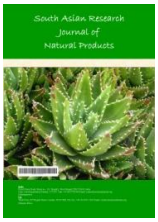

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Citric Acid Production using Induced Mutant *Aspergillus niger* and *Aspergillus awamori* Isolated from Soil

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Authors' contributions

This work was carried out in collaboration among all authors. Author IKE and MDM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JEO, IHN and FUA managed the analyses of the study. Author SOO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed at citric acid production using mutation induced *Aspergillus niger* and *Aspergillus awamori* isolated from soil in Keffi, Nigeria. Soil samples were collected from different location in Keffi. *Aspergillus* species were isolated and identify using standard microbiological method. Citric acid was produced using a batch fermentation system. Citric acid yield highest by *A. awamori A1* was at 30^{O}C with 4.23 mg/l and *A. awamori* A2 produced highest citric acid 30 ^{O}C with 3.93 mg/l whereas *A. niger* F4 yield highest at 26 ^{O}C with 5.03 mg/ml and . *A. niger* F5 yield the highest at 26^{O}C with 5.75 mg/l. the yield of citric acid at different fermentation duration showed that *A. awamori* A1 highest after 120hrs with the 5.20 mg/l and *A. awawori* A2 yield highest after 120hrs with 6.00 mg/l while *A. niger* F4 yielded 6.09 mg/ml after 144hrs and *A. niger* F5 produced highest after 144hrs with 6.80 mg/ml. by *A. awamori* A1 yield highest 5.88 mg/ml at pH of 4.0 and *A. awamori* A2 produced highest also at pH of 4.0 with 5.84mg/ml. *A. niger* F4 yields the highest citric acid at pH of 4.5 with 4.99 mg/ml and *A. niger* F5 yield highest at pH of 4.5 with 7.14mg/ml. *A.*

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awamori yields highest at substrate concentrations of 125mg/l with 8.97mg/ml, *A. awamori* A2 yielded 8.80mg/ml in 150 mg/l substrate concentration while *A. niger* F4 produced highest in substrate concentration of 175 mg/l with 9.17mg/ml and *A. niger* F5 yielded highest in substrate concentration of 175 mg/l with 10.02 mg/ml. Strain development will play important role in citric acid and developing strains of *Aspergillus* species.

Keywords: mutation; yield; Aspergillus species; fermentation; citric acid.

1. INTRODUCTION

The intermediate of the tricarboxylic acid cycle Citric acid (CA), is known for its importance as one of the most valuable organic acids used commercially because of its high demand in food industry (70%), pharmaceuticals (12%), and others (18%) [1].The global citric acid market exceeded the volume of 2 Million Tons in 2018. The market is further projected to reach a volume of nearly 3 Million Tons by 2024. As a result of its general uses, the quantity of citric acid produced by fermentation continually increases at a high rate of 5% per year [2] which has also led to continuous increase in consumption. The varying market conditions have led to decrease in the price of Citric acid, which is about $2.0 to $2.3 per kilogram. The demand rate increases day after day due to its various applications or use; considering the increase in demand and price, the market value for this commodity will exceed $8 billion in 2020 [3].

It is accepted globally, generally recognized as safe (GRAS), as approved by the Joint FAO/WHO Expert Committee on Food Additives [4]. Sodium and potassium which are primarily Citric acid salts are used in a large number of industries: as a chelating agent, buffer, pH adjustment, and derivatization agent. Applicable in laundry detergents, shampoos, cosmetics, enhanced oil recovery and chemical cleaning [5]. Liquid solutions of Citric acid is a good buffer when partially neutralized as citric acid, it is a weak acid and has three carboxylic groups. This study aimed at citric acid production using induced mutant *Aspergillus niger* and *Aspergillus awamori* isolated from soil in Keffi, Nigeria.

2. MATERIALS AND METHODS

2.1 Methods

2.1.1 Study area

The study was carried out in Keffi Local Government Area, Nasarawa State, Nigeria. Keffi is approximately 68km from Abuja, the Federal Capital Territory and 128km from Lafia, the Capital of Nasarawa State. Keffi is located in latitude $8^{o}5'$N of the equator and longitude $7^{o}8'$E and situated at an altitude of 850m above sea level [6].

