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SCREENING AND IMPROVEMENT OF LOCAL ISOLATES OF ASPERGILLUS NIGER FOR CITRIC ACID PRODUCTION

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ABSTRACT
The study involved the screening of fourteen isolates of Aspergillus niger for citric acid production from glucose. The study was aimed at screening and improving local strains of Aspergillus niger with potential for citric acid production. All the isolates screened produced varying amounts of citric acid, the highest was by the isolate designated CP3 which produced 12.81g/l and the least was B11 and O12 which produced 3.42g/l all after 6 days of submerged fermentation. The isolate CP3 was chosen for further studies. Strain improvement studies which was based on exposure of CP3 to ultraviolet irradiation for 10, 30 and 45 minutes increased citric acid yield to 22.20g/l (48.89% increase) by mutant M45 followed by a citric acid yield of 16.22g/l by the 30 minutes mutant (M30) and the least being M10 which produced 13.66g/l in all cases after 6 days of fermentation. The mutant (M45) also yielded higher amount of citric acid (19.64g/l) without methanol as additive compared to the parent strain (CP3) which gave a citric acid of 14.94g/l with the addition of methanol after six days (144h) of fermentation in both cases. Finding from this study showed that all the isolates have potential for citric acid production.
Keywords: Isolation, Screening, Improvement, Aspergillus niger, Citric acid

INTRODUCTION

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is the most important commercial product, which is found in almost all plant and animal tissues. It exists widely in the nature and is one of the organic acids found in juice of lemon, orange, pineapple, plum, peas, peach and in animal bones, muscles and blood. It has many applications in food, pharmaceutical and cosmetic industries as an acidulant, flavour enhancer, preservative, antioxidant, emulsifier and chelating agent (Ramseh and Kalanselvan, 2011). In 1880, citric acid had been synthesized from glycerol and since that time a number of syntheses from other raw materials have been published. In 1893, Wehmer, discovered that *Penicillium spp* could produce citric acid from sugar. However, microbial production of citric acid did not become industrially important until World War I disrupted Italian citrus exports.

In 1917, the American food chemist James Curie discovered that certain strains of the mould *Aspergillus niger* could be efficient citric acid producer, and Pfizer began industrial level production using this microorganism two years later (Wikipedia, 2006).

Citric acid production by fermentation is the most economical and widely used way of obtaining this product. More than 90% of the citric acid produced in the world is obtained by fermentation. The industrial citric acid fermentation can be carried out in three different ways: submerged fermentation, surface fermentation and solid-state fermentation or Koji process (Madigan *et al.,* 1997).

The improvement of citric acid producing strains had been carried-out using mutagenesis and selection. The most employed technique has been by inducing mutations in parental strains using mutagens. Mutants of *Aspergillus niger* are used for commercial production of citric acid. Among mutagens used, gamma (γ) radiation, ultraviolet (UV) radiation and chemical mutagens are often used (Soccol *et al*, 2006). The study was aimed at screening and improving local strains of *Aspergillus niger* with potential for citric acid production.

MATERIALS AND METHODS

Isolation and Identification of *Aspergillus niger*

Isolates of *Aspergillus niger* were obtained from bread, onions, mango, orange pulp, wheat bran and soil. The soil samples were seeded in sterile distilled water, serially diluted and inoculated on Potato Dextrose Agar (PDA).The bread, onions, mango, orange pulp and wheat bran were moistened and kept at room temperature to develop fungal growth which were plated on PDA. Fungal growth suspected to be *Aspergillus niger* based on macroscopic observation (carbon black or dark brown conidia) were further subcultured on fresh PDA plates. Those that exhibited characteristic *A. niger* growth (initially white, quickly turning black) were subjected to microscopic observation with reference to the manuals of Barnett and Hunter (1972) and Mycology online of Ellis, (2006). For the microscopic identification a drop of lactophenol blue was placed on a clean slide, a bit of the fungal growth was removed and placed inside the drop of lactophenol blue, covered with a cover slip and observed under the microscope using x10 and x40 objective lens.

Cellulase producing isolates of *Aspergillus niger* were also obtained from the Microbiology Department of the Ahmadu Bello University, Zaria. All isolates were maintained on Potato Dextrose Agar slants at 4^0C.

Inoculum Preparation and Standardization
The spores of the isolates were harvested from slant bottles of 6 days old cultures by washing with sterile distilled water containing 0.8% Tween 80 (Poolyoxyethylene- sorbitanmonooleate) and enumerated using a haemocytometer to give a spore suspension of 10^7 spores /ml (Haq *et al*., (2003).

