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Research article

Citric Acid Fermentation by *Aspergillus niger*

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Abstract

From twenty soil samples collected, ninety six isolates were identified as *Aspergillus niger*. All *A. niger* isolates understudying were varied in their capability to produce citric acid on Czapek-Dox broth medium. Based on the productivity of citric acid in shake flask cultures after 3 days, the highest local *A. niger* MH368137, was selected for further experiments with the reference strain, *A. niger* EMCC132. The optimal time incubation for the maximum citric acid production reached statistically 5 days of fermentation. Through series of experiments were designed on various physicochemical fermentation parameters to establish the optimal conditions for citric acid overproduction, it could be summarized as: optimum temperature, 28˚C; initial pH 6.5; inoculums size 3.0×10^6 spores/ml; shaking culture (100rpm); maltose as a carbon source 40 g/L; peptone as a nitrogen source 3 g/L; Sodium phosphate as phosphorus source; 2% of methanol as enhancer. Through these environmental and nutritional parameters, citric acid production was clearly improved and gave 14 times higher that reached to 13.33 mg/1ml comparing to the original yields 0.907 mg/ ml by *Aspergillus niger* MH368137 (FQW). When local agricultural wastes and byproducts (pineapple peel, sugarcane bagasse, potato peels; sugarcane molasses and dates molasses) utilized for citric acid production, local strain *A. niger* MH368137 (FQW) produced highest amount of citric acid, reached up to 256.94 mg/ ml when grew on sugarcane molasses as a sole carbon source.

Introduction

Citric acid, or 2-hydroxy-propane-1, 2, 3-tricarboxylic acid ($C_6H_8O_7.H_2O$) is a naturally occurring weak organic acid, with pH 0.2 found in all citrus fruits [1]. Citric acid in its pure form is readily soluble in water and colorless [2]. It is solid at room temperature. Citric acid has a melting point of 153°C and it decomposes at higher temperatures. Citric acid has a molecular weight of 210.14 g/mol and possesses three different pKa values, at pH 3.1, 4.7 and 6.4, owing to the presence of three functional groups of carboxylic acid in its structure [3].Citric acid fermentation is one of the primitive fermentations but still its production is increasing with passage of time.

In 1916, a study conducted by James Currie made a breakthrough for successful economic industrial production of citric acid from *Aspergillus niger*. He discovered that significant amounts of citric acid could be obtained from various strains of *A. niger*. The most important findings were the ability of *A. niger* to grow at a pH of around 2.5–3.5, which curbed the formation of gluconic and oxalic acid, and the increase in citric acid production with increasing sugar concentration. This single piece of research laid the foundation for present-day industrial citric acid production, which was established in the USA by the pharmaceutical company Pfizer in 1923 [4].

Materials and methods

Sample collection and isolation

Soils were collected into a clean polythene bag from King Saud University. Ten gram of each soil sample was suspended in 90 ml of sterilized distilled water. Subsequently, fungal cultures were isolated by serial dilutions in sterilized saline and plated onto Petri dishes with potato dextrose agar medium. The plates were incubated at 28-30°C for 4-7 days. Individual colonies were picked by repetitively streaked through a series of plates to isolate the individual member. For purification, the growing single colonies were picked up and streaked on slants of Potato Dextrose Agar, PDA (plus 4 ml per liter of chlorampenicol, 25 mg/ml, after autoclaving) and kept at 4°C until using. The isolated colonies were microscopically examined and identified according to their observed morphological characteristics [5].

Fungal cultures maintenance

Aspergillus niger strains used in the study include the local isolates which were obtained from various locations

in Riyadh region in addition to fungal strain, *Aspergillus niger* EMCC132 that used throughout the experiments as a reference strain for citric acid production and it was obtained officially from Cairo Microbiological Resources Centre (Cairo MIRCEN), Ain Shams University, Cairo, Egypt. The cultures of *Aspergillus niger* were maintained on sterilized potato dextrose agar medium (PDA) and stored at 5ºC in the refrigerator. In general, all the used media, unless otherwise stated, were sterilized in autoclave at 15-lbs/inch2 pressure (121°C) for 15 min.

Preliminary screening

The *A. niger* culture was screened qualitatively for citric acid production by plate method on czapeck dox agar (contained (g/l): Sucrose: 20; $NaNO_3$: 2; K_2HPO_4: 1; KCl: 0.5; $MgSO.7H_2O$: 0.5; $FeSO_4.7H_2O$: 0.01 and agar: 15. Streptomycin (30µg/ml) was added to the above medium after sterilization and cooling to suppress bacterial growth, containing Bromocresol green as indicator for citric acid detection. The medium was prepared by dissolving all of the ingredients except agar; the pH was maintained at 6.0. Then, the medium was sterilized in the autoclave after the agar addition [6-7].

Citric acid production in submerged culture

The medium used was Czapek-Dox broth medium. The experiments were performed in shaking incubator using 50 ml of Czapek-Dox broth medium contained in 250 ml Erlenmeyer flask. All media were sterilized at 121°C for 15 min after cooling at room temperature and seeded with 1ml of inoculums (conidia suspensions). The flasks were incubated in a rotary shaker operating 100 rpm at 28°C. After incubation periods, the culture broth from each flask was filtered through Whatman filter paper for mycelia separation, dried and the yield of dry fungal biomass was recorded. The clear culture filtrate was employed for determination of citric acid and recorded final pH values.

Effect of some cultural conditions on extracellular citric acid production by *Aspergillus niger*

Eight factors were investigated to study their effects on citric acid production by *A. niger*. Those factors were, incubation periods at 24, 48, 72, 96, 120, 144 hrs; incubation temperature at 12, 20, 28, 36 and 44°C; initial pHs 3, 5, 8 and 10; Inoculum Size at (1.0, 2.0 and 3.0) $x10^6$ spores/ml carbon source: sucrose, maltose, glucose, fructose, lactose and arabinose with initial concentration of carbon source (3% w/v); selected carbon source concentrations at 20, 25, 30, 35, 40 g/l; nitrogen source: NaNO3 (2g/l), NH_4NO_3 (1.9 g/l), KNO_3 (2.4 g/l), NH_4Cl (2 g/l), $(NH_4)_2SO_4$ (2.1 g/l), glycine (1.8 g/l), or peptone (2.64 g/l); selected nitrogen source at 1, 2, 3, 4 or 5 g/l; Phosphorus Source (1.0 g/l) :NaH_2PO_4, Na_2HPO_4, Na3PO4, KH_2PO_4 and alcohol source (1%) Methanol, Propanol and Ethanol; selected alcohol concentrations at 1.0, 2.0, 0.0, 4.0, 0.0% added after sterilization. Triplicate flasks containing the optimized Czapek-Dox,s medium were prepared. The medium was inoculated with fungal discs and incubated under the previous successive conditions.

Usage of raw materials for citric acid production

Raw material sources

Five different agricultural waste products (pineapple, sugar cane bagasse, sugar cane molasses, dates molasses, potato peels) used in this study was kindly obtained from various sources, sugarcane bagasse from sugarcane juice shops; potato peel from Saudi factory for the manufacture of potato and food – Riyadh - third industrial region; pineapple peel from vegetables markets – alraboa – Riyadh; dates molasses from Tuwaijri farm – Qassim and sugar cane molasses from the United Company for sugar – Jeddah.

Preparation of raw materials

Samples of sugar cane bagasse, potatoes peels and pineapple peels were dried in outside air (at the roof) for a week with a daily continuous stirring. After the samples completely dried, were milled using blinder, then sieved using sieve with narrowness holes. All samples were kept in clean bottles at a temperature of 25-28 until use.

Chemical analysis of the raw materials

All wastes samples were dried in oven at 50°C. For CHNS (carbon, hydrogen, nitrogen and sulfur) analysis, the samples were passed through 75 Micron Sieve and CHNS were analyzed by high combustion method using PERKIN ELMER Series II, CHNS/O Analyzer. Phosphorus (soluble) was analyzed by Ascorbic Acid method using HACH DR/U 4000 Spectrophotometer. Soluble components of Potassium, Calcium and Magnesium were analyzed by Ion Chromatography using Shimadzu Ion Chromatograph.

Raw materials and saccharification

The saccharification of waste materials used for the production of citric acid consisted of 5% in distilled water (w/v or v/v) of selected raw materials (i.e. sugarcane bagasse, pineapple peel, dates molasses and sugarcane molasses) except only 3% (w/v) of potato peel because of high viscosity of the suspended materials. Autoclaving (sterilization) of all prepared solutions were conducted for mild hydrolysis the raw materials. After cooling, the suspended insoluble materials were removed by filtration and the obtained filtrates were used in preparation of fermentation media for citric acid production.

Raw materials and production medium

Fermentation experiments were performed in 250 ml Erlenmeyer flasks containing Czapek-Dox broth medium components dissolved in 50 ml of the previous prepared filtrate (5 or 3% of the waste), then they were cotton plugged and sterilized at 15 lbs for 15 minutes at 121°C. Flasks were inoculated with isolated fungal, 1 ml of spores suspension used as inoculums. The flasks were incubated in rotary shaker operating at 100 rpm at the different temperatures for 5 days. After that the mycelia separation by filtration to assay the biomass dry weight and the filtrate was analyzed for pH and citric acid amounts.