2.2 Sample Collection

The sample collection was carried out by the method adopted by [7]. Soil samples were randomly collected from the top 10 cm of the soil profile from four different locations in Keffi metropolis using a sterile spatula and placed in sterile sample bags and clearly labeled and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.3 Isolation of *Aspergillus* species

Isolation of *Aspergillus* species was carried out using a method described by [8]. Briefly, One (1) gram of the soil sample was suspended in a test tube containing 9 ml of sterile distilled water to make a soil suspension and a ten-fold serial dilution was made by transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. These steps were repeated seven times to obtain a dilution of 10^{-7}. From the fourth test tubes, 0.2 ml of the aliquot was spread on Potato Dextrose agar plates, Malt extract agar and Yeast extract agar and incubated at 28^{o}C for 4 days.

2.4 Identification of *Aspergillus* species

Identification of *A. niger* and *A. awamori* were carried out by the method adopted by [8]. Briefly, the cultural characteristics of *A. niger* and *A. awamori* were determined by their growth appearance on culture plates and the morphological features were determined microscopically using lactophenol cotton blue staining technique, where lactophenol cotton blue strain was dropped on a clean grease-free microscope slide, a small portion of mycelium or colony from the fungi culture plate was dropped on the lactophenol cotton blue with the aid of a

mounted needle, the mycelium was spread well with the two mounted needle and covered with cover slip. The slide was then viewed under the microscope at x40 and x100 lens. The images were identified with reference to the work of [8] and fungi standard chart.

2.5 Screening for Citric Acid-Producing *Aspergillus niger* and *Aspergillus awamori*

Screening for Citric Acid-Producing *A. niger* and *A. awamori* were carried out as described by [8]. The isolates were screened qualitatively for citric acid production. Potato dextrose agar plate method containing Bromocresol green as an indicator 1% at pH 6 was used. *A. niger* and *A. awamori* were inoculated on the plates and incubated for 48 hours, yellow color zone around the *A. niger* and *A. awamori* growth indicate citric acid production.

2.6 Strain Improvement Techniques

2.6.1 Chemical mutagens treatment on *Aspergillus niger* and *Aspergillus awamori*

Induction of mutation in *A. niger* and *A. awamori* isolates were carried out by the modification of a method described by [9]. Briefly, To develop mutant *A. niger* and *A. awamori* strains with ethyl methane-sulfonate, eight test tubes with two millimeter (2 ml) of spore suspension (10^6) each were taken and one of them was kept aside as control and rest of them were incubated with ethyl methane-sulfonate concentrations varying from 2mg, 3mg, 4mg, 5mg, 6mg, 7mg 8mg and 9mg for 30 and 60 minutes at room temperature (32±2^{O}C). After required period of incubation, the spore cells were centrifuged at 3000 rpm for 10min, washed with sterilized phosphate buffer (pH 7.0) twice. A volume of 0.1 ml of ethyl methane-sulfonate treated *A. niger* and *A. awamori* suspension was poured into sterilized petri plates containing potato dextrose agar medium and incubated (26±2^{O}C). At the end of 7th day, morphological changes of *A. niger* and *A. awamori* suspension were noticed and examined from changes.

Those with significant cultural and morphological changes such as color change from black to grey were found in spore suspension exposure to ethyl methane-sulfonate concentration of 6mg for one hour incubation and then suspension was diluted and subcultured on a Potato dextrose agar plate for further use.

2.7 Preparation of Starch Substrates

The Starch substrate was prepared using a method described by [10]. Substrates corn grains were collected and grind into powdered form using clean grinding machine and sieved. Five hundred gram (500 g) powdered form was added into 4 liter of distilled water and sieved to form a homogenous mixture and placed at 4ºC for 24 hours. The starch settled down was separated from liquid and oven-dried at 60ºC, overnight. A starch solution of 20 g/l was dissolved and autoclaved at 5.0 lbs/in2 pressure (115°C) for 5 min. To liquefy starch, alpha-amylase (2.0 µ/ml) was added and heated at 95ºC in a water bath for 15 min. For saccharification, amyloglucosidase (2.0 µ/ml) was added and heated at 55ºC while constantly stirring for about 4 hours and stored for further use.

2.8 Production of Citric Acid

2.8.1 Preparation of inoculum for fermentation

Preparation of inoculum for fermentation was carried out as described by [8]. Seven milliliter (7ml) of peptone water and glass beads was prepared and autoclaved at 5.0 lbs/in2 pressure (115°C) for 5 min and three milliliter (3 ml) of tween 80 was added into medium. Five milliliter (5ml) of the medium containing 3% tween 80, peptone water and glass beads were transferred into four (4) days' slant culture of *A. niger* and *A. awamori;* was shaken thoroughly until spores was dissolved and the spore suspension incubated at room temperature (28±1oC) for 6 hours and stored for further use.