Screening of Isolates for Citric Acid Production
Fourteen isolates of *Aspergillus niger* (CP1, CP2, CP3, CP4, OP5, OP6, S7, S8, M9, B10, B11,O12, O13, WB14) were screened for citric acid production using submerged fermentation in shake flask. The fermentation medium consisted of 150g of glucose/litre, 3.1g of ammonium nitrate (NH_4NO_3)/litre, 0.15g of potassium in hydrogen phosphate (KH_2PO_4)/litre and 2.2g of magnessium sulphate heptahydrate ($MgSO_4.7H_2O$)/litre. The pH of the medium was adjusted to 6.0 with 1M sodium hydroxide (NaOH) using pH metre (HANNA HI 991000). Fermentation was carried out at temperature of between 26^o C -29.5^o C on an orbital shaker at a speed of 450rpm and length of fermentation of 168h (7days). Throughout the work the fermentations were carried-out in 500ml Erlenmeyer flasks and the working volume was 60% (300ml) of the flask capacity. The inoculum size was 10ml of the spores suspension corresponding to 10^7spores per ml (Al-Delaimy and El-Holi, 2003).

Analytical Methods
Citric acid was determined daily by filtering 10ml of the culture medium through Whatman filter paper no. 41. Two to three drops of phenolphthalein as indicator was added and the filtrate was titrated against 0.1M NaOH and calculated as % citric acid according to the following formula (Al- Delaimy and El-Holi, 2003):

$$\% \text{ Citric acid} = \frac{192.13 \times M_{NaOH} \times V_{NaOH}}{\text{Weight of substrate}}$$

Where,
192.13 = molar mass of citric acid.
M_{NaOH} = molarity of NaOH
V_{NaOH} = volume of NaOH consumed during titration.
Dry mycelia weight was determined as described by Al – Delaimy and El-Holi, (2003), Haq *et al*., (2003).
Residual sugar was determined from the filtrate obtained above using the 3, 5-Dinitrosalycilic acid (DNS) method (Haq *et al*., 2003).

Preliminary Strain Improvement Studies
The spore of the wild *Aspergillus niger* isolate (CP3) was subjected to 254 nm (short wavelength) of ultraviolet (UV) irradiation for 10, 30 and 45minutes using Phillips germicidal lamp and tested for citric acid production from glucose.

UV Irradiation Procedure
The UV irradiation was carried-out using a modification of the method of Kubicek *et al*, 1986. Five millilitres of sterile distilled water containing 0.1%(w/v) Tween 80 was pipetted into three slant bottles of 7 day old cultures of the wild *Aspergillus niger* isolate and shaken vigorously to break clumps. This was further diluted to give a final density of 2 x 10^6 spores per ml. Ten millilitres of the suspension were pipetted into three different Petri dishes (13.8 cm diameter). The three dishes labelled 10, 30 and 45 minutes were placed separately inside the dispensing cabinet 20cm away from the centre of the germicidal UV lamp source for the period corresponding to the minutes on their labels. The Petri dishes were covered with alluminium foil for one hour and used for the isolation of potential mutants.

Presumptive Mutant Isolation
This was also based on the method of Kubicek *et al*, 1986. Their identification was based on the assumption that mutants lacking citrate control of phosphofructokinase would grow faster on a sucrose medium containing citrate. The diluted mutagenized spore suspensions were plated on solid medium containing 20% (w/v) sucrose and 5% (w/v) citrate as carbon sources. Other inorganic nutrients and nitrogen source were essentially as used in the citric acid fermentation medium except that the pH was adjusted to 3.5 with 1M HCl.
The fastest-growing colonies were considered to be less affected in carbohydrate breakdown by citrate and thus selected for further work.

Screening of Mutants for Citric Acid Production
The selected mutants exposed to UV irradiation for 10, 30, and 45 minutes were screened for citric acid production using 15% glucose. Other nutrients include 0.15g of KH_2PO_4/litre, 2.2g of $MgSO_4.7H_2O$/litre, 3.1g of NH_4NO_3/litre, 3% (v/v) methanol. The pH used was 5.5, agitation rate was 450rpm and length of fermentation was 7 days. These conditions were based on findings of an earlier optimization of cultural conditions studies by the authors of this paper.

Statistical Analysis
Analysis of variance (ANOVA) was carried out to determine the difference in the mean citric acid yield between the three mutants (M45, M30, M10).

