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Screening of *Aspergillus* and *Candida* Species with Utmost Potential to Synthesize Citric Acid

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Authors' contributions

This work was carried out in collaboration among all authors. Author GAU designed the study, managed the analyses of the study and literature searches, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NPA and NNO thoroughly supervised the study and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Citric acid production through fermentation is economical but meeting its increasing global demand has been challenging in recent times.
Aim: This study aimed to screen *Aspergillus* and *Candida* sp. isolated from different sources with potentials of producing citric acid.
Methodology: *Aspergillus* and *Candida* spp. were isolated from compost soil and fruits (cucumber and banana) and their morphological characteristics were described using standard microbiological methods. The isolates were quantitatively screened for citric acid production based on appearance of yellow zone of clearance for 3 days. All the isolates which had acid unitage (AU) values> 5.0 were selected for further characterization using molecular methods.
Results: *Candida tropicalis, Aspergillus* sp*. A. niger* and *Penicillium* sp. were isolated from the soil and fruit samples. The isolates screened for citric acid production displayed varying diameters of yellow zones around their colonies is indicative of varying capability of the microbial strains. *A. niger* from compost soil which had highest AU value of 8.5 at Day 3 demonstrated greatest potential to yield citric acid. Molecular characterization revealed high citric acid producing strains as *Aspergillus niger* (EU440768.1) and *Aspergillus welwitschiae* (MG669181.1).

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Conclusion: Although *Aspergillus niger* is widely utilized for industrial production of citric acid, this study has demonstrated that *A. welwitschiae* is a specie of *Aspergillus* capable of synthesizing citric acid reasonably.

Keywords: Citric acid; Aspergillus sp.; Candida sp.; molecular characterization.

1. INTRODUCTION

In recent times, the demand for citric acid has been on the increase. Yearly, worldwide demand for citric acid is about 6, 000000 tons. In 2004, global production of citric acid was estimated to be 1.4 million tonnes which later increased to 1.6 million tonnes in 2007 [1,2]. It is projected that growth in annual demand for citric acid globally is 3.5–4% [3].

Citric acid is a weak organic acid, solid at room temperature, has a melting point of 153^{o}C and molecular weight of 210.14 g/mol. This weak organic acid occur naturally in all citrus fruits. At pH 3.1, 4.7 and 6.4, citric acid has three different pKa values. The demand for citric acid globally is high due to its low toxicity when compared with other acidulants which has useful applications mainly in pharmaceutical and food industries [2,4,5].

This weak organic acid has several applications. They include pharmaceutically active substances, pharmaceuticals, personal care and cosmetic products, food, flavouring agent, diuretic, blood anticoagulant, environmental remediation and beverage. Emerging uses of citric acid involves manufacturing of household detergents, dishwashing cleaners, disinfectants etc [6]. Citric acid is Generally Recognized as Safe (GRAS). This status was as a result of approval given to the product by Joint FAO/WHO Expert Committee on Food Additives [7].

In the past, citric acid was naturally obtained from orange, lemon and lime. However, quantity of citric acid produced through this means is grossly insufficient to meet global demand for the product. To achieve production of citric acid in commercial quantity, the use of microorganisms which involves fermentation is preferable than chemical methods. This is because, chemical method is not economically competitive compared with fermentation method [2]. Some species of fungi such as *Aspergillus* sp., *Acremonium* sp*., Botrytis* sp*., Eupenicillium* sp*., Mucor* sp. *Penicillium* sp. and *Trichoderma* sp. could be utilized for citric acid production. Similarly, some species of yeast mainly of the genera *Candida* sp. as well as bacteria such as *Bacillus licheniformis, Arthro- bacter paraffinens, Corynebacterium* sp*.* and *Bacillus subtillis* have also been used for citric acid production [8,9].