2.8.2 Starter culture

The starter culture (broth inoculum) was prepared as described by [11]. 10ml of the spore suspension was inoculated into 90 ml of freshly prepared potato dextrose broth and incubated at 35^{O}C for 12 hours.

2.8.3 Media formulation and fermentation technique

The batch fermentation was carried out as described by [10] with modification. The prepared Starch hydrolysate, M [starch (20 g/L) $(NH4)_2SO_4$ 1.5 g, and KH_2PO_4 4.1 g,] was added into

mineral solution containing 0.09 g/l $ZnSO_4.7H_2O$, 0.1 g/l $CuSO_4.5H_2O$, 0.4 g/l $MnSO_4$ and 5 g/l $MgSO_4$, was transfered to conical flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min.

2.8.4 Optimization of citric acid production

2.8.4.1 Effect of ph on citric acid on production

The effect of pH was carried out by adoption of a method described by [10,12]. Ninety milliliters (90 ml) of the prepared fermentation substrate was transferred into different conical flasks. The pH ranges was adjusted to, 4.0 4.5, 5.0 5.5, 6.0, 6.5, 7.0 and 7.5 of fermentation media using 1.0 N HCl to adjust the pH of the media before autoclaving and the ten milliliters (10ml) of prepared broth inoculum was added and incubated at (28±1o C) for 5 days.

2.8.4.2 Effect of temperature on citric acid production

The effect of temperatures was carried out following a method described by [13]. Ninety milliliters (90 ml) of the fermentation substrate was transferred into different conical flasks and ten milliliters (10 ml) of prepared broth inoculum was added into the fermentation media and incubated at different temperature of 26^oC, 28^oC, 30^oC and 32^oC for 5 days and pH 4.5

2.8.4.3 Effect of fermentation duration on citric acid production

The effect of fermentation duration was carried out as described by [10]. Briefly, Ninety milliliters (90 ml) of the fermentation substrate was transferred into different conical flasks; ten milliliters (10ml) of prepared broth inoculum was added into the fermentation media and incubated (28±1oC). Different time intervals were monitored during the fermentation ranging from 24 hours, 48hours, 72 hours, 96 hours, 120hours, 144hours, 168hours and pH 4.5 all the experiment was carried out in three replicates

2.8.4.4 Effect of different substrate concertation on citric acid production

The effect of substrate concertation was carried out as described by [14]. Briefly, Ninety milliliter (90 ml) of the fermentation substrate of different concentration was transferred into different conical flasks; ten milliliters (10 ml) of prepared broth inoculum was added into the fermentation media and incubated (28±1o C). Substrate concertation of 75 mg/l, 100 mg/l, 125 mg/l, 150 mg/l, 175 mg/l and 200 mg/l

2.9 Quantification of Citric Acid

2.9.1 Estimation of citric acid

The citric acid produced during fermentation was determined by Gas Chromatography and Mass Spectrometry (GC and MS) [15] as detailed below;

2.9.2 Sample preparation

During sample preparation, 7 mL of fermented media was added to 40 mL of buffer-acetonitrile mobile phase (0.5% (w/v) (NH4)2HPO4 (0.038 M) - 0.4% (v/v) acetonitrile (0.049 M), at pH 2.24 with H3PO4), extracted for 1 hour in orbital shaker and centrifuged at 6000 x *g* for 5 min. The supernatant was collected and filtered once through filter paper Whattman No. 1 and twice through a 0.45 µm membrane filter, and then used directly for GC and MS analysis. Duplicate analysis was performed on all samples.

2.10 GC and MS Analysis

Chromatograph equipped with flame-ionization detector. The column used for the separation of solvent PEG (2.1 m x 3.0 mm). The operating conditions were mobile phase, aqueous 0.5% (w/v) $(NH_4)2HPO_4$ (0.038 M) - 0.2% (v/v) acetonitrile (0.049 M) adjusted to pH 2.24 with H_3PO_4; flow rate 0.3 mL min-1; ambient column temperature. The mobile phase was prepared by dissolving analytical-grade $(NH_4)2HPO_4$ in distilled deionized water, GC and MS -grade acetonitrile, and H_3PO_4. GC and MS -grade reagents was used as standards (Sigma Chemical Co., St. Louis, MO). Solvents were filtered through a 0.45 µm membrane filter and One hundred and twenty degrees centigrade (120^oC), Nitrogen gas (30 mL/minutes) were used as carrier gas. The temperatures of injector and detector were 150^oC and 200^oC respectively. The Peaks were recorded on "SHIMADZU C-R-4_A, Chromatograph", and was identified by comparison of the retention times with that of standard mixture. The experiment was carried out in duplicate and the means ± standard deviations of the yield of citric acid were recorded.

