates was also done, *S. cerevisiae* produced 55.90 mg/l when agitated compared to when it was static with 26 mg/l. This was also observed with *P. guilliermondii* and *C. tropicalis* which produced highest when the fermentation medium was agitated with 19.0 mg/l and 75.70 mg/l respectively compared to when it was static with 16.30 mg/l and 38.90 mg/l. This was as shown in Fig. 4.5. It is known that agitation increases the area available for oxygen transfer by dispersing air and insoluble substrate in the culture fluid in the form of fine bubbles and an optimum agitation speed is required to maximize product production (Kamzolova *et al.*, 2003).

Temperature is also one of the factors that could affect both the growth of yeasts and the production of citric acid. The temperature that favoured the highest yield of citric acid for *S. cerevisiae* and *C. tropicalis* was 30°C yielding, 40.80 mg/l and 39.80 mg/l while *P. guilliermondii* gave the highest yield at 35°C with 50.90 mg/l. This was presented in Fig. 4.6. Temperature reported in various researches on citric acid production by yeasts was between 22-35°C and that optimum temperature for citric acid production may change relative to the used strain and medium conditions (Crolla and Kennedy, 2001).

The day on which the yeast isolates produced highest after incubation for *S. cerevisiae* was observed to be on day 5 with 24.32 g/l, while both *P. guilliermondii* and *C. tropicalis* produced the highest on day 6 with 20.48 g/l and 23.02 g/l yield of citric acid. Similar findings were obtained and reported by previous researchers such as Karasu-Yalcin *et al.*, 2010 where they worked on the effect of fermentation conditions on growth and citric acid production kinetics of two *Yarrowia lipolytica* strains.

Lye and Woodley (1999) observed that many fermentation processes have low productivity and yield which may be due to product inhibition or hydrolysis of product by further catalytic reactions, which can be said of the yield of the 4.231 g of citric acid from 300 ml of the fermentation medium in the course of this research.

A recovery of 72% (2.159 g) was obtained after the recrystallization of 3g of the extracted citric acid. This is in agreement with Karklin *et al.* (1984) when he reported that the yield of citric acid was between 64.8 - 92.9% when the classical method, precipitation was used in the extraction of citric acid from the fermentation of n-alkanes.

The solubility of the compound in the solvent used for recrystallization is important. In the ideal case, the solvent would completely dissolve the compound to be purified at high temperature, usually the boiling point of the solvent, and the compound would be completely insoluble in that solvent at room temperature or at 0 °C. In addition the impurity either would be completely insoluble in the particular solvent at the high temperature, or would be very soluble in the solvent at low temperature. In the former case, the impurity could be filtered off at high temperature, while in the latter case the impurity would completely stay in solution upon cooling. A suitable recrystallization solvent should also be partially volatile in order to be easily removed from the purified crystals. The solvent should not react with the compound being purified and it should have the boiling point below the melting point of the compound being purified because solid melts before it dissolves (oiling out) because recrystallization is a technique that has to be practiced and perfected. Hence, the choice of acetone in the course of this work, which has been reported by Vandenberghe *et al.* (1999) that better results was also achieved using extraction technique at normal temperature when acetone was used, followed by water, ethanol and then methanol.

A pure substance will always have the same melting point, no matter how it was prepared. An impure substance will melt differently than a pure substance in two ways (a) it will melt at a different, usually lower temperature than the pure sample (b) it will usually melt over a broad temperature range rather than a range of 1-3 degrees that is expected for a pure substance. Which as shown in table 4.11, the melting temperature of the crystals before recrystallization was between 140°C and 141°C, whereas the melting temperature of the recrystallized citric acid ranged between 151°C to 152°C and this is comparable to the standard melting point of citric acid, which is 153°C.

5. CONCLUSION

From the present study, it can be concluded that pineapple wastes, plantain wastes and sugarcane cane are cheap agricultural wastes that could be used as substrates for citric acid production using yeasts. In addition to the isolation and screening of yeast isolates for their ability to produce citric acid, the optimization of fermentation conditions such as the carbon sources, nitrogen sources, temperature, pH, agitation and the incubation period are of primary importance in the development of any fermentation process owing to their impact on the yield of citric acid obtained.

Funding: This study received no specific financial support.

Competing Interests: The authors declare that they have no competing interests.

Contributors/Acknowledgement: All authors contributed equally to the conception and design of the study.

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