Among the microorganisms which have the capability to produce citric acid, *Aspergillus niger and Candida tropicalis* is preferable because they are capable of synthesizing considerable amount of citric acid with minimal formation of undesirable toxic by-products [10]. *A. niger* can utilize varieties of cheap materials and result in high yield of citric acid due to its well-developed enzymatic system [11]. Almost all industrial processes use *A. niger* as the producing organism for citric acid production due to its simplicity of handling, rapid proliferation, tolerance to acidic pH and high yield [12]. This fungus is GRAS except in rare occasions when humans develop hypersensitivity reactions following exposure to the spore dust. United States Food and Drug Administration have considered many *Aspergillus niger* enzymes to be GRAS [10]. However, *Candida tropicalis* is less used in research and industrial purposes for citric acid production. According to Surkesh et al. [13], citric acid could be produced using *A. niger* isolated from decayed fruit and agronomic wastes such as grapes, orange, apple, vegetable, tapioca or coconut husk as substrate. Different strains of *Aspergillus niger* and *Candida tropicalis* could have varying abilities of producing citric acid. Therefore, this study seeks to screen the capability of *Aspergillus* and *Candida* spp. to produce citric acid as well as use molecular methods to identify the fungal and yeast isolates with great potential of synthesizing citric acid.

2. MATERIALS AND METHODS

Fresh fruits (cucumber and banana) were purchased from markets within Port Harcourt metropolis. Soil samples around Biology Green House as well as compost soil samples inside the campus were obtained using the method described by Pepper et al. [14]. All the samples were used for isolation of *Aspergillus niger* and *Candida tropicalis* in the Postgraduate

Microbiology Research Laboratory, Department of Microbiology, Rivers State University, Port Harcourt, Nigeria. The isolates obtained from banana fruit, cucumber fruit, soil around Biology Green House and compost soil was coded numerically using FB, FC, SB and SC, respectively.

2.1 Isolation and Subculture of Fungal Isolates

Serial dilution was performed using the soil samples and agricultural products (cucumber and banana) in accordance with the procedure described by Jalal et al. [15]. Using standard microbiological methods, potato dextrose agar (PDA) containing 10% lactic acid to suppress bacterial growth was used to isolate and subculture the fungal and yeast isolates.

2.2 Screening and Selection of Fungal Isolates for Citric Acid Production

The capabilities of fungal isolates to produce citric acid were determined using the methods adopted by Patil and Patil [16]. After l20 h incubation period at 28 ± 2°C, the culture plate which comprise Czapek-Doxagar medium, 0.5 g Ca_2CO_3 added to the medium and bromocresol green as an indicator inoculated with spores from each fungal isolate was quantitatively screened for citric production for 3 Days. Positive isolates were identified based on presence of yellow zones around the colonies. The ability of isolated fungi and yeast to produce citric acid was determined by measuring zones of clearance of each isolate. The isolates which showed wide zone of clearance were selected because they were assumed to be the highest producers of citric acid.

2.3 Acid Unitage (AU) Test

The method used by Shaikh and Qureshi [17] was adopted. In determining the acid unitage (AU) of each isolate, the diameter of the yellow zone was divided by the diameter of the colonies. The isolates which recorded high AU were selected for molecular characterization. All the isolates were transferred aseptically into potato dextrose agar (PDA) slants maintained at 4°C throughout the duration of the study.

2.4 Molecular Identification of Screened Isolates

DNA extraction PCR amplification of the fungi 18S rRNA PCR gene and gel electrophoresis of the screened isolates were carried out at the Biotechnology Research Centre, University of Port Harcourt. The PCR product was sent to International Institute of Tropical Agriculture (IITA), Ibadan for sequencing the 18S rRNA.

2.4.1 DNA extraction of screened fungal isolates

DNA extraction was carried out using a ZR fungal / bacterial DNA mini prep extraction kit supplied by Biotechnology Research Centre, University of Port Harcourt. A heavy growth of the pure culture of the isolates was suspended in 200 µL of isotonic buffer and enough quantity of fungi inoculum was placed in a ZR Bead^{TM} Lysis tubes, into a ZR Bashing Bead Lysis tubes and 750 µL of lysis solution was added to the tubes. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly (Disruptor Genie TM) and processed at maximum speed for 5 min. The ZR bashing bead TM lysis tube was centrifuged in a micro-centrifuge at 10,000 rpm for 1 min.