Molecular identification of fungal isolates

Identification of *Aspergillus niger* species was identified according to Gilman, Joseph 1998, then identification was further confirmed using nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing by CEQ 8000. The genomic DNA was obtained using the protocol of GeneJet Plant genomic DNA purification Kit (Thermo) **# K0791 (**http://www.thermoscientificbio.com/). Internal transcribed spacer (ITS) region of 5.8S rRNA was amplified using the primers **ITS1 (5′TCC GTA GGT GAA CCT TGC GG 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′)**. PCR sequencing of the amplified product was carried out at GATC Company (Germany). The resulting sequence was entered into the BLAST algorithm of National Centre of Biological Information (NCBI) database to obtain closely related phylogenetic sequences. A phylogenetic tree was constructed using MEGA 6 software. The obtained sequence was then submitted to the GenBank of National center for biotechnological information (NCBI) database. A strain identifier and an accession number were obtained for each isolate.

Results and discussion

Microfloral picture

The aim of this part is to isolate and get pure cultures of local fungus, *A. niger* strains. From twenty soil samples collected from different sites in Riyadh region, several *Aspergilli* strains were isolated and purified.

Among the colonies characteristics observed, the identification process was performed based on their morphological characteristics. *Aspergillus niger*, it was confirmed by Gillman fungi Manual Based on morphological characterization, ninety six isolates of *Aspergillus niger* from different sources were selected as mentioned in Table (1), the twenty sites and number of *A. niger*isolates from each site with an abbreviation cods.

Preliminary screening of *A. niger* isolates producing citric acid

The experiment was conducted to choose citric acid producing-fungal isolates using plates of Czapek-Dox agar containing Bromocresol green as an indicator and yellow zones indicated citric acid production. To check from efficacy of the obtained isolates for citric acid production, the experiment was conducted using plates of Czapek-Dox agar containing Bromocresol green as an indicator and yellow zones indicated citric acid production. Ninety six isolates of *Aspergillus niger* were tested for their capability to produce citric acid using an indicator plates medium. All *A. niger* isolates understudying were varied in their capability to produce citric acid and in productivity amount at different time intervals i.e. 24, 48, 72, 96 hours incubation on indicator plats medium through the diameter of the formed yellow zone (mm). Most of *A. niger* isolates (70 isolates) reached to the maximum acid production, where yellow color filled the plates medium (90 mm), after 72 hour incubation, represented 66 72.9 % of the total isolates (Figure 1). However, some isolates (14 isolates) were slowly released acid in the indicator medium and acid production gave a maximum yellow zone diameter (90 mm) after 96 h incubation, represented 14.6 % of the total isolates. In the same time, twelve *A. niger* isolates proved to be the best citric acid producers, where gave a widest yellow zone diameter (90 mm) after only 48 hour incubation, represented 12.5 % of the total isolates.

Table 1. Total count (colony/g dry soil), relative density percentage (R. D. %) and species diversity (H) of *Aspergillus* isolates from different sources in KSU.

Soil Source Isolates (Codes)	Total count
1. South Wadi Hanifa SWH	210
2. North Wadi Hanifa NWH	164
3. Slam Park in the center of Riyadh SPR	276
4. King Saud University Malaz, Garden KSUM	170
5. King Saud University Diriya, Garden KSUD	174
6. Rawabi garden east of Riyadh EGR	148
7. Farm in Thumama (A) FTHA	162
8. Farm in Thumama (B) FTHB	116
9. Farm way Qassim (A) FQWA	168
10. Farm way Qassim (B) FQWB	112
11. Farm in Mansuriya FMN	114
12. A Farm in AL-Muzahmiyya FMZ	130
13. A Farm in AL-Ghad FGD	148
14. A Farm in Dirab (A) FDRA	168
15. A Farm in Dirab (B) FDRB	160
16. A Farm in the dam Diriya FDD	104
17. A Farm in AL-Kharj FKH	102
18. A Farm in Dharme FDR	88
19. Farm in Hayer FHA	98
20. Farm in Arka	82
Total number of colonies	2894

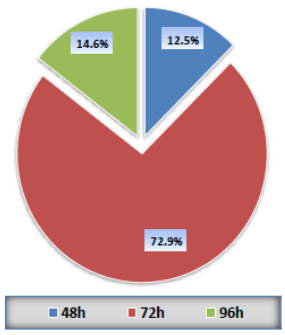

Figure 1. Distribution of all *A. niger* isolates (96) producing citric acid after different incubation periods (sruoh).

On the basis of obtained results of the preliminary screening through used an easily and quick technique, It could be able to select six *A. niger* isolates, that obtained from different isolation sources, gave highest productivity of citric acid, ranged from 49 to 60 mm zone diameter, after 24 hours incubation. The favorable isolates of *A. niger* for citric acid production were SWH4, KSUD4, FTH1, FQWA1, FDRA3 and FAR4 selected for further experiments. In view of other workers, the citric acid is being produced by microorganisms during fermentation through their metabolic citric acid cycle [6, 8]. When microbes concern, there are many organisms which are producing citric acids. However, fungus is considered to be suitable producer of citric acid, especially, *Aspergillus niger* is utilized as much as it produces.

Selection and time course

One of the most important fungi used in industrial microbiology, *Aspergillus niger*, has been employed for many years for the commercial production of citric acid. However, the worldwide demand for citric acid is increasing faster than its production and more economical processes are required [9]. Citric acid production synthesis 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, which has its own advantages: operations are simple and stable, the plant is generally less complicated and needs less sophisticated control systems, technical skills required are lower, energy consumption is lower and frequent power failures do not critically affect the functioning of the plant [10]. Especially, the submerged technique is widely used for citric acid production. It is estimated that about 80 % of world production is obtained by submerged fermentation. This fermentation process employed in large scale requires more sophisticated installations and rigorous control. On the other hand, it presents several advantages such as higher productivity, lower labour costs and lower contamination. Submerged fermentation can be carried out in batch, fed batch or continuous systems, although the batch mode is more frequently used. Normally, citric fermentation is concluded in 5 to 12 days, depending on the process conditions [11]. In addition, the submerged process has become the method of choice in the industrialized countries, because it is less labor intensive, yields higher production rate, and uses less space [4]. As well, the choice of a suitable strain is of great importance. Among the different fungal isolates of *A. niger*has the highest ability to produce citric acid [12].

Selection off citric acid producers

Therefore in this part, the best six citric acid producing-fungal isolates (which selected after primary qualitatively screening for their potential as citric acid producers on plates of Czapek-Dox agar with Bromocresol green as an indicator) were selected from a group of indigenous *A. niger* population to study their capability for citric acid production in submerged culture, shake flask cultures, during three days incubation. The selected six *A. niger* isolates are FDR, SWH, FAR, FQW, KSUD and FTH. In addition to *A. niger* EMCC132 was used as a reference fungal strain for citric acid production. Data recorded in Table (2) shows that the production of citric acid by six locally isolated and selected reference strain *A. niger* EMCC132 were significantly varied. Citric acid production was in the range from 4.52 to 16.34 mg/10 ml culture filtrate. A highest significant citric acid concentration (16.34 mg/10ml) was produced by *A. niger* EMCC132 followed by KSUD, FQW and FDR that produced not significantly different amounts of citric acid, 9.98, 9.07 and 8.84 mg/10ml respectively. Dry fungal mass measurements were ranged from 0.24 to 0.41 g/50 ml. It noticed that no correlation between fungal biomass of all tested isolates and produced citric acid amounts (Table 2). The pH measurements of the tested fungal cultures after 3 days incubation reduced and reached to acid range (3.19 – 3.63) as shown in Table (2). The submerged fermentation process is desirable and fermentative production of citric acid arises from a primary energy metabolism although it is non-growth associated [13]. Based on the productivity of citric acid under these conditions, higher two local *A. niger* isolates, KSUD and FQW, were selected for further experiments comparing to the reference strain, *A. niger* EMCC132.

Time course of citric acid production under submerged fermentation condition

Although citric acid can be produced by submerged and solid state fermentation, industry still prefers submerged fermentation because of its continuous operation [14] [15]. For that, submerged fermentation in shaking flask cultures was used in all over current experiments for citric acid production.

Table 2. Production of citric acid (mg/10ml) by most potent isolates of *Aspergillus niger* comparing with reference stain (EMCC132) in submerged culture. Data represented as mean ± standard error.

Isolate	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
EMCC132	$3.61^{a} \pm 0.06$	$0.29^{ac} \pm 0.03$	$16.34^{a} \pm 0.09$
FDR	$3.26^{cd} \pm 0.06$	$0.41^{a} \pm 0.05$	$8.84^{b} \pm 0.04$
SWH	$3.34^{bc} \pm 0.03$	$0.38^{ab} \pm 0.03$	$6.76^{c} \pm 0.10$
FAR	$3.49^{ab} \pm 0.12$	$0.28^{bc} \pm 0.01$	$7.05^{c} \pm 0.33$
FQW	$3.63^{a} \pm 0.02$	$0.40^{ab} \pm 0.06$	$9.07^{b} \pm 0.29$
KSUD	$3.19^{c} \pm 0.04$	$0.24^{c} \pm 0.06$	$9.98^{b} \pm 0.13$
FTH	$3.37^{bd} \pm 0.02$	$0.38^{ab} \pm 0.01$	$4.52^{d} \pm 0.09$
LSD $_{0.05}$	0.1789	0.1207	1.2674

In Czapek-Dox broth medium.Initial pH was 6.5. Inoculum density was ~$1.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28oC for 3 days.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

To determine the effect of fermentation period on citric acid productivity and to define the optimum incubation time, fermentation was carried out for various time periods (1 – 6 days). As shown in figure 3. Shaking flask cultures were incubated at 28˚C. Samples were withdrawn every 24 hours. Citric acid production, change in pH and dry fungal biomass was also determined. The obtained results of citric acid production, pH values and fungal biomass that determined in shake flask cultures of the three molds under investigation (*A. niger* KSUD, FQW and EMCC132) are expressed in Table (3). The obtained data show that citric acid amounts produced by all *A. niger* tested isolates were gradually and significantly increased by increment of the incubation periods.