3. RESULTS AND DISCUSSION

The cultural characteristics of the *Aspergillus* species is as giving in Table 1.

Table 2 shows the screening of production of citric acid by *Aspergillus* species isolated from soli in Keffi, Nigeria.

The effect of temperature on citric acid production by mutation induced *A. awamori* isolates is as given in Fig. 1. It was observed that the highest citric acid was produced by *A. awamori A1* was at temperature of 30^{O}C with the yield of 4.23 mg/l and the least was at temperature of 26^{O}C with the yield of 2.00 mg/l while *A. awamori* A2 produced highest citric acid at temperature of 30^{O}C with 3.93 mg/l and the least was observed at temperature of 26^{O}C with citric acid yield of 2.01 mg/l. While the highest citric acid that was produced by *A. niger* F4 was observed at temperature of 26^{O}C with the yield of 5.03 mg/ml and the lowest was at 32 ^{O}C with yield of 2.33 mg/l. *A. niger* F5 yield the highest citric acid at temperature of 26^{O}C with 5.75 mg/l and the lowest was at temperature of 28^{O}C with 3.12 mg/l respectively, this is similar to studies earlier reported by [16;17] high yield of citric acid at temperature of 20^{O}C. Temperature is one of the critical factors that have direct effect on the production of citric acid. When the temperature of medium was low, the enzyme activity was high, giving a significant impact on the enhancement of citric acid production as shown in this study.

The effect of fermentation duration on citric acid production by mutant *A. awamori* is as shown in Fig. 2. The highest yield of citric acid by *A. awamori* A1 was after 120 hrs with the yield of 5.20 mg/l and the lowest was after 24 hr with 1.21 mg/l. *A. awamori* A2 yield highest after 120 hrs with 6.00 mg/l and the least was after 24 hrs with yield of 1.40 mg/l while The yield of citric acid produced by mutant *Aspergillus niger* isolates at different fermentation duration is as given in Fig. 2. The highest yield produced by *A. niger* F4 was after 144hrs yielded 6.09 mg/ml and the least was after 24hrs with 1.81 mg/ml whereas *A. niger* F5 produced highest after 144hrs with yield of 6.80mg/ml and the lowest was after 24hrs with the yield of 1.91mg/ml. The optimum time of incubation for high citric acid production varies both with the organism and fermentation conditions. In batch-wise fermentation of citric acid like this study, the production increase after a lag phase of one day and reached maximum at the onset of stationary phase or late exponential phase [17,10].

Table 1. Cultural and morphological characteristics of fungi isolated from soil in Keffi

	Characteristics	
Fungal Isolate	**Cultural**	**Morphological**
Aspergillus awamori	colonies are typically black in colour with wihte cleistothecia developing within and upon the conidial layer. Reverse may be olive to drab-grey or brown	Branched conidiophores with chains of conidia like a brush
Aspergillus niger	Yellow or yellowish green colonies with distinct margin, the colony reverse is brownish to dark in colour.	Conidiophores arise from a foot cell. Club shaped vesicles at top of conidiophores. Conidia are found in chains and surface is irregularly rough.

Table 2. Screening for citric acid producing *Aspergillus* species isolated from soil

Isolates	Sample code	Citric Acid mg/ml
A. niger	F4	+
A. awamori	A3	-
A. awamori	A1	+
A. awamori	A2	+
A. niger	B1	-
A. niger	F5	+
A. awawori	C2	-
A. niger	B2	+

KEY: + = Positive; - = Negative; A = Angwan zakara; B = Angwan Jarme; C = Tudun Wada; D = Pyanku

Fig. 3 shows the yield of citric acid by *Aspergillus awamori* isolates at different pH. The highest citric acid produced by *A. awamori* A1 was 5.88 mg/ml at pH of 4.0 and the least was 2.68 mg/ml whereas *A. awawori* A2 produced highest also at pH of 4.0 with yield of 5.84mg/ml and the least was 2.62mg/ml while The yield of citric acid by *Aspergillus niger* at different pH is indicated in Fig. 3. *A. niger* F4 yield the highest citric acid at pH of 4.5 with 4.99mg/ml and the lowest was at pH of 7.5 which had 2.94 mg/ml however *A. niger* F5 had the highest yield at pH of 4.5 which yielded 7.14mg/ml and least was 3.10 mg/ml at pH of 7.5.