Four hundred microliters (400 µL) of supernatant was transferred to a Zymo-Spin ^{TM} IV Spin Filter (orange top) in a collection tube and centrifuged at 7000 rpm for 1 min. One thousand two hundred microliters (1200 µL) of fungal DNA binding Buffer was added to the filtrate in the collection tubes which brings the final volume to 1600 µL. Eight hundred microliters (800 µL) was then transferred to a Zymo-Spin ^{TM} IIC Column in a collection tube and centrifuged at 10,000 rpm for 1 min. The flow through was discarded from the collection tube, the remaining volume was transferred to the same Zymo-Spin^{TM}IIC and centrifuged at 10,000 rpm for 1 min. Two hundred microliters (200 µL) of the DNA Pre-wash Buffer was added to the Zymo-Spin^{TM} IIC Column in a new collection tube and spun at 10,000 rpm for 1 min. followed by addition of 500 µL of fungal DNA wash Buffer and centrifuged at 10,000 rpm for l min.

The Zymo-Spin ^{TM} IIC Column was transferred to a clean 1.5 µL centrifuge tube (Eppendorff tubes), and 100 µL of the DNA elution buffer was added directly to the column matrix, centrifuged at 10,000 rpm for 30 sec to elute the DNA. The extracted DNA was then stored at -200^{o}C for other downstream reactions. The concentration and purity of the extracted genomic DNA of the fungal isolates were estimated using a Nanodrop 1000 spectrophotometer. The absorbance was taken at 260 nm and 280 nm for each sample and the ratio of absorbance at 260 nm and 280

nm were used to assess the purity of the DNA. A ratio of -1.8 is generally accepted as "pure" for DNA while a ratio of -2.0 is generally accepted as "pure" for RNA.

2.4.2 PCR amplification of fungi 18S rRNA gene

PCR amplification of the 18S rRNA genes of the isolates were amplified using the primer set ITS4 5 – TCCTCCGCTTATTGATATGS -3 and ITS5 5-GGAAGTAAAAGTCGTAACAAGG -3. The reaction was carried out using 25 µL volume containing 6.6 µL of the cocktail mix (Zymo Master Mix), 1 µL each of the forward and reverse primer, mixed with 3 µL of DNA template and sterile 13.4 µL nuclease free water. The sequencing machine used was 3130XL genetic analyzer from Applied Biosystems while the PCR thermal cycler used was GeneAmp PCR system 9700. The PCR cycling parameters were: Initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94v°C for 30 sec, annealing at 54°C for 30 sec and elongation at 72°C for 45 sec. followed by a final elongation step at 72°C for 7 min. and hold temperature at 10°C. Amplified fragments were visualized on safe view - stained 1% agarose electrophoresis gel.

2.4.3 Agarose gel electrophoresis

After the PCR reaction, five microliters (5 µL) of the amplified products were separated on a 1% agarose gel. Six hundred base pair (600 bp) DNA ladder was used as DNA molecular weight marker. Electrophoresis was done at 120 V for 20 min. and the gel was visualized using UV *transilluminator* to determine the size of the DNA of the isolates.

2.4.4 Sequencing of amplified fungi 18S rRNA

Sanger method and 3130XL genetic analyzer from Applied Biosystems was used to sequence the amplified 18S products. The sequence generated by the sequencer were visualized using Bioformatic Algorithms such as Chromoslite for base calling. BioEdit was used for sequence editing before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Center for Biotechnology Information) database (https://blast.ncbi.nim.nih.gov/Blast.cgi). Similar sequences were downloaded and aligned with Cluster X and phylogenic tree was drawn with MEGA 6 software.