A. niger FQW was grown on Czapek-Dox broth medium and gave maximum biomass, 0.397 mg DW/50 ml, at the 6th day of incubation. In the same time, citric acid was detected and quantified starting from the 1st day and reached to a statistically maximum value, 13.19 mg/10ml, at 5th day shaking incubation (Table 3). Also the fungal biomass was grown progressively and recoded highest growth after 6 day incubation that was 0.40 g DW/50 ml. In contrast, pH value was gradually reduced that recorded lowest value, 2.29, at the 6^{th}day. The recorded results in Table (3) indicate that the time course analysis of submerged culture of *A. niger* KSUD was slowly propagated in the first day, then gradually increased and gave a maximum fungal biomass (0.40 g DW/50 ml) in the 4th day of incubation. Although the fungal growth started reduces at 5th day incubation, citric acid amount increased and assayed statistically high value, 16.91 mg/10ml among 5 days incubation. The estimated pH values appeared to obviously decrease after first 24 hours and gradually reduced in parallel with acid production, that recorded lowest value, 2.43, at the 6th day incubation (as mentioned in Table 3). Comparing the previous data of local fungal isolates with the reference one, it was found that *A. niger* EMCC132 was produced highest amount of citric acid and reached to 25.51 mg/10ml at the 6th day incubation (Table 3), moreover, gave a maximum growth, 0.38 mg DW/50 ml, where pH values gradually reduced and recorded 2.73 after 6 days. Although, an international strain *A. niger EMCC132*, used as a reference fungal strain for citric acid production, had a pronounced capability to produce highest amount of citric in flask culture after 6 days incubation. However in case of *A. niger* KSUD and FQW, the maximum amounts of citric acid were at the 5th day and the higher amounts of citric acid at sixth day were insignificantly increased. But the variation was not significantly. Moreover, the quantity of citric acid produced varies with both microorganism and fermentation period. According to the pervious results, it is indicated that among all the tested fungal strains, citric acid production was varied. Citric acid was ranged between 14.09 – 25.51 mg/10ml. The tested fungal strains were produced citric acid in the following order, *A. niger* EMCC132 >*A. niger* KSUD >*A. niger* FQW. On comparing the time course of the citric acid production in the fermentation medium to that of incubation period where citric acid amounts reached statistically to maximum values on 5th day of fermentation. The optimal time of incubation for maximum citric acid production varies with both the organism. The obtained results of the time course analysis of tested fungal cultures indicated that optimal time incubation for the maximum citric acid production varies with both the tested fungal strains under the same condition and ranged between 5-6 days incubation period. Similarly, it was found by [16] who reported that viability increases with time of incubation, but higher production of citric acid was achieved and reached maximum at the stationery phase in less than 7 days incubation. While study of [17] revealed that the optimum time course is 192 hr (8 days) for citric acid production. In addition, our

result in agreement with the most recent study [18] where the quantity of citric acid produced varies with the type of tested microorganism. The rate of citric acid biosynthesis was studied and the maximum yield of citric acid (0.61 g/L) was after 5 days of fermentation. Extension of the fermentation period brought about depletion in the yield of citric acid produced. In batch fermentation of citric acid, the production started after a lag phase of one day and reached maximum at the onset of stationary phase or later. It might be due to the decreased available nitrogen in fermentation medium, the age of fungi, and depletion of sugar contents. These explanations were also in corroboration with earlier reports by [19] stated that much time causes the decrease of nitrogen and sugars in the substrate, thereby a reduction in citric acid production. This implies that the fermentation period used was suitable for the production of citric acid as higher yield is harvested at early stage thereby cutting down the cost needed to maintain the fermentation for longer time. The local fungal strains, *A. niger* KSUD and FQW which gave the maximum amounts of citric acid at the 5th day, therefore, this incubation period was applied in the further experiments.

Table 3. Time course of citric acid production in shake submerged culture of *Aspergillus niger* Strains. Data represented as mean ± standard error.

A. niger Strains	Time (days)	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	0	6.43^{b} ±0.03	0.06^{e} ±0.00	3.32^{d} ±0.16
	1	5.95^{a} ±0.05	0.01^{e} ±0.02	3.00^{d} ±0.07
	2	4.04^{c} ±0.08	0.20^{d} ±0.01	5.21^{c} ±0.33
	3	3.61^{d} ±0.00	0.26^{c} ±0.01	9.57^{b} ±0.43
	4	3.37^{e} ±0.04	0.25^{c} ±0.035	9.19^{b} ±0.97
	5	2.54^{f} ±0.01	0.31^{b} ±0.01	13.19^{a} ±0.51
	6	2.29^{g} ±0.06	0.40^{a} ±0.00	14.09^{a} ±0.53
	$LSD_{0.05}$	0.13959	0.04538	1.5396
KSUD	0	6.50^{a} ±0.00	0.03^{e} ±0.00	4.54^{c} ±0.58
	1	6.12^{b} ±0.16	0.06^{e} ±0.00	5.70^{c} ±0.71
	2	4.12^{c} ±0.11	0.23^{c} ±0.02	6.59^{c} ±0.13
	3	3.56^{d} ±0.06	0.26^{cd} ±0.01	9.34^{b} ±0.66
	4	3.32^{de} ±0.04	0.40^{ab} ±0.03	11.77^{b} ±1.29
	5	3.02^{e} ±0.19	0.30^{d} ±0.02	16.91^{a} ±0.06
	6	2.43^{f} ±0.05	0.36^{a} ±0.00	17.16^{a} ±1.55
	$LSD_{0.05}$	0.3244	0.04712	2.64969
EMCC132	0	6.07^{a} ±0.00	0.08^{e} ±0.00	2.00^{e} ±0.00
	1	5.32^{b} ±0.02	0.12^{e} ±0.01	2.96^{e} ±0.32
	2	4.13^{c} ±0.05	0.22^{d} ±0.01	8.96^{d} ±0.71
	3	3.90^{cd} ±0.10	0.25^{cd} ±0.03	15.18^{c} ±0.92
	4	3.77^{d} ±0.11	0.28^{c} ±0.02	16.96^{c} ±0.08
	5	2.98^{e} ±0.03	0.33^{b} ±0.02	22.15^{b} ±1.20
	6	2.73^{e} ±0.02	0.38^{a} ±0.01	25.51^{a} ±0.87
	$LSD_{0.05}$	0.266	0.04909	2.1889

In Czapek-Dox broth medium. Initial pH was 6.5. Inoculum density was ~$1.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Optimization of fermentation condition for citric acid production

In developing a fermentation process for obtaining a valuable product, the optimization of cultivation conditions and selection of appropriate substrates and the most favorable of their concentrations have primary importance due to their impact on the economy and feasibility of the process. Many studies have been intended to obtain high-yielded production using optimization processes with low cost as possible. The improvement strategies used in citric acid production by *Aspergillus niger* were generally interested in classical optimization methods, and there are many reports about classical optimization methods for citric acid production based on varying one-factor-at-a time [11, 17, 20].

Effect of incubation temperature

Temperature is known to influence the metabolic rate of the organism involved in the process, which in turn determines the amount of the end product. Therefore, the optimum temperature for citric acid production was defined by assaying the citric acid amounts at different incubation temperatures ranging from 12 to 44˚C, after 5 days as a favorite parameter refereeing to the pervious

experiment results. Data represented in Table (4) show a significant increment in citric acid production with the increase of incubation temperature up to 28˚C for the three tested fungal strains (*A. niger* FQW, KSUD and EMCC132) and thereafter, higher temperature resulted in a gradually lowering of citric acid formation. Maximum citric acid production by tested fungal strains, *A. niger* FQW, KSUD and EMCC132 were reached to 11.26, 15.25 and 22.02 mg/10ml, respectively at 28˚C. Obtained results indicated also that changing in pH values was accompanied by a noticeable of the yield of citric acid. Moreover, growth of fungal strains were significantly lowered at both 12 and 44˚C, but changing in fungal growth were insignificantly different in the temperature range 20-36˚C (Table 4). In general, temperature obviously influences the metabolic activity of cells. Where, the obtained results for growth of fungi at various incubation temperatures reveal that all tested *A. niger* strains could not tolerate higher temperature, that was appeared in the dry weight of the fungal biomass. Incubation temperature at 28˚C was considered as an optimum cultivation temperature for the growth. Temperatures between 25-30˚C are usually considered optimal for fungi. However, a temperature above 35˚C inhibits mycelial growth and favours oxalic accumulation. There is an exception for the thermo tolerant *A. niger* cultures which can produce citric acid even at 29-40˚C [21]. In addition, [22] reported that the temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid. Temperatures lower than 27°C slowed down growth and production substantially. When the temperature of medium was low, the enzyme activity was also low, giving no impact on the citric acid production. However when the temperature of medium was increased above 30°C, the biosynthesis of citric acid was decreased.

