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ISOLATION AND SCREENING OF MICROORGANISMS FOR CITRIC ACID PRODUCTION

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Abstract

Citric acid is a weak acid and naturally occurring antioxidant. Due to its wide application in the food industry, cosmetics and manufacturing cleaning products, Citric acid is artificially synthesized by exploiting microorganisms, especially fungi. With an aim of isolating and screening the potential microorganisms for citric acid production, the present study was carried out by collecting samples of decaying fruits and vegetables from the local market of Kalaburagi, Karnataka, India viz., *Citrus limon, Citrus sinensis, Vitis vinifera, Malus pumila, Ananas comosus, Solanum tuberosum, Punica granatum, Citrullus lanatus, Musa paradisiaca, Solanum lycopersicum, Phaseolus vulgaris, Mangifera indica, Manilkara zapota, Beta vulgaris, Saccharum officinarum, Ipomoea batatas, Daucus carota, Psidium guajava, Solanum melongena* and *Abelmoschus esculentus.* A total of 20 isolates have been isolated from the samples, out of 20 fungal isolates, 7 isolates viz., KLV03, KLV04, KLV07, KLV10, KLV11, KLV13 and KLV14 showed significant acid unitage values of 3.00, 1.88, 3.36, 1.31, 2.35, 1 and 1 respectively. Among these, KLV07 isolate showed the highest acid unitage value of 3.36. Molecular identification of the isolate KLV07 was done by 18S rRNA gene sequencing and confirmed the isolate to be *Aspergillus niger* strain KRF9 small subunit ribosomal RNAgene with Sequence ID: MK611800.1. The next closest homologue was found to be *Aspergillus awamori* isolate RN 206 smallsubunit ribosomal RNA gene with Sequence ID: MK530131.1.

Keywords: *Aspergillus niger*, Citric Acid, Isolation, Fruit wastes and Screening.

Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) occurs in many plants, especially citrus plants, and it is an intermediate in the tricarboxylic acid cycle (TCA) of aerobic metabolism. Citric acid monohydrate has a molecular weight of 210.14 g mol^{-1} (Dhillon et al., 2011). Citric acid contains three carboxyl groups with three different pKa values (3.13, 4.76, and 6.40). Commercial citric acid is a colourless, transparent, or translucent crystal, or particle powder that is readily soluble in water and ethanol. It is widely used in food (75%), pharmacy (10%), and other industries (15%) (Ates et al., 2002; Tran et al., 2008). Citric acid can be produced through microbial fermentation, or it can be extracted from a natural source. Chemical syntheses of citric acid are possible but uneconomical because the starting chemical is usually more expensive than the desired product. The production of citric acid by microbial fermentation has many advantages compared to direct extraction from citrus fruits.

Citric acid manufacturing has grown significantly over the past century since the advent of biotechnology, which gives adequate comprehension of fermentation procedures and product recovery. Biochemistry, which gives understanding of several factors affecting citric acid synthesis and blockage, has also improved the production of citric acid and efficiency of recovery. In addition to biotechnology and biochemistry, molecular regulatory mechanisms and techniques had a significant role in increasing citric acid synthesis. Extensive literature studies and hundreds of publications on citric acid manufacturing have been published during the last 60 years (Prescott and Dunn 1959; Lockwood and Schweiger 1967; Miall 1978; Dawson 1986; Vandenberghe et al. 1999; Papagianni 2007; Max et al.
2010; Show et al. 2015; Tong et al. 2019).