2.4.5 Construction of phylogenetic tree

The evolutionary history was inferred using the Neigbor-Joining method [18]. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed [19]. The trees were drawn to scale and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Jukes-Cantor method considering units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [20].

2.5 Statistical Analysis

Two readings were taken and average calculated for acid unit age value of each isolate.

3. RESULTS AND DISCUSSION

Table 1 shows the morphological characterization of fungal and yeast isolates. The isolate code FB1, FC1, FC3, FC4, FC5, SB4, SC1, SC2 and SC3 were identified as *Aspergillus niger*. Isolate SB1, SB2 and SB3 were identified as *Candida tropicalis* while Isolate FB2 and FC2 were identified as *Aspergillus* sp. and *Penicillium* sp., respectively. Isolate FB1 and FB2 was gotten from fresh banana fruit; isolates FC1, FC2, FC3, FC4 and FC5 from fresh cucumber fruit; isolate SB1, SB2, SB3 and SB4 from soil around Biology Green House while isolates SC1, SC2 and SC3 from compost soil. All the isolates obtained were screened for citric acid production. Our result shows that most of the isolates screened were *Aspergillus niger* gotten from fresh cucumber.

Several studies have demonstrated that *Aspergillus niger* is chiefly used as industrial species for the production of citric acid. *Candida tropicalis* has also been implicated in production of citric acid [21,22]. Other fungal species namely *Aspergillus carbonarius, A. aculeatus, A. awamori, A. fonsecaeus, A. foetidus, A. Phoenicis* and *Penicillium janthinellum* as well as yeasts such as *Candida oleophila, C. guilliermondii, C. citroformans, Hansenu laanamola* and *Yarrowia lipolytica*) and bacteria (*Bacillus licheniformis, Arthrobacter paraffinens* and *Corynebacterium* spp.) are capable of producing citric acid [9].

The ability of *Aspergillus niger, Aspergillus sp., Penicillium* sp. and *Candida tropicalis* to produce citric acid was evidenced by yellow zones of clearance observed during screening of the isolates for citric acid production. Our results shows that isolates belonging to the same species had different diameters of clearance, colour change and progression in acid unitage (AU). The differences observed could be attributed to different fungal and yeast strains belonging to the same species [21]. Shown in Table 2 is the AU value of fourteen (14) isolates obtained from various sources with potentials of producing citric acid. Isolates SC1, SC2 and SC3 identified as *Aspergillus niger* from compost soil, isolate FB2 identified as *Aspergillus sp.* from banana fruit and SB3 identified as *Candida tropicalis* from soil around Biology Green House had acid unitage value of zero at Day 1. At Day 2, all the isolates had AU values above zero. Isolate SC3 identified as *Aspergilllus niger* from compost soil had the highest AU value of 8.5 at Day 3. On the contrary, isolate FC2 which is *Penicillium* sp. from cucumber had the lowest AU value of 2.5. This result is an indication that *Penicillium* sp. is not preferable for citric acid production. In a related study, Abonama et al. [23] optimized citric acid production using *Candida tropicalis*. The researchers were able to achieve highest citric acid concentration of 30.0 g/L using most effective conditions.

The AU values obtained in this study is similar with the results reported by Lingappa et al. [24] which stated that AU value of ≥ 5.0 should be demonstrated by any good citric acid producing strain. Furthermore, microbial strains which had AU of 3 - 5 were regarded as being moderate while those that were below 3 were referred as poor citric acid producers. Their result shows that AU of *Aspergillus niger* isolates from fruit waste, irrigated soil, municipal solid waste and onions were within the range 4.5 - 6.6, 2.0 - 4.0, 2.0 – 5.6, 3.0 – 5.0, respectively. In reference to Lingappa et al. [23], we can categorize isolate SC1, SC3 and FC4 as high citric acid producing strains at Day 3. Meanwhile, isolates FB1, FB2, FC1, FC3, FC5, SB1, SB2, SB4 and SC2 are regarded as moderate citric acid producing strains at Day 3. Only isolate FC2 which is *Penicillium* sp. at Day 3 is considered as a poor citric acid producing strain. Our result is in agreement with a related study carried out by Lingappa et al. [23] which reported highest AU value for *Aspergillus niger* isolated from fruit waste which was within the range 4.5 – 6.6 compared with AU values of isolates obtained from other sources.