Table 4. Effect of incubation temperature on citric acid production in shake flask culture of *Aspergillus niger* strains. Data represented as mean ± standard error.

A. niger Strains	Incubation Temperature (˚C)	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	12	6.45^{a} ±0.02	0.19^{b} ±0.02	4.00^{c} ±0.43
	20	2.73^{d} ±0.02	0.31^{a} ±0.01	7.01^{b} ±0.10
	28	2.39^{d} ±0.01	0.31^{a} ±0.04	11.26^{a} ±0.01
	36	3.79^{c} ±0.09	0.30^{a} ±0.03	7.86^{b} ±0.87
	44	5.12^{b} ±0.49	0.20^{b} ±0.02	3.22^{c} ±0.01
	$LSD_{0.05}$	0.6951	0.0750	1.3719
KSUD	12	6.44^{a} ±0.03	0.20^{b} ±0.03	7.04^{c} ±0.73
	20	2.71^{d} ±0.01	0.29^{a} ±0.01	6.52^{cd} ±0.09
	28	2.19^{e} ±0.00	0.30^{a} ±0.02	15.25^{a} ±0.08
	36	3.67^{c} ±0.00	0.32^{a} ±0.01	11.19^{b} ±0.35
	44	4.76^{b} ±0.07	0.16^{b}±0.01	5.10^{d} ±0.64
	$LSD_{0.05}$	0.1121	0.0507	1.4574
EMCC132	12	6.49^{a} ±0.00	0.21^{b} ±0.00	6.33^{d} ±0.11
	20	3.4^{cd} ±0.10	0.39^{a} ±0.01	8.55^{c} ±0.53
	28	3.08^{d} ±0.03	0.35^{a} ±0.015	22.02^{a} ±0.14
	36	3.70^{c} ±0.14	0.40^{a} ±0.06	20.90^{b} ±0.06
	44	4.63^{b} ±0.53	0.21^{b} ±0.02	5.09^{e} ±0.02
	$LSD_{0.05}$	0.4990	0.0953	0.7894

In Czapek-Dox broth medium.Initial pH was 6.5. Inoculum density was ~$1.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm for 5 days. Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Moreover, *Aspergillus niger* has grown well at 30°C and produced maximum amount of citric acid (80.68 g l-1) followed by 20°C with 74.21 g l-1 of citric acid [17]. Also, [23] have reported maximum amount of citric acid production (99.56 ± 3.59 g l-1) achieved by *Aspergillus niger* GCBT7 at 30°C. [24] has also reported that maximum citric acid production was recorded at 30°C using *Aspergillus niger* NCIM 705. The effect of temperature plays an important role in the production of citric acid by *Aspergillus niger* [25]. According to obtained data in our experiments, incubation temperature of 28°C was selected for further experiments for improvement of citric acid production.

Effect of initial pH

The metabolic activity of fungus is very sensitive to pH level of media. The initial pH of the medium was found to have an impact on citric acid production by *A. niger* [26]. Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 to 6, but some fungi are able to growth at a pH value below 2 to better compete against bacteria [27].

The favorable initial pH is one of the most important steps for the successful progression of citric acid fermentation. The objective of this experiment was to

evaluate the effect of the initial pH of the nutrient solution on citric acid production by *A. niger* local strains and the reference strain. The influence of initial pH value of the culture medium on growth and citric acid production by *Aspergillus niger* strains (FQW, KSUD and *A. niger* EMCC132) was investigated at initial pH values 3, 5, 6.5, 8 and 10. The initial pH values were adjusted by diluted acid or alkali. The flask cultures were incubated on rotary shaker for five days at optimal temperature 28^{o}C attained in the foregoing experiments. The results on the effects of different initial pH values on citric acid production, mycelial dry weight and changing in cultures' pH by *A. niger* strains are recorded in Table (5). The obtained results indicated that maximum citric acid concentrations by *A. niger* FQW, KSUD and EMCC132 (11.26, 15.25 and 22.27 mg/10 ml, respectively), was obtained at pH 6.5. Moreover, a very sharp decrease in citric acid yield was observed when the initial pH values were increased to 8.0 and to 10.0. This reduction was also significantly noticed and accompanied by a similar reduction in mycelia dry weight. The effect of the initial pH of nutrient solution on citric acid production was evaluated and concluded that the initial pH had a considerable effect on the growth of *A. niger* strains and production of citric acid. From the results of our work, it could be concluded that citric acid production and growth yield were strongly affected by initial pH level and apparently the maintenance of a favorable initial pH 6.5 is essential for successful production of citric acid in further experiments. In general, initial pH value of the culture medium mainly controls the fungal growth and citric acid production as well. Different pH optima have been report by several researchers. The obtained results are in line with [28, 29] studied the influence of low and high initial pH values and reported that decrease in initial pH value lower than 3.5 results in reduction in citric acid production. It might be due to that at low pH, the ferrocyanide ions were more toxic for the growth of mycelium. A higher initial pH leads to the accumulation of oxalic acid. Also Oxalic acid is an undesirable by-product of citric acid fermentation and is considered to be a main impurity in the commercial citric acid process from *A. niger* at higher initial pH [30]. In addition, [23] insured that the maintenance of a favorable pH is very essential for the successful production of citric acid. Effect of different pH (4.5-7.0) on the citric acid production was studied and the highest value of citric acid yield (96.12 ± 3.5 g/l) when initial pH of the fermentation medium was kept at 6.0 by culture of *Aspergillus niger* GCBT7. A lower initial pH was reported to inhibit the growth of *A. niger* and is thus expected to negatively affect citric acid production. Similar results were explained in previous studies. Where the effect of different initial pH (4.5 - 7.0) of the fermentation media, it was found that maximum citric acid (47.63 g/l) were obtained when the initial pH of the fermentation medium was adjusted to 5.5. Decrease in pH caused reduction in both protein and citric acid yield [31].

Table 5. Effect of initial pH value on citric acid production in shake flask culture of *Aspergillus niger* strains. Data represented as mean ± standard error.

A. niger Strains	Initial pH	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	3.0	2.39^{b} ±0.02	0.47^{a} ±0.04	6.12^{b} ±0.02
	5.0	2.46^{a} ±0.01	0.43^{a} ±0.01	6.32^{b} ±0.30
	6.5	2.39^{b} ±0.01	0.34^{b} ±0.02	11.26^{a} ±0.01
	8.0	2.14^{c} ±0.01	0.24^{c} ±0.04	6.59^{b} ±0.77
	10.0	2.34^{b} ±0.03	0.32^{b,c}±0.03	6.15^{b} ±0.49
	LSD $_{0.05}$	0.0579	0.0920	1.3539
KSUD	3.0	2.41^{b} ± 0.04	0.26^{bc} ±0.01	9.31^{c} ±0.77
	5.0	2.54^{a} ± 0.06	0.49^{a} ±0.03	11.31^{b} ± 0.35
	6.5	2.18^{c} ± 0.01	0.31^{b} ±0.01	15.25^{a} ±0.08
	8.0	2.07^{d} ± 0.01	0.26^{bc} ±0.01	8.24^{cd} ±0.29
	10.0	2.12^{cd} ± 0.01	0.24^{c} ±0.01	7.14^{d}±0.05
	LSD $_{0.05}$	0.1062	0.0553	1.2619
EMCC132	3.0	3.44^{a} ±0.07	0.33^{ab} ±0.01	8.72^{d} ±0.52
	5.0	3.22^{ab} ±0.07	0.29^{bc} ±0.03	15.93^{b} ±0.51
	6.5	3.09^{b} ±0.29	0.37^{a} ±0.01	22.27^{a} ±0.01
	8.0	2.55^{c} ±0.12	0.26^{c} ±0.00	11.15^{c} ±0.03
	10.0	3.00^{b} ±0.04	0.3^{bc} ±0.00	8.38^{d} ±0.01
	LSD $_{0.05}$	0.2257	0.0443	1.025

In Czapek-Dox broth medium.Inoculum density was ~1.0X10^{6} spore/ml. Cultures were grown with shaking at 100 rpm at 28oC for 5 days. Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Effect of inoculums density