There are various accounts of production of synthetic citric acid by several microorganisms. Reports on Bacteria: *Bacillus licheniformis, Arthrobacter paraffinens, Corynebacterium* sp., *Bacillus subtilis, Brevibacterium flavum* and *Corynebacterium* sp. Fukuda et al. (1970). Reports on Fungi: *Aspergillus niger, A. aculeatus, A. awamori, A. carbonarius, A. wentii, A. foetidus, Penicillium janthinelum* Karow and Waksman (1947), Roukas (1991), El Dein and Emaish (1979), Grewal and Kalra (1995), Chen (1994). Reports on Yeast: *Saccahromicopsis lipolytica, Candida tropicalis, C. Oleophila, C. Guilliermondii, C. Parapsilosis, C. Citroformans, Hansenula anamola, Yarrowia lipolytica, Torulopsis, Hansenula, Debaromyces, Torula, Pichia, Kloekera, and Zygosaccharomyces* by Gutierrez et al., (1993); Kapelli et al. (1978); Oh et al., (1973); Omar and Rehm (1980); Rane and Sims (1993); Uchio et al., (1975).

Because of its vast use, citric acid synthesis via fermentation continues to be of interest for major studies. Over the years, more substrates were developed for citric acid fermentation, allowing the industry to scale it up and boost production to meet the growing demand for citric acid. Most common substrates used for citric acid production includes Pineapple waste (Kumar et al. 2003), Apple pomace (Shojaosadati and Babaeipour, 2002), Banana peels (Karthikeyan and Sivakumar 2010), Coffee husk (Shankaranand and Lonsane, 1994), Corn cob (Hang and Woodams 1998), Grape pomace (Hang and Woodams, 1985), Corn (Hang and Woodams, 2000), Kiwi fruit (Hang et al., 1987). Brewery waste (Roukas and Kotzekidou, 1986 and 1987), Turnip whey (supplemented with molasses) (Chanda et al. 1990), Sweet potato (Anwar et al., 2009), Beet molasses (Lesniak et al., 2002), Date syrup (Roukas and Kotzekidou, 1997), Coconut oil (Soccol et al., 2006), Soybean oil (Soccol et al., 2006), Orange peel (Rivas et al., 2008). Therefore, the present study was aimed to isolate and screen the potential fungal isolate for citric acid production and to identify it using gene sequencing. (B.Manjunatha et al.,2020) it is due to the fact that nanosized particles show more cell membrane permeability and effective

diffusion in the cell, which will result in extermination of harmful bacterium.

MATERIAL AND METHODS

1. **Collection of samples**

   Decaying fruits and vegetables samples were collected from the local market of Kalaburagi, Karnataka, India. The samples were collected in the sterile sealed polythene bags and brought to the laboratory and stored at room temperature for further studies.

2. **Isolation of fungi for citric acid production**

   1 gram of sample was taken in a 250 ml conical flask containing 100 ml of sterile distilled water and shaken well for 20 minutes. From the stock, Serial dilutions of the samples were prepared as per the standard method (Cochrane et al., 1961), up to 10-6 dilutions. 0.5ml of 10^{-5} and 10^{-6} dilutions, 10^{-4} and 10^{-3} dilutions were inoculated on Nutrient Agar and Sabouraud Dextrose Agar respectively for the isolation of fungi. All the inoculated plates were kept for incubation in the incubator at 30°C for 5 days.

3. **Screening of fungi for citric acid production**

   Isolated cultures were subjected for screening of citric acid production by determining acid unitage value (AU). A loop full of fungal sporulation solution was inoculated on petri plates containing mineral salt agar acid indicator medium (Park *et al.*,1998) supplemented with bromoscerol green and incubated for five days for the formation of yellow zone around the mycelia growth. The acid unitage value for selection of potential fungal isolate was calculated using the formula given below.

$$\mathbf{AU} = \frac{\textbf{Zone diameter (mm) of yellow halo}}{\textbf{Zone diameter (mm) of fungal colony}}$$

4. **Morphological and microscopic observation**

   Lactophenol cotton blue was used as a mounting medium and staining agent for preparation of slides and microscopic examination of fungi. Nagajyoti @el .Depending upon the morphology of the particles, there are two categories in Suspension.

5. **Molecular identification**

   a. DNA Extraction was carried out based on the standard protocol described by Rogers and Bendich, (1994).

   b. PCR Amplification of 18s Gene: 170 ng of Extracted DNA is used for amplification along with 10pM of each primer.