Agarose electrophoresis of amplified 18S rRNA gene of fungal isolates is shown in Fig. 1 shown in Fig. 2 is the evolutionary relationship of fungal isolates and their closest Genbank relative screened for production of citric acid while Table 3 shows the sequence identification from NCBI BLAST hits and their percentage relatedness. The evolutionary distances computed in this study is in agreement with the phylogenetic placement of 18S rRNA of isolate ITS4 21 with *Aspergillus niger* while that of isolate ITS4 22 and ITS4 23 were found to be closely related to *Aspergillus welwitschiae* and *Candida tropicalis,* respectively.

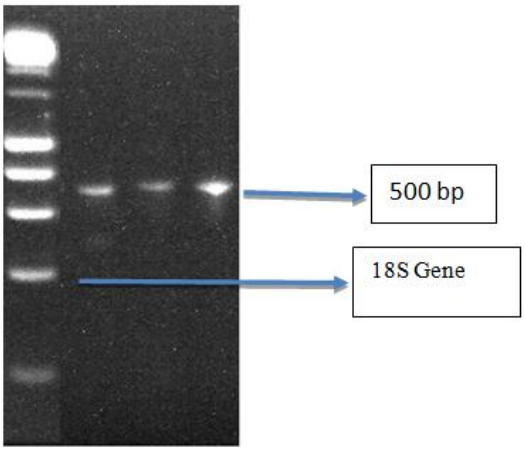

Fig. 1. Agarose electrophoresis of amplified 18S rRNA gene of fungal and yeast isolates
Lane 1 – 3 represent the isolates

Table 1. Morphological characterization of fungal and yeast Isolates from fruits and soil

Isolates	Macroscopy	Microscopy	Probable organism
FB1	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
FB2	Surface colony colour is Light green lawn surrounded by white lawn-like growth without radial symmetry	Septate hyphae with septate conidiophores bearing conidia	*Aspergillu* ssp.
FC1	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
FC2	Green powdery surface surrounded by white lawn, brown reverse symmetry	Septate hyphae with septate conidiosphores bearing conidia	*Penicillium* sp.
FC3	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
FC4	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
FC5	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
SB1	Textures of colonies are powdery and brown spores with white flabby edge while the reverse is pale yellow.	Pseudo-hyphae with budding blastoconidia	*Candida tropicalis*
SB2	Textures of colonies are powdery and brown spores with white flabby edge while the reverse is pale yellow.	Pseudo-hyphae with budding blastoconidia	*Candida tropicalis*
SB3	Textures of colonies are powdery and brown spores with white flabby edge while the reverse is pale yellow.	Pseudo-hyphae with budding blastoconidia	*Candida tropicalis*
SB4	Growth rate is rapid and textures of colonies are powdery and	Septate hyphae with globose and radiate conidia	*Aspergillus*

	produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*niger*
SC1	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
SC2	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*
SC3	Growth rate is rapid and textures of colonies are powdery and produced radial tissues in the agar. Surface colony colour was initially white becoming deep brown with conidial production while the reverse is pale yellow.	Septate hyphae with globose and radiate conidia heads with metulae that support the phialides; conidiosphores are hyaline and smooth-walled	*Aspergillus niger*

Key: FB = Banana fruit, FC = Cucumber fruit, SB = Soil around Biology Green House, SC = Compost soil