The yield of citric acid obtained from the species of *A. awamori* isolates at various substrate concentrations is as shown in Fig. 4, the yield of citric acid ranged from 8.97mg/ml in substrate concentration of 125mg/l, and 5.77 mg/ml in 75mg/l substrate concentration for *A. awamori* A1, while *A. awamori* A2 yielded 8.80 mg/ml in 150mg/l substrate concentration, least was in substrate concentration of 75 mg/l. Citric acid produced by *A. nger* in different concentration of media growth is as given in Fig. 4, with *A. niger* F4 produced highest in substrate concentration of 175mg/l with 9.17mg/ml and the least was in 75mg/l substrate concentration with 5.88 mg/ml whereas *A. niger* F5 yielded highest in substrate concentration of 175 mg/l with 10.02 mg/ml and the lowest was in 75 mg/l with 6.02 mg/ml.

The finding in this study is in agreement with the observations of a higher initial pH and low substrate concentration leads to the accumulation of oxalic acid as reported by [18]. In addition, low substrate concentration and pH has been found inhibitory for the growth of *A. niger*.

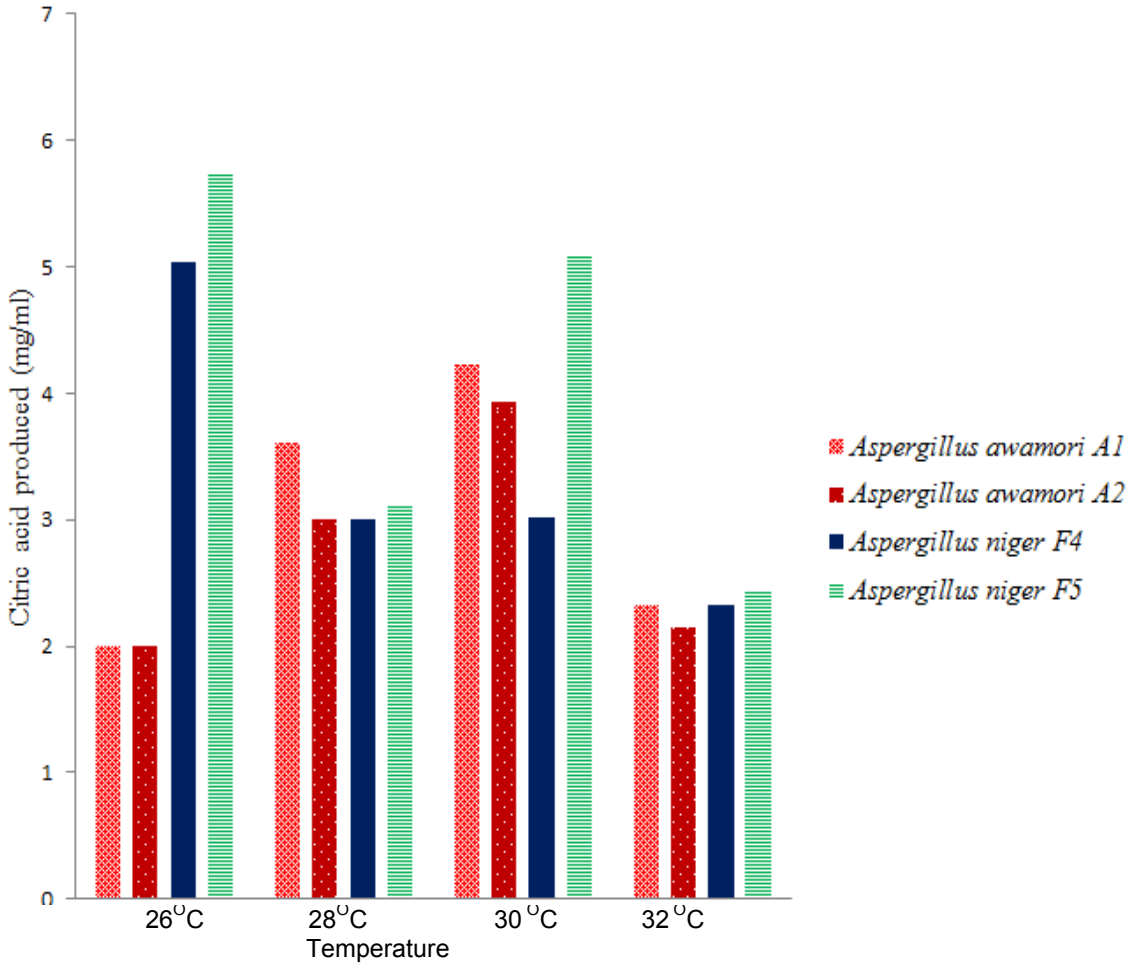

Fig. 1. Effect of different temperature on citric acid production by mutant *Aspergillus isolates*

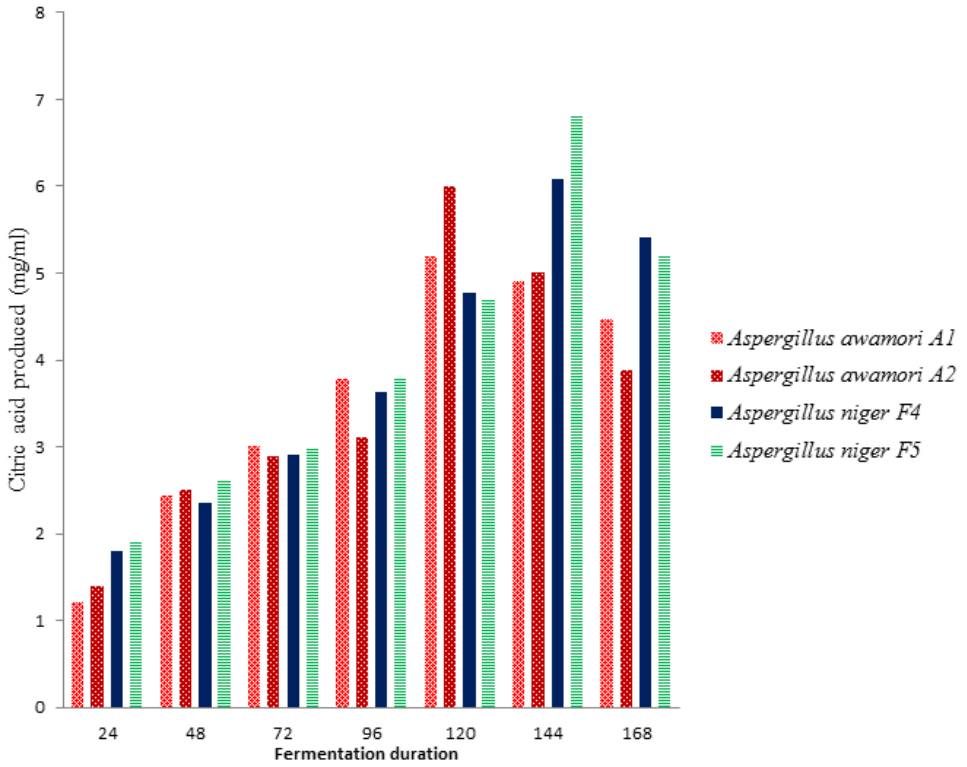

Fig. 2. Effect fermentation duration on citric acid production by mutant *Aspergillus isolates*