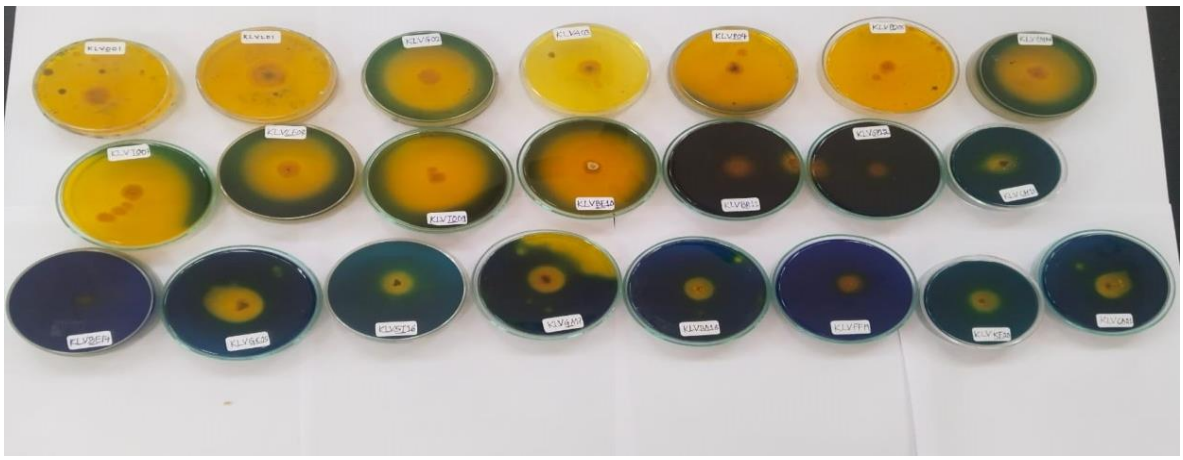
   c. Composition of TAQ Master MIX:

   - High-Fidelity DNA Polymerase
   - 2.5mM dNTPs
   - 3.2mM $MgCl_2$
   - PCR Enzyme Buffer

   d. The ~2kb, 18s-rDNA fragment was amplified using high–fidelity PCR polymerase (Table 1 and Table 2). The PCR product was sequenced Bi- directionally (Table 3). The sequence data was aligned and analyzed to identify the organism and its closest neighbors.

Table 1. Cycling Conditions

Initial Denaturation	3 minutes at 94°C	
Denaturation	1 minutes at 94°C	30 Cycles
Annealing	1 minute 50°C	
Extension	2 minutes at 72°C	
Final Extension	7 minutes at 72°C	



Amplification conditions

PCR Amplification conditions	Volume
DNA	1 µl
18s Forward Primer	2 µl
18s Reverse Primer	2 µl
dNTPs (2.5mM each)	4 µl
10X Taq DNA polymerase Assay Buffer	10 µl
Taq DNA Polymerase Enzyme (3U/ ml)	1 µl
Water	30 µl
Total reaction volume	50 µl

Table 3. Primer details of KLV07 isolate

No.	Oligo Name	Sequence (5`à 3`)	Tm(°C)	GC-Content
1	18sForward	GTAGTCATATGCTTGTCTC	42	42.11%
2	18sReverse	GAAACCTTGTTACGACTT	38	38.89%

RESULT AND DISCUSSION

Isolation of fungal strains from various sources

A total of 20 samples of decaying fruits and vegetables (Table 4) were subjected to isolation of fungi for citric acid production on SDA medium and incubated under 25 °C for 120hrs. The prominent colonies of the fungi were selected for further screening.