Table 2. Acid unitage value

Isolates	Days			Colour reaction	Substituting bromocresol purple with Na_2CO_3
	1	2	3		
FB1	1	2	3	Y	+
FB2	0	2	5	Y	+
FC1	1	25	4	Y	+
FC2	1.2	1.5	2.5	P	+
FC3	1	1.7	3.5	P	+
FC4	2.33	4	5.67	Y	+
FC5	2.5	4	5	Y	+
SB1	1.6	2	3	Y	-
SB2	1.5	4	5	Y	+
SB3	0	2	3.5	Y	+
SB4	1.2	3	5	Y	+
SC1	0	2.86	5.71	Y	+
SC2	0	2	3	Y	-
SC3	0	2.5	8.5	Y	-

Key: FB = Banana Fruit, FC = Cucumber fruit, SB = Soil around Biology Green House, SC = Compost Soil, Y = Yellow, P = Purple

Our results presented in Table 3 confirmed that percentage similarity of *Aspergillus niger* (EU440768.1), *Aspergillus welwitschiae* (MG669181.1) and *Candida tropicalis* (KT356204.1) to other species was 97.74%, 99.30% and 83.11%, respectively. One interesting finding from this study is an indication that *Aspergillus welwitschiae* has the potential to synthesize citric acid in addition to *A. niger* which has been widely used for the same purpose. This result is in agreement with Almousa et al. [5] which reported the use of molecular methods to characterize *Aspergillus niger* MH368137 which demonstrated ability to produce citric acid in large quantity. Their phylogenetic tree showed *Aspergilllus welwitshiae* (KT826632.1), *Aspergillus welwitschiae* (KT826638.1) *Aspergillus welwitschiae* (KT826640.1), *Aspergillus welwitschiae* (MH035989.1) and other strains of *Aspergillus niger* and *Aspergillus awamori* as potential strains capable of producing citric acid in large quantity.

Table 3. Sequence identification from NCBI BLAST hits and their percentage relatedness

S/N	Sequence code	NCBI BLAST relative	Accession number	E value	% relatedness
1	ITS4 21	*Aspergillus niger*	EU440768.1	0.0	97.74
2	ITS4 22	*Aspergillus welwitschiae*	MG669181.1	0.0	99.30
3	ITS4 23	*Candida tropicalis*	KT356204.1	5e-102	83.11

Key: ITS4 21 (FC4) = Aspergillus niger; ITS4 22 (FB2) = Aspergillus welwitschiae; ITS4 23 (SB2) = Candida tropicalis

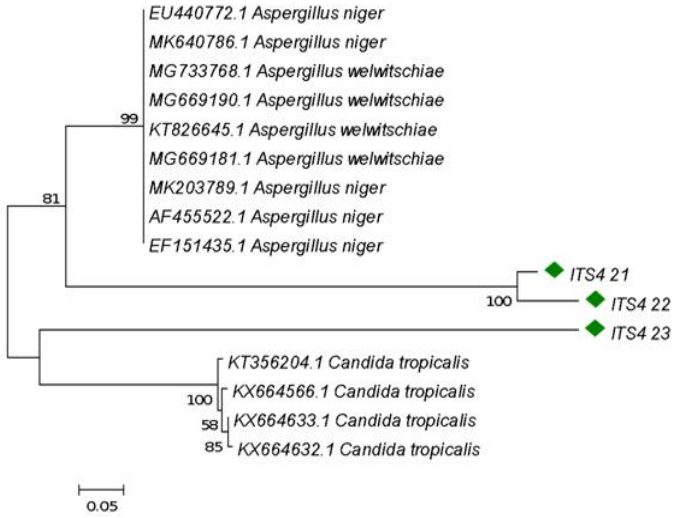

Fig. 2. Evolutionary relationship of fungal and yeast isolates and their closest Genbank relative
ITS4 21 (FC4) = Aspergillus niger; ITS4 22 (FB2) = Aspergillus welwitschiae; ITS4 23 (SB2) = Candida tropicalis

4. CONCLUSION

Among the various sources fungal and yeast isolates screened for citric acid production were obtained from, *Aspergillus niger* isolated from compost soil demonstrated greatest capability to produce citric acid but *Penicillium* sp. obtained from cucumber showed poorest ability to do same.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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