Spore inoculum level is another parameter that influences citric acid fermentation and improvement. The objective of the present experiment was to evaluate the effect of the inoculums density on citric acid production by *A. niger* local strains and the reference strain. The influence of inoculums density on fungal growth and citric acid production by *Aspergillus niger* strains (FQW, KSUD and *A. niger* EMCC132) was investigated. The conidial density (from 5 days old cultures) of the used inoculums were (1.0, 2.0 and 3.0) x10^6 spores/ml. The flask cultures were incubated on rotary shaker. The initial pH, incubation temperature and incubation period were maintained at optimal levels that attained in the foregoing experiments. Data present in Table (6) obviously indicate that citric acid yields by the three strains of *A. niger* were significantly increased by increment of the conidial density in the inoculums after 5 days of flask fermentation at 28^{o}C. Among different density of used inoculums, citric acid productivity was proportionally increased by conidial density increasing. Where citric acid yields were reached to the highest values with 3.0X10^6 inoculums size of all tested *A. niger* strains. Changing in pH values and fungal growth of *A. niger* FQW, KSUD and EMCC132 after 5 days of shaking flask cultures were not significantly appeared by increasing the inoculums density (as shown in Table 6). According to obtained data, inoculums size 3.0X10^6 spores/ml was selected for further experiments for improvement of citric acid production. In agreement with our findings of inoculums density between 1x10^4 to 1x10^9 spores/ml was found to be suitable for citric acid production by *Aspergillus niger* in submerged fermentation [32, 33]. According to the previous literatures, citric acid production is known to be affected by inoculums density. About 1.0% vegetative inoculums are adequate for optimal production of citric acid. The citric acid yield was lower at 5% of the vegetative inoculum [34]. This may be due to the clumping of *Aspergillus niger* at higher inoculum concentration. The increase in the number of inoculated spores primarily increases the level of acid production; however, on the long run this increase tends to force the process toward cellular crowding and to favor the consumption of sugar for biomass reproduction, thus resulting in a reduction in production of citric acid [31]. The excessive reduction in the level of inoculation also causes prolongation in the adaptation phase, therefore decreasing an ideal outcome. This result suggests that there appears to be an optimum amount of inoculum required for citric acid production. This disagrees with other findings who reported that the 5% conc. Of inoculum was optimum for maximum citric acid production (96.55 g l-1) using *Aspergillus niger* under submerged fermentation condition. The 5% conc. of the inoculums is seems to be optimum for citric acid production [6]. A high inoculum density leads to population over-crowding, higher nutrient competition and rapid exhaustion of nutrients. Up to a specific limit, metabolite production generally increases with inoculum density [35]. At the lower inoculum density, metabolite production drops and contamination risks increase due to an insufficient cell population. In addition, the highest level of citric acid is produced when the age of spores is less than seven days, because older spores tend to consume the initially produced acid before the fermentation process is completed [36]. Otherwise, the increase in the number of inoculated spores primarily increases the level of acid production, however, in the long run this increase tends to force the process toward cellular crowding and to favor the consumption of sugar for biomass reproduction, thus resulting in a reduction in production of citric acid. The excessive reduction in the level of inoculation also causes prolongation in the adaptation phase, therefore decreasing an ideal outcome [37, 38]. By studying the effect of vegetative inoculum size (1–5%) on citric acid production by *Aspergillus niger*. They mentioned that the maximum citric acid production of 0.53 g/L was obtained with 3% inoculum size. As the inoculum size increased, citric acid production decreased [18].

Effect of carbon sources

Citric acid accumulation is strongly influenced by the type of carbon source. The carbon source for the citric acid fermentation has been the focus of much study, frequently with a view to the utilizing polysaccharide sources. Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for good production of citric acid [10]. In general, only sugars that are rapidly taken up by the fungus allow a high final yield of citric acid. Polysaccharides, unless hydrolyzed, are generally not a useful raw material for citric acid fermentation because they are broken down too slowly to match the high rate of sugar catabolism required for citric acid production. The slow hydrolysis of polysaccharides is due to the low activity of the hydrolytic enzymes at the low pH that is necessary for producing citric acid [44]. There is several factors affect citric acid production and among these, carbon source has been found to play an important role in citric acid biosynthesis. So that for enhancing the production of citric acid in the present study, *A. niger* strains (*A. niger* FQW, KSUD and EMCC132) were cultivated on the Czapek-Dox broth medium amended with different carbon sources (sucrose, maltose, glucose, fructose, lactose and arabinose) in flask shack culture at 28˚C for 5 days to search for inducers of the citric acid overproduction.

Table 6. Effect of inoculums density on citric production in shake flask culture of *Aspergillus niger* strains. Data represented as mean ± standard error.

A. niger Strains	Inoculum Density (X10^{6}spore/ml)	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	1.0	2.65^a ± 0.04	0.31^a ±0.05	10.89^b ±0.34
	1.5	2.55^{ab} ±0.05	0.39^a ±0.02	11.82^b ±1.34
	2.0	2.47^b ±0.01	0.35^a ±0.02	16.97^a ±0.04
	LSD $_{0.05}$	0.1274	0.1130	2.755
KSUD	1.0	2.66^a ±0.09	0.55^{ab} ±0.00	10.19^c ±0.03
	1.5	2.85^a ±0.10	0.44^{bc} ±0.07	14.16^b ±0.06
	2.0	2.59^a ±0.00	0.59^a ±0.00	18.62^a ±0.11
	LSD $_{0.05}$	0.2778	0.1385	0.2507
EMCC132	1.0	3.04^a ± 0.01	0.33^{ab} ±0.05	14.35^b ±0.11
	1.5	3.04^a ±0.10	0.38^a ±0.03	21.53^a ±0.04
	2.0	2.94^a ±0.01	0.23^b ±0.01	23.02^a ±3.37
	LSD $_{0.05}$	0.1976	0.1209	6.7424

In Czapek-Dox broth medium.Initial pH was 6.5. Cultures were grown with shaking at 100 rpm at 28°C for 5 days. Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Data represented in Table (7) indicated that maltose followed by sucrose have been employed as the preferred carbon sources for citric acid production by *A. niger* FQW (23.02 and 17.97 mg/10 ml, respectively) and *A. niger* KSUD (20.99 and 16.25 mg/10 ml, respectively) however in the case of reference strain *A. niger* EMCC132, maltose followed by glucose as the preferred carbon sources for citric acid production, 41.74 and 24.61 mg/10 ml, respectively. All of the other used sugars showed lower citric acid yield. Moreover, arabinose and lactose had shown significantly an inhibitory effect on citric acid production. In general, maltose served as the best carbon source for citric acid production by all tested *A. niger* strains (*A. niger* FQW, KSUD and EMCC132). It is clear that citric acid production by fungal tested strains is dependent on nutritional conditions and especially on carbon source. The fungal biomass was varied widely among the carbon sources utilized. Where, the highest biomass values were obtained in the presence of maltose and sucrose, while the fungal growth were suppressed in the presence of lactose and arabinose that is reflect on the acid production. Among the used carbon substrates (which are mostly sugars of hexoses, mannoses, mono- or disaccharide nature), the previous studies were varied. For instance, [45] explained that the nature of the sugar source has marked effect on citric acid production by *A. niger*. He also showed that sucrose was the most favorable carbon source followed by glucose, fructose and galactose. Galactose contributed to a very low growth of fungi and did not favor citric acid accumulation. Other sources of carbon such as sorbose, ethanol, cellulose, manitol, lactic, malic and a-acetoglutaric acid, allow a limited fungal growth and low citric acid production. Starch, pentoses (xyloses and arabinoses), sorbitol and pyruvic acid slow down growth, though the production is minimal [46]. Sucrose is the traditional commercial substrate for citric production although glucose, fructose and maltose have also been used as substrates for citric acid production [46]. Generally, the presence of carbohydrates which are rapidly taken up by microorganisms has been found essential for a good production of citric acid [47]. The superiority of sucrose over glucose and fructose has been documented by [48]. Moreover, among the easily metabolized carbohydrates, sucrose is the most favourable carbon source followed by glucose, fructose and galactose for citric acid production [47, 49, 9]. However, it is also found that glucose is not suitable for higher production of citric acid [50]. In contrary of the obtained results [51] found that maximum citric acid production was recorded in the glucose incorporated medium and the low amount of citric acid was recorded in the maltose supplemented medium.

Maltose sugar which gave the highest citric acid production was used in further experiments for optimization of other medium components.

Effect of nitrogen sources

Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also basic part of cell proteins. This report agreed with [25] that fermentation media for citric acid biosynthesis should consist of substrates necessary for the growth of microorganism primarily the carbon, nitrogen and phosphorus sources. This experiment was performed to evaluate the influence of different organic (urea, peptone, beef extract) and inorganic (sodium nitrate as a control, ammonium nitrate, ammonium chloride, ammonium sulfate) nitrogen sources on citric acid production by tested strains of *A. niger* (FQW, KSUD and EMCC132) at the previous favorable parameters.

Table 7. Citric acid production by *Aspergillus niger* strains on various carbon sources in shake flask culture. Data represented as mean ± standard error.