The colour transition from purple to yellow on the fermentation medium indicates positive results for citric acid synthesis. Plate 2 illustrates the synthesis of organic acid on a modified mineral salt medium; a yellow halo forms around the citric acid-producing colonies. Out of 20 fungal isolates, 7 isolates viz., KLV03, KLV04, KLV07, KLV10, KLV11, KLV13 and KLV14 showed significant acid unitage values of 3.00, 1.88, 3.36, 1.31, 2.35, 1 and 1 respectively (Table 5). Among these, KLV07 isolate (Plate 3) showed the highest acid unitage value of 3.36 and was selected for morphological and molecular identification (Table 5). The culture of KLV07 isolate was maintained in slant (Plate 4) and was stored in 4°C for further use. (Rahul jabnoor @ el) The contrasting in the image is due to the difference in spreading from variety surfaces area as an effect of geometrically difference and dispersed oxide sample.

Plate 2. Screening of potential fungal isolate for citric acid production

Table 4. Fungal strains isolated from various sources

Sl. No.	Source of microorganism	Isolates
1	*Citrus limon* (Lemon)	KLV01
2	*Citrus sinensis* (Orange)	KLV02
3	*Vitis vinifera* (Grapes)	KLV03
4	*Malus pumila* (Apple)	KLV04
5	*Ananas comosus* (Pineapple)	KLV05
6	*Solanum tuberosum* (Potato)	KLV06
7	*Punica granatum* (Pomegranate)	KLV07
8	*Citrullus lanatus* (Watermelon)	KLV08
9	*Musa paradisiaca* (Banana)	KLV09
10	*Solanum lycopersicum* (Tomato)	KLV10
11	*Phaseolus vulgaris* (Beans)	KLV11
12	*Mangifera indica* (Mango)	KLV12
13	*Manilkara zapota* (Chiku)	KLV13
14	*Beta vulgaris* (Beetroot)	KLV14
15	*Saccharum officinarum* (Sugarcane)	KLV15
16	*Ipomoea batatas* (Sweet Potato)	KLV16
17	*Daucus carota* (Carrot)	KLV17
18	*Psidium guajava* (Guava)	KLV18
19	*Solanum melongena* (Brinjal)	KLV19
20	*Abelmoschus esculentus* (Lady Finger)	KLV20

Table 5: Acid unitage values of Fungal isolates after 120hrs of Incubation on mineral salt medium

Cultures used	Zone diameter (mm) of fungal colony	Zone diameter (mm) of yellow halo	Acid unitage value (au)
KLV03	6 mm	18 mm	3.00
KLV04	17 mm	32 mm	1.88
KLV07	11 mm	37 mm	3.36
KLV10	16 mm	21 mm	1.31
KLV11	17mm	40 mm	2.35
KLV13	6 mm	6 mm	1.00
KLV14	6 mm	6 mm	1.00

Plate 3. KLV07 isolate on supplemented with bromocersol green

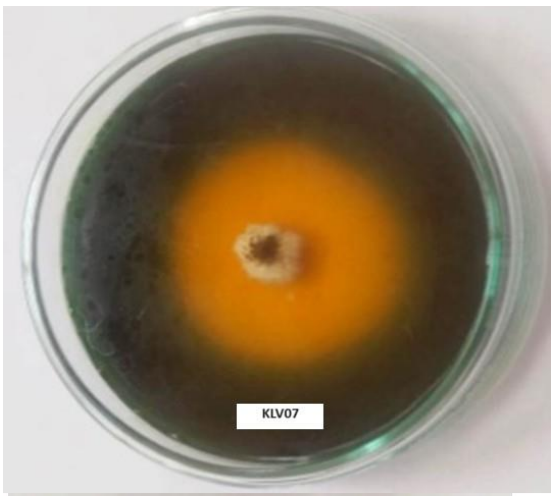
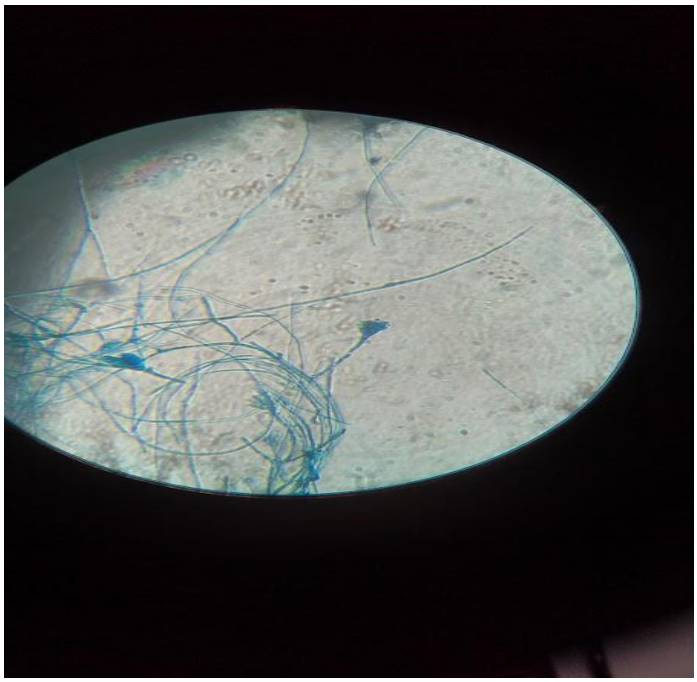