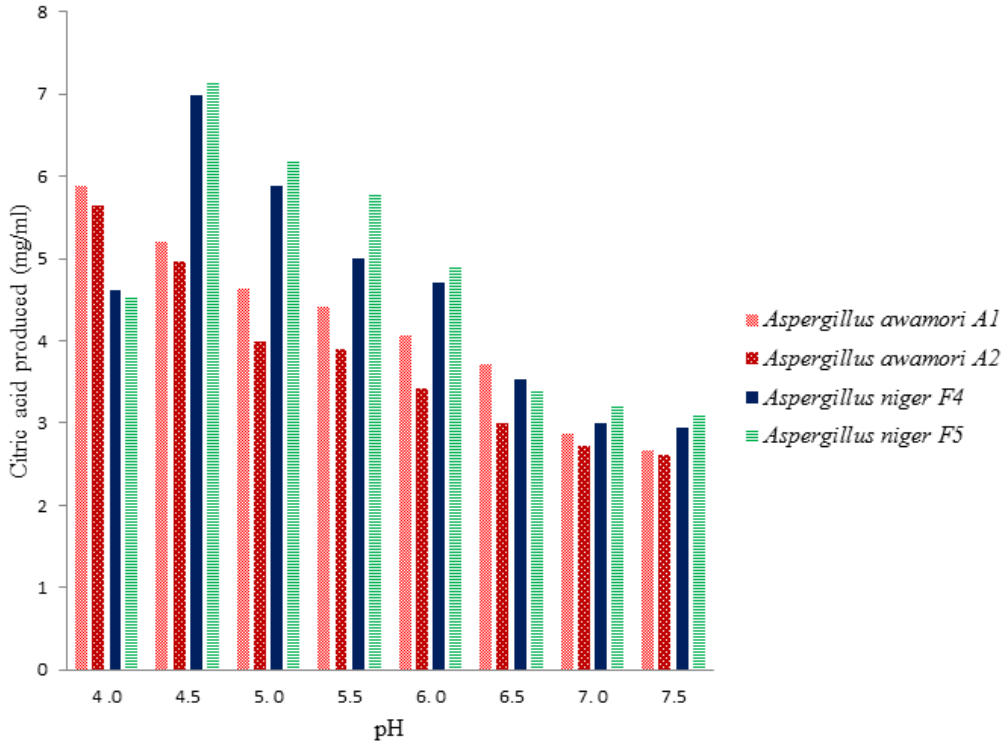

Fig. 3. Effect pH on citric acid production by mutant *Aspergillus isolates*

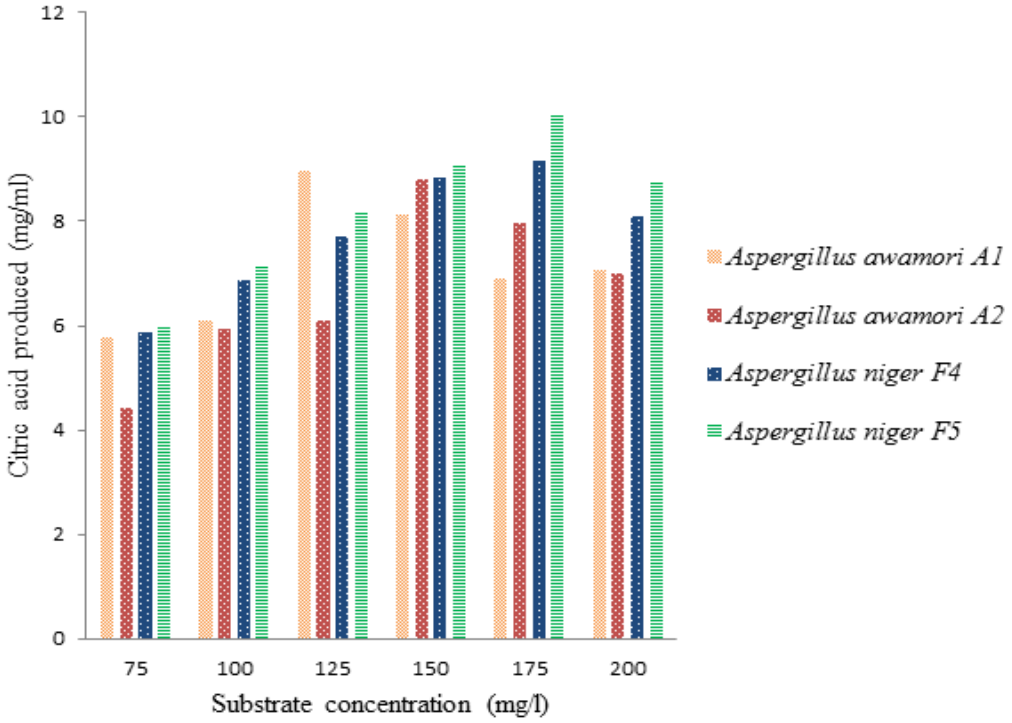

Fig. 4. Effect substrate concentrations on citric acid production by mutant *Aspergillus isolates*

4. CONCLUSION

Stable variant of *Aspergillus species*, resistant to higher concentrations of ethyl methane-sulfonate were obtained. Those mutants showed high production of citric acid using liquid medium. In the fermentation studies, those mutant, showed high yields of citric acid in different parameter studied. The productivity was considerably higher.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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