A. niger Strains	Carbon Sources	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	Control	2.95^{c} ±0.05	0.35^{b} ±0.01	17.97^{b} ±0.29
	Maltose	2.59^{d} ±0.02	0.40^{a} ±0.01	23.02^{a} ±0.03
	Glucose	2.78^{cd} ±0.08	0.29^{c} ±0.00	9.96^{c} ±0.12
	Fructose	2.93^{c} ±0.07	0.19^{e} ±0.02	3.29^{f} ±0.04
	Lactose	3.60^{b} ±0.12	0.25^{d} ±0.01	3.91^{e} ±0.11
	Arabinose	4.24^{a} ±0.02	0.24^{d} ±0.00	5.22^{d} ±0.10
	$LSD_{0.05}$	0.2153	0.03248	0.4424
KSUD	Control	2.78^{c} ±0.01	0.30^{cd} ±0.00	16.25^{b} ±0.11
	Maltose	2.95^{c} ±0.11	0.52^{a} ±0.03	20.99^{a} ±0.27
	Glucose	2.77^{c} ±0.12	0.32^{c} ±0.01	10.70^{c} ±0.30
	Fructose	2.84^{c} ±0.07	0.40^{b} ±0.01	11.20^{c} ±0.43
	Lactose	4.51^{a} ±0.09	0.26^{d} ±0.01	5.30^{d} ±0.77
	Arabinose	3.64^{b} ±0.14	0.27^{d} ±0.02	5.97^{d} ±0.24
	$LSD_{0.05}$	0.30529	0.05066	1.2680
EMCC132	Control	2.94^{c} ±0.01	0.43^{a} ±0.01	23.31^{c} ±0.14
	Maltose	3.16^{c} ±0.19	0.31^{b} ±0.01	41.74^{a} ±0.38
	Glucose	3.43^{c} ±0.10	0.26^{c} ±0.00	24.61^{b} ±0.23
	Fructose	3.40^{c} ±0.06	0.26^{d} ±0.00	7.90^{d} ±0.07
	Lactose	5.15^{b} ±0.64	0.18^{d} ±0.01	5.89^{e} ±0.32
	Arabinose	5.10^{a} ±0.00	0.13^{e} ±0.01	3.22^{f} ±0.17
	$LSD_{0.05}$	0.8459	0.02965	0.7449

In modified Czapek-Dox broth medium containing sucrose as control.Initial pH was 6.5. Inoculum density was ~$2.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C for 5 days.
Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

The results were given in Table (8) demonstrated that peptone as an organic nitrogen significantly enhanced citric acid production and showed superiority over all organic and inorganic nitrogen sources. Where maximum citric acid yield were reached to 49.78, 56.07 and 52.23 mg/10 ml by *A. niger* FQW, KSUD and EMCC132, respectively. Mostly presences of organic nitrogen sources acted as enhancer for both citric acid production and fungal growth. However, inorganic nitrogen supplements had varied impact on production of the citric acid by all tested strains. Moreover, ammonium chloride significantly suppressed citric acid production that gave only 5.53, 5.32 and 3.65 mg/10 ml by *A. niger* FQW, KSUD and EMCC132, respectively (as shown in Table 8). Mostly complex nitrogen sources were better than inorganic nitrogen sources. This may be due to other nutrients and growth enhancers present in such organic compounds. In addition, mycelia growth had been found high and reached to a maximum values in the presence of organic nitrogen source especially peptone and lowest fungal growth in the presence of inorganic nitrogen source, ammonium chloride. Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, peptone, malt extract, etc. Acid ammonium compounds are preferred because their consumption leads to pH decrease, which is essential for the citric fermentation. However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea has a tampon effect, which assures pH control [43]. The effect of nitrogen source on citric acid production has been intensively studied in solid substrate and submerged fermentation. Ammonium chloride, ammonium sulphate, ammonium nitrate, peptone and yeast extract were the most suitable nitrogen source for production of citric acid

by fungus [56] Other sources of nitrogen that have been used include urea and yeast/malt extract [46]. Complex media such as molasses are rich in nitrogen-containing compounds and rarely need to be supplemented with a nitrogen source. The high purity media that are used mainly in research laboratories are generally supplemented with ammonium salts, particularly ammonium nitrate and ammonium sulfate, to provide the necessary nitrogen. An advantage of using ammonium salts is that the pH declines as the salts are consumed and a low pH is a requirement of citric acid fermentation [44].

Table 8. Citric acid production by *Aspergillus niger* strains on various nitrogen sources in shake flask culture. Data represented as mean ± standard error.

A. niger Strains	Nitrogen Sources	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	Control (Sodium nitrate)	2.96^{b} ±0.08	0.68^{b} ±0.02	25.63^{b} ±0.53
	Ammonium nitrate	2.69^{c} ±0.02	0.58^{c} ±0.02	11.03^{d} ±0.69
	Ammonium chloride	1.99^{d} ±0.00	0.28^{g} ±0.00	5.53^{f} ±0.01
	Ammonium sulfate	2.10^{b} ±0.01	0.47^{e} ±0.01	10.41^{de} ±0.36
	Beef extract	2.88^{bc} ±0.06	0.41^{f} ±0.02	9.39^{e} ±0.06
	Peptone	2.77^{bc} ±0.02	0.85^{a} ±0.02	49.78^{a} ±0.53
	Urea	4.25^{a} ±0.16	0.53^{d} ±0.01	18.12^{c} ±0.91
	$LSD_{0.05}$	0.2157	0.0463	1.6328
KSUD	Control (Sodium nitrate)	2.51^{b} ±0.04	0.62^{b} ±0.00	29.68^{d} ±0.25
	Ammonium nitrate	2.77^{cd} ±0.01	0.41^{c} ±0.04	12.33^{e} ±0.04
	Ammonium chloride	2.09^{e} ±0.02	0.22^{e} ±0.01	5.32^{f} ±0.03
	Ammonium sulfate	2.86^{c} ±0.02	0.28^{de} ±0.00	6.27^{f} ±0.12
	Beef extract	2.59^{bd} ±0.06	0.64^{b} ±0.01	50.05^{b} ±0.49
	Peptone	2.62^{bd} ±0.02	0.82^{a} ±0.03	56.07^{a} ±0.18
	Urea	3.38^{a} ±0.14	0.29^{d} ±0.02	43.27^{c} ±0.89
	$LSD_{0.05}$	0.1870	0.0588	1.2251
EMCC132	Control (Sodium nitrate)	3.84^{b} ±0.07	0.52^{d} ±0.01	47.00^{b} ±0.16
	Ammonium nitrate	3.10^{d} ±0.09	0.46^{d} ±0.01	13.97^{d} ±0.65
	Ammonium chloride	2.06^{f} ±0.02	0.29^{f} ±0.00	3.65^{e} ±0.30
	Ammonium sulfate	2.84^{e} ±0.03	0.68^{b} ±0.01	48.59^{b} ±0.71
	Beef extract	3.17^{d} ±0.02	0.37^{e} ±0.00	12.55^{d} ±0.67
	Peptone	3.33^{c} ±0.08	0.74^{a} ±0.00	52.23^{a} ±2.58
	Urea	4.07^{a} ±0.01	0.57^{c} ±0.01	25.86^{c} ±0.04
	$LSD_{0.05}$	0.1608	0.01793	3.2708

In modified Czapek-Dox broth medium.Initial pH was 6.5. Inoculum density was ~$2.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C for 5 days.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Effect of phosphorus sources

Presence of phosphate in the medium has a great effect on the yield of citric acid. Low levels of phosphate have positive effect on citric acid production. This effect acts at the level of enzyme activity and not at the level of gene expression. On the other hand, the presence of excess of phosphate leads to a decrease in the fixation of carbon dioxide, which in turn increases the formation of certain sugar acids, and the stimulation of growth [9, 25, 43]. Several factors affect citric acid production and among of these, phosphorus source has been found to play an important role in citric acid biosynthesis. So for enhancing the production of citric acid in the present study, *A. niger* strains (*A. niger* FQW, KSUD and EMCC132) were cultivated on the Czapek-Dox broth medium amended with different phosphorus sources (NaH_2PO_4, Na_2HPO_4, Na_3PO_4 and KH_2PO_4) in the same amount of the initial phosphorus source, K_2HPO_4 (1.0 g/l) in flask shack culture at 28˚C for 5 days to search for inducers of the citric acid overproduction. Data represented in Table (9) indicated that Na_3PO_4 significantly employed as the preferred phosphorus source for citric acid production by *A. niger* FQW (96.60 mg/10 ml) and *A. niger* KSUD (77.81 mg/10 ml) however in the case of reference strain *A. niger* EMCC132, KH_2PO_4 was significantly as the preferred carbon sources for citric acid production, 91.33 mg/10 ml. All of the other forms of phosphate showed lower citric acid yields. Moreover, phosphate in the form of Na_2HPO_4 had shown significantly an inhibitory effect on citric acid production. In general, Na_3PO_4 served as the best phosphate form for citric acid production by local tested *A. niger* strains (*A. niger* FQW and KSUD). It is clear that citric acid production by fungal tested strains is dependent on nutritional conditions including phosphorus

salt source. The fungal biomass was varied widely among the various phosphorus sources utilized (Table 9). From results of the present experiment, phosphorus salt in the form Na_3PO_4 has been recommended for the maximal citric acid production from *A. niger* FQW, KSUD and EMCC132 strains and was selected as a control for the subsequent experiments. Phosphate is known to be essential for the growth and metabolism of *A. niger* [59]. The concentration of exogenous phosphorus in medium had significant effect on cell multiplication and metabolite production [43]. Presence of phosphate in the medium has a great effect on the yield of citric acid. In the present study, phosphorus salt in the form Na_3PO_4 was the favorite phosphorus source by the tested fungal stains however certain previous research mentioned that Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source. For instance KH_2PO_4 and K_2HPO_4 proved to be the best phosphorus source [56]. It was found that phosphorous at concentration of 0.5 to 5.0 g/L was required by the fungus in a chemically defined medium for maximum production of citric acid [60]. In addition, phosphorus limitation induced higher citric acid production and yield [58].

Table 9. Citric acid production by *Aspergillus niger* strains on various phosphorus sources in shake flask culture. Data represented as mean ± standard error.