Plate 4. Slant of KLV07 isolate

Morphological identification

Basic morphological features of aerial mycelium, substrate mycelium and nature of colonies were observed under microscope as per the methods described in the Bergey's Manual of determinative Bacteriology (Goodfellow, 1989). Fungal elements are stained intensely blue. Under the microscope, several fruiting bodies and mycelia were observed (Plate 5).

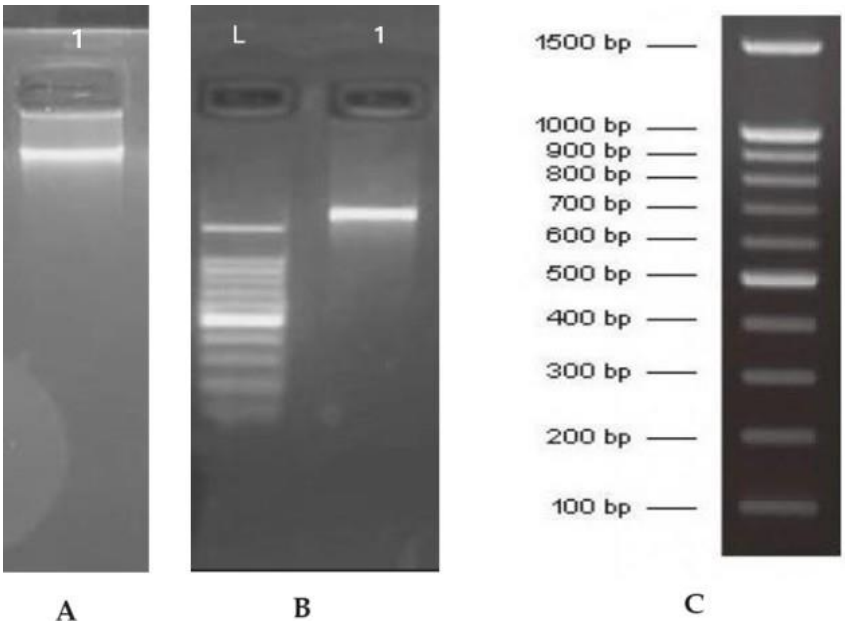
Plate 5. KLV07 isolate under microscope



Molecular Identification

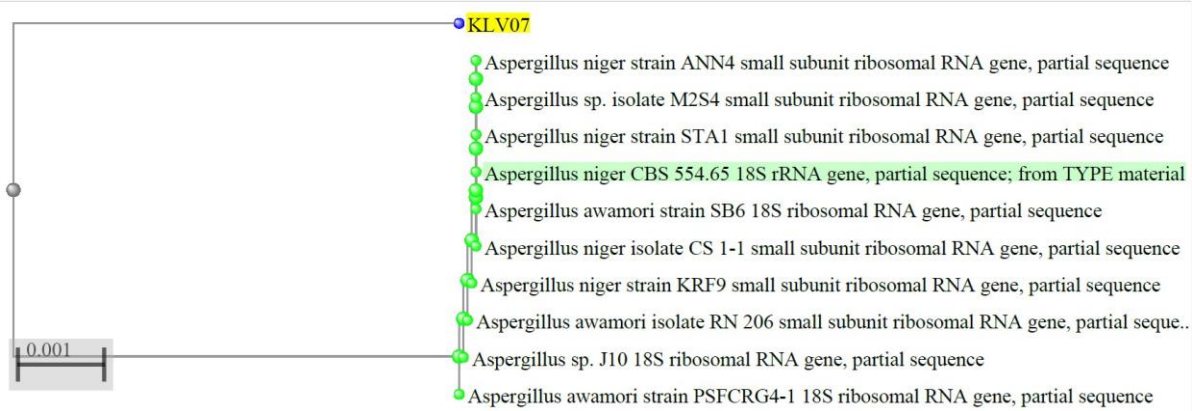
Plate 3 depicts the genomic DNA of KLV07, PCR amplified gene and 100bp ladder for the isolate.

Plate 3. QC report for KLV07 isolate: A, genomic DNA of KLV07; B, PCR amplified gene; C, 100bp ladder for the isolate.



18S rRNA gene sequence confirmed the isolate to be *Aspergillus niger* strain KRF9 small subunit ribosomal RNA gene with Sequence ID: MK611800.1 (Table 6). The next closest homologue was found to be *Aspergillus awamori* isolate RN 206 small subunit ribosomal RNA gene with Sequence ID: MK530131.1 (Plate 6).

Palte 6. Phylogenetic tree of KLV07 isolate using neighbour joining method



Aligned Sequence Data of Sample –KLV07 (1558 bp)

>KLV07
TGGAACACTTTAATAACGCTATTGGAGGTGGAATTACCGGGGTGCTGGCCCAAC
TTGCCTCCAATGGTCCTCGTAAGGGTTTAATTGGCTTTTTCCATTCCGGACCCAA
AAAGCCCTTTTTCTTTTATACATAAATTTATAACATTTTTTTTTCCCCGGTGTTAA