A. niger Strains	Phosphorus Sources	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	Control (K_2HPO_4)	2.54^{a} ±0.04	0.76^{a} ±0.00	55.60^{d} ±0.98
	Na_2HPO_4	2.43^{b} ±0.03	0.84^{a} ±0.06	16.66^{e} ±0.09
	Na3PO4	2.44^{b} ±0.02	0.70^{a} ±0.04	96.60^{a} ±0.49
	NaH_2PO_4	2.46^{b} ±0.01	0.78^{a} ±0.12	75.53^{c} ±0.90
	KH_2PO_4	2.45^{b} ±0.01	0.71^{a} ±0.01	80.91^{b} ±0.34
	LSD $_{0.05}$	0.0758	0.2351	2.0615
KSUD	Control (K_2HPO_4)	2.33^{a} ±0.01	0.74^{c} ±0.01	52.26^{c} ±0.35
	Na_2HPO_4	2.24^{c} ±0.00	0.86^{b} ±0.01	10.93^{e} ±0.12
	Na3PO4	2.27^{bc} ±0.03	1.01^{a} ±0.01	77.81^{a} ±1.79
	NaH_2PO_4	2.25^{bc} ±0.00	0.86^{b} ±0.06	68.41^{b} ±0.42
	KH_2PO_4	2.29^{ab} ±0.02	0.89^{b} ±0.01	47.86^{d} ±0.34
	LSD $_{0.05}$	0.0440	0.0941	2.6519
EMCC132	Control (K_2HPO_4)	2.86^{bc} ±0.015	0.54^{c} ±0.03	53.30^{c} ±0.76
	Na_2HPO_4	2.90^{b} ±0.02	0.55^{bc} ±0.04	11.60^{d} ±0.08
	Na3PO4	2.86^{bd} ±0.01	0.72^{a} ±0.00	81.31^{b} ±0.39
	NaH_2PO_4	2.82^{cd} ±0.02	0.62^{b} ±0.03	81.46^{b} ±0.47
	KH_2PO_4	2.96^{a} ±0.00	0.02^{d} ±0.01	91.33^{a} ±0.44
	LSD $_{0.05}$	0.0488	0.0799	1.5038

In modified Czapek-Dox broth medium. Initial pH was 6.5. Inoculum density was ~$2.0X10^6$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C for 5 days.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Effect of enhancer (Alcohol addition)

Among all of the previous studied parameters were utilized as optimum conditions which led to enhance of citric acid production by *A.niger* local strains FQW and *A. niger* KSUD (Table 10). Besides the optimization of basal environmental parameters and nutrients as well as their concentrations, higher citric acid production levels can be obtained by applying stimulators, such as alcohols and chelating agents according to [61]. Several reports have shown the stimulatory effects of additives on fungal citric acid accumulation and secretion [26]. To improve citric acid production, stimulators have been used, such as organic solvents, phytate and lipids [26, 62, 63]. The objective of the present research was to determine the potential increase in citric acid production by *A. niger* selected strains (*A. niger* FQW, KSUD and EMCC132) in the presence of stimulators (1%) such as propanol, ethanol and methanol. Data recorded in Table (10) indicate that after 5 days of fermentation, impact of alcohols addition on citric acid production by all tested *A. niger* strains was significantly varied. Where methanol had obviously pronounced stimulatory effect, on contrary propanol was suppressed citric acid production while the effect of ethanol addition varied among the tested strains. Without methanol, the control produced 82.24 and 80.58 mg/10 ml while the addition of methanol significantly stimulated citric acid production to 128.2 and 103.58 mg/10 ml by *A. niger*

FQW and KSUD, respectively. By comparing the effect of both ethanol and methanol on biomass growth of tested local fungal strains it was observed that methanol has fruitful effect on biomass growth but there is no stimulatory effect of ethanol on biomass, which means that ethanol was solely used by *A. niger* FQW and KSUD as a carbon source for citric acid production. Similar

findings were also reported that Alcohols have been shown to act principally on membrane permeability in microorganisms by affecting phospholipid composition [64]. Other studies showed that alcohols stimulate citric acid production by affecting growth and sporulation on space organization of the membrane or changes in lipid composition of the cell wall [65] The obtained results are in harmony with pervious data that showed the inductive effect of methanol in the fermentation media for citric acid by *A. niger* [66, 50] the stimulation of citric acid production by methanol in synthetic media is affected by cultural conditions, and especially by the mold strain used. The age and the amount of mycelia inoculum which is probably a reflection of its surface area may be critical. They reported that these factors must be investigated in applying the effect of the alcohol in any individual case [63]. From results of this experiment, methanol had significant favorable effect for the maximal citric acid production by local fungal strains (*A. niger* FQW and KSUD) and selected for the subsequent experiments.

Table 10. Citric acid production by *Aspergillus niger* strains in the presence of various alcohol sources (1%) in shake flask culture. Data represented as mean ± standard error.

A. niger Strains	Alcohol Sources	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	Control	2.61^{b} ±0.04	0.50^{b} ±0.00	82.24^{c} ±0.80
	Propanol	3.01^{a} ±0.07	0.29^{c} ±0.02	12.66^{d} ±0.13
	Ethanol	2.72^{b} ±0.03	0.45^{b} ±0.03	101.16^{b} ±0.99
	Methanol	2.68^{b} ±0.01	0.63^{a} ±0.06	128.20^{a} ±0.75
	$LSD_{0.05}$	0.1372	0.1080	2.4110
KSUD	Control	2.43^{d} ±0.02	0.56^{b} ±0.01	80.58^{b} ±0.90
	Propanol	3.21^{a} ±0.02	0.26^{d} ±0.00	8.61^{d} ±0.22
	Ethanol	2.54^{c} ±0.00	0.42^{c} ±0.00	73.20^{c} ±0.81
	Methanol	2.67^{b} ±0.01	0.68^{a} ±0.03	103.58^{a} ±3.74
	$LSD_{0.05}$	0.0456	0.0515	6.415
EMCC132	Control	2.90^{c} ±0.01	0.75^{b} ±0.02	79.89^{b} ±1.03
	Propanol	3.42^{a} ±0.02	0.32^{d} ±0.01	14.79^{c} ±0.07
	Ethanol	3.42^{a} ±0.03	0.44^{c} ±0.02	15.35^{c} ±0.09
	Methanol	3.02^{b} ±0.00	0.92^{a} ±0.01	90.30^{a} ±0.48
	$LSD_{0.05}$	0.05324	0.0492	1.8600

In modified Czapek-Dox broth medium. Initial pH was 6.5. Inoculum density was ~$2.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C for 5 days.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Utilization off agricultural wastes for citric acid production by *A. niger* strains

A. niger a filamentous fungus is most commonly used for citric acid production due to ease of handling, its ability to utilize varieties of substrates because of its well-developed enzymatic system in addition to its ability to ferment a variety of cheep raw materials and high yield of citric acid [51, 68, 69]. Utilization of refined sugars such as glucose, maltose and sucrose are the most commonly used substrates for commercial production of citric acid by fermentation process; while they are expensive and can be replaced by various cheap and abundant substrates like agro-industrial wastes or by-products [4]. Several raw materials can be employed successfully for citric acid production. However, there are some critical factors that should be taken into account such as costs or need of pretreatment for choosing the type of substrate [10]. Thus, the objective of this part was to adopt the use of certain local raw materials as a cheap medium for the production of citric acid by tested *A. niger*. In this production technique, which is still the major industrial rout to commercial citric acid production nowadays, tested strains of *A. niger* are cultivated on a raw material-containing modified Czapek-Dox broth medium to produce citric acid. Citric acid productivity by *A. niger* tested strains (*A. niger* FQW, KSUD and EMCC132) was determined after 5 days growing cultures in which 5% (w/v or v/v) of selected raw materials (pineapple peel, sugarcane bagasse, sugarcane molasses and dates molasses) or 3% (w/v) of potato peel was supplemented to the fermentative medium either carbon free or carbon and nitrogen free. The estimated results present in Table (11) indicate citric acid production by tested fungal strains in the presence of selected raw materials as a sole carbon source. The results showed that sugarcane molasses was significantly superior for citric acid production (256.94 and 188.45 mg/10 ml) by *A. niger* FQW and *A. niger* EMCC132, respectively.

Table 11. Citric acid production by *Aspergillus niger* strains grown in fermentative medium carbon free and supplemented with various waste sources. Data represented as mean ± standard error.

A. niger Strains	Waste Sources	pH	Growth (DW g/50 ml)	Citric Acid (mg/10ml)
FQW	Pineapple peels	$3.83^{c} \pm 0.04$	$0.94^{b} \pm 0.06$	$180.25^{b} \pm 0.66$
	Sugarcane bagasse	$7.73^{a} \pm 0.13$	$0.46^{d} \pm 0.04$	$6.61^{e} \pm 0.51$
	Sugarcane molasses	$3.03^{d} \pm 0.11$	$1.74^{a} \pm 0.10$	$256.94^{a} \pm 1.09$
	Date molasses	$2.91^{d} \pm 0.05$	$0.71^{c} \pm 0.05$	$142.88^{c} \pm 0.38$
	Potato peels	$6.36^{b} \pm 0.19$	$0.22^{e} \pm 0.01$	$11.07^{d} \pm 0.16$
	$LSD_{0.05}$	0.3723	0.1937	2.0161
KSUD	Pineapple peels	$2.40^{d} \pm 0.04$	$1.33^{a} \pm 0.03$	$238.19^{a} \pm 1.14$
	Sugarcane bagasse	$6.20^{b} \pm 0.05$	$0.56^{d} \pm 0.02$	$10.53^{d} \pm 0.08$
	Sugarcane molasses	$2.56^{c} \pm 0.02$	$1.01^{b} \pm 0.03$	$168.87^{b} \pm 1.18$
	Date molasses	$2.50^{cd} \pm 0.00$	$0.71^{c} \pm 0.06$	$162.67^{c} \pm 0.34$
	Potato peels	$7.01^{a} \pm 0.01$	$0.24^{e} \pm 0.01$	$12.64^{d} \pm 0.11$
	$LSD_{0.05}$	0.1016	0.1051	2.3762
EMCC132	Pineapple peels	$3.36^{c} \pm 0.02$	$0.74^{c} \pm 0.02$	$127.73^{c} \pm 0.41$
	Sugarcane bagasse	$7.63^{a} \pm 0.12$	$0.62^{d} \pm 0.01$	$7.24^{e} \pm 0.11$
	Sugarcane molasses	$3.15^{cd} \pm 0.02$	$1.06^{a} \pm 0.01$	$188.45^{a} \pm 0.65$
	Date molasses	$2.91^{d} \pm 0.01$	$0.82^{b} \pm 0.03$	$182.14^{b} \pm 0.06$
	Potato peels	$6.73^{b} \pm 0.17$	$0.12^{e} \pm 0.01$	$14.873^{d} \pm 0.25$
	$LSD_{0.05}$	0.29505	0.05478	1.1507

In modified Czapek-Dox broth medium.Initial pH was 6.5. Inoculum density was ~$2.0X10^{6}$ spore/ml. Cultures were grown with shaking at 100 rpm at 28°C for 5 days.

Means in the same column followed by the same letter are not significantly different based on LSD at p = 0.05 according to Duncan's multiple range test.

Molecular identification of fungal isolates

The identification of the isolates was further confirmed using nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. The traditional Sanger technology and the new 454 technology were combined for sequencing the PCR products. The obtained nucleotide sequence was deposited at the NCBI GenBank and a strain identifier was given to each isolate. Thus, they were identified as *Aspergillus niger* MH368137 as shown in figure 2.

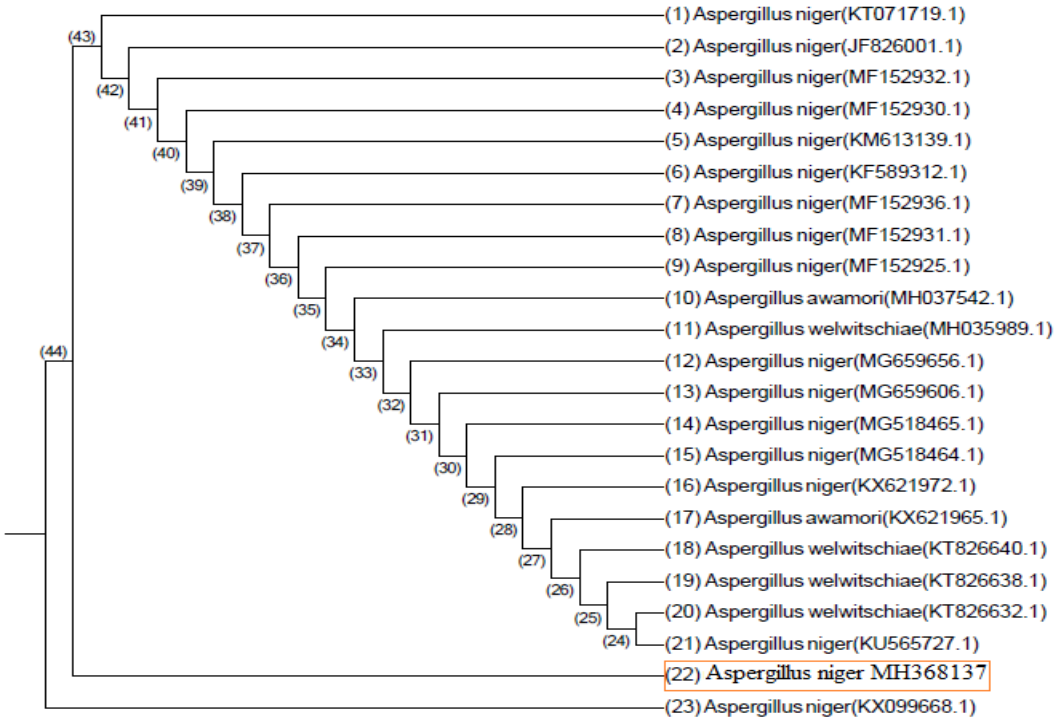

Figure 2. Phylogenetic tree of *Aspergillus niger* MH368137 from NCBI database.

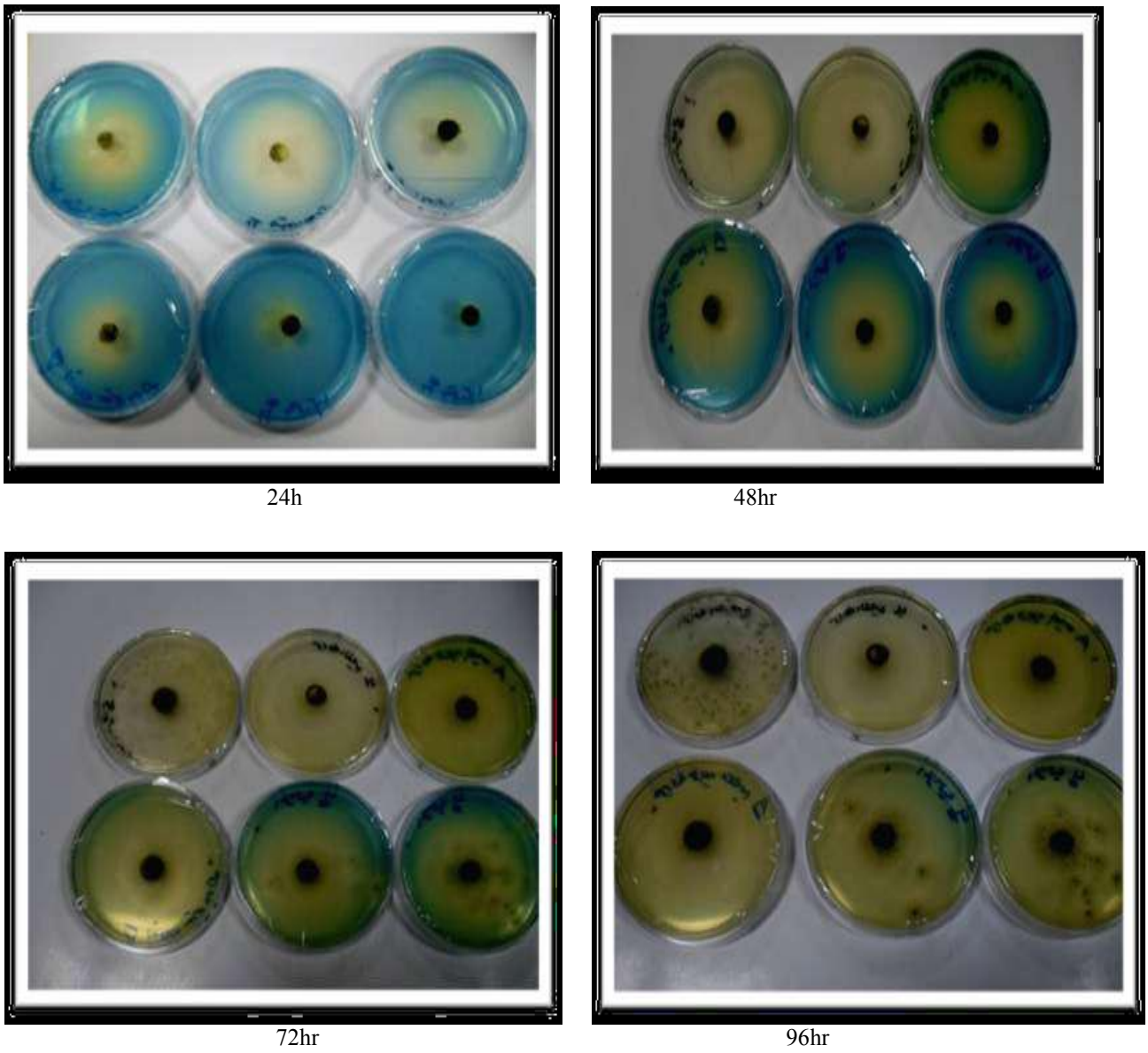

Figure 3. Detection for citric acid production on solid medium for various *A. niger* at different time intervals.

Conclusion

Fermentation parameter for citric acid production by *A. niger* depend on the type of process. The fermentation conditions are key to high and consistent production of citric acid. In the present study, *Aspergillus niger* MH368137 isolated from Farm way Qassim supported maximum production of citric acid. The optimal conditions for the maximum citric acid production reached statistically 5 days at 28˚C; initial pH 6.5; inoculums size 3.0×106 spores/ml; shaking culture (100 rpm); maltose as a carbon source 40 g/L; peptone as a nitrogen source 3 g/L; Sodium phosphate as phosphorus source; 2% of methanol as enhancer. It was also observed in this study that Potato peels supported the maximum production of citric acid.

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