AAAAAACCCGGGGGTTAAAAAACCCCGCGGGCGGAAAAAAAAAAACCCCCCC
CCGGGGTCTATTTCCCCCCCTTAGGGGGAGGCCCCCCGGGGACGGGGCGGGGA
TTTGTTTTTTTCGGGGGGGACCAAAAACCCTTTCCCCCCCAAAGAGGGCCGGC
CCAATTTTCCCCTCCCCCCCGGGGGTTGCCAAAAATTTTATAGGGGCTTTTTGG
GTCCGGAATGGAAAAAGCCAATTAAACCCTTACGAGGACCATTGGAGGCAAGT
TGGGCCAGCACCCCGGTAATTCCACCTCCAATAGCGTTATTAAAGTGTTCCAGT
AAAAAGTCGTAGTGAACCTTGGTCTGGCTGGCGGTCCGCTCCCCGCGAGTATT
GGTCCGACTGACCTTTCCTTCTGGGAATCTCATGGCTTCACTGGCTGTGGGGGA
ACCAGGACTTTTACTGTGAAAAAATTAGAGTGTCAAAGCAGGCCTTTGCTCGA
ATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCT
AGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTC
AGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCA
AGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGAT
ACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGACGGTGTTTCT
ATTATGACCCGTTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGG
AGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCA
GACAAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATCTTTTGGATGGTGG
TGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAA
CGAGACCTCGGCCCTTAAATAGCCCGGTCCGCATTTGCGGGCCGCTGGCTTCTT
AGGGGGACTATCGGCTCAAGCCGATGGAAGTGCGCGGCAATAACAGGTCTGTG
ATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAG
TACATCACCTTGACCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGG
GGATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAG

TCATCAGCTCGTGCCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTA
CTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGACTCAGGAGTTTTTAC

Table 6. BLAST Data generated for first ten closet of KLV07 isolate

Sl. No.	Organism Name	Accession No.	% Match
1	*Aspergillus niger* strain KRF9 small subunit ribosomal RNA gene	MK611800.1	96.85
2	*Aspergillus awamori* isolate RN 206 small subunit ribosomal RNA gene	MK530131.1	96.85
3	*Aspergillus* sp. J10 18S ribosomal RNA gene	KU170626.1	96.85
4	*Aspergillus awamori* strain PSFCRG4-1 18S ribosomal RNA gene	HQ393868.1	96.85
5	Aspergillus niger strain ANN4 small subunit ribosomal RNA gene	MN420840.1	96.79
6	*Aspergillus* sp. isolate M2S4 small subunit ribosomal RNA gene	MN328341.1	96.79
7	*Aspergillus niger* strain STA1 small subunit ribosomal RNA gene	MK895556.1	96.79
8	*Aspergillus niger* CBS 554.65 18S rRNA gene from TYPE material	NG_065763.1	96.79
9	*Aspergillus niger* isolate CS 1-1 small subunit ribosomal RNA gene	MK564721.1	96.79
10	*Aspergillus awamori* strain SB6 18S ribosomal RNA gene	MG211804.1	96.79

Results shows that strain KLV07, together with the above-mentioned species do not form a distinct phyletic line with the neighbour-joining method. The summary of the accession number of the first ten closest strains to KLV07 is listed in table 6.

In the present study, the potential isolate for citric acid production is identified as *Aspergillus niger* with acid unitage value 3.36 isolated from pineapple substrate. Citric acid has recently been commercially produced through fungal fermentation, primarily by *Aspergillus niger*. It is also produced commercially using mutant fungal strains of *Saccharomycopsis lipolytica* (Good et al., 1985), *Penicillium simplicissimum* and *Aspergillus. foeitidus* (Tran et al., 1998). *A. niger* are principally used to produce citric acid, however in culturing these fungi, the growth of *Aspergillus* in pellet form is desirable and this can be achieved by process optimization (Rohr 1998; Wayman and Mattey, 2003). *Yarrowia lipolytica* can produce citric acid from raw glycerol (Rymowicz, 2007), a by- product of biodiesel production from rapeseed oil. Citric acid is commercially generated using various cost-effective and accessible raw materials such as molasses, carob pod extract, rape seed oil, corncobs, apple pomace, grape pomace, kiwi-fruit peel, mandarin orange, and brewery wastes as carbon sources (Roukas, 1998).

Due to increasing demand of citric acid, and there is a dire need for cost-effective and sustainable citric acid production methods. As therefore, from the standpoints of bioeconomy and environmental (white) biotechnology, it is crucial to develop a novel bioprocess for citric acid synthesis that has higher productivity and a lower environmental impact.

A. niger's ability to produce citric acid biochemical and physiological processes have attracted both biological and biotechnological interests, and studies have been performed by many research groups (Usami, 1978; Ruijter et al., 1996 and Roehr, 1998). Citric acid

fermentation by *A. niger* is the most economical and exclusively used method to produce citric acid (Usami, 1978; Rohr *et al.,* 1996; Ruijter et al., 1996; Roehr, 1998; Bentley and Bennett, 2008; Berovic, 2004 and Karaffa & Kubieck, 2003). There reports on *A. niger's* full genome sequences and comparative genomics studies by Pel et al., (2007) and De Veries et al., (2027) making the organism open to targeted growth through genetic and metabolic engineering. Genetic selection of citric acid producing strains, or the development of strains with desirable traits, has been investigated by numerous research groups. Honda et al. (2011) established a high-frequency homologous transformation method for gene disruption and tailored gene substitution in citric acid generating *A. niger.*

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