RESULTS

Screening Tests
Table 1 shows the results obtained during the screening of fourteen isolates of *Aspergillus niger* for citric acid production. All the isolates screened produced varying amounts of citric acid, the highest being CP3 which produced 12.81g/l and the least was B11 and O12 which produced 3.42g/l both after 6 days of fermentation. All the other isolates produce the highest amount of citric acid after six days of fermentation except OP5, OP6, M9, B11 and O13 which yielded the highest amount of citric acid after 120h (five days) of fermentation.

There was a progressive decrease in residual sugar concentration, the least being that of CP3 (2.50g/l) and the highest being that of O12 (30.85g/l) both after 168h (seven days) of fermentation. Mycelia weight increased steadily for all the isolates with the highest values obtained after seven days of fermentation.

Preliminary Strain Improvement Studies

Figures 1, 2 and 3 represent citric acid yield, residual sugar concentration and mycelia weight of *A. niger* (CP3) after exposure to ultraviolet irradiation for 10, 30 and 45 minutes respectively. The mutant (M45) obtained after exposure of parent *A. niger* to 45 minutes of ultraviolet radiation gave the highest citric acid yield (22.20g/l) after 144h of fermentation followed by a citric acid yield of 16.22g/l by the 30 minutes mutant (M30). The mutant obtained from 10 minutes (M10) exposure to ultraviolet irradiation produced the least amount of citric acid (13.66g/l). There was no significant difference in mean citric acid yield between the M45 and M30 and also between M30 and M10 ($P \geq 0.05$), but there was significant difference in mean citric acid yield between M45 and M10 ($P \leq 0.05$). In all cases, the concentration of residual sugar steadily declined throughout the seven days of fermentation, from 124.50g/l to 4.70g/l, 126.00g/l to 10.80g/l and 118.55g/l to 2.20g/l after exposure to ultraviolet irradiation for 10, 30 and 45 minutes respectively while mycelia weight increased throughout the seven days of fermentation, from 2.99g/l to 12.58g/l, 3.44g/l to 11.44g/l and 3.01g/l to 12.48g/l after exposure to ultraviolet irradiation for 10, 30 and 45 minutes respectively.

Figure 4 shows comparison of citric acid yield between parent and mutant *A. niger* (CP3) with and without methanol as additive. The mutant yielded higher amount of citric acid 19.64g/l without methanol and 22.20g/l with methanol compared to the parents (14.09g/l from parent without methanol and 14.94g/l from parent with methanol) after six days (144h) of fermentation. Residual sugar concentration decreased all through the fermentation period, from 109.90g/l to 2.85g/l, 122.60g/l to 15.90g/l, 127.80g/l to 2.60 and 118.50 to 2.20g/l from parent without methanol, parent with methanol, mutant without methanol and mutant with methanol respectively while mycelia weight increased steadily up to day seventh of the fermentation, the highest value obtained being 12.48g/l from mutant with methanol.

Table 1: Citric acid yield (g/l), residual sugar concentration (g/l) and mycelial weight (g/l) of fourteen isolates of *A. niger* during the screening tests

Isolates	**Days**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
CP1							
CAY	8.11	8.54	8.96	9.39	9.39	9.82	7.69
RSC	125.65	108.55	86.20	62.70	34.55	17.46	6.50
MW	3.11	4.50	7.01	10.15	13.67	14.03	16.60
CP2							
CAY	5.98	8.54	9.39	9.39	10.25	10.67	8.54
RSC	132.00	106.40	87.60	60.50	45.55	20.50	4.65
MW	2.80	4.52	6.80	9.95	11.05	15.00	16.95
CP3							
CAY	7.26	10.25	11.10	11.95	12.38	12.81	11.53
RSC	124.50	98.95	76.70	52.86	28.10	10.50	2.50
MW	3.05	4.45	6.80	9.00	11.00	13.05	15.05
CP4							
CAY	6.40	10.25	10.25	11.10	11.53	11.95	10.67
RSC	124.40	98.70	78..25	50.50	32.50	18.70	7.00
MW	3.79	4.65	5.98	7.56	10.77	12.09	14.97
OP5							
CAY	6.40	6.83	8.54	9.82	10.67	10.67	8.54
RSC	130.80	117.15	98.35	78.70	62.70	45.60	15.05
MW	4.98	7.00	8.50	11.40	14.11	16.75	19.00
OP6							
CAY	4.70	4.70	5.12	6.40	7.69	7.26	6.40
RSC	131.20	112.40	99.60	80.40	57.35	39.10	19.90
MW	4.50	6.14	8.00	11.68	16.77	19.01	20.00
S7							
CAY	2.99	5.12	5.12	5.55	6.40	7.26	6.40
RSC	135.05	112.00	91.53	72.10	49.70	27.92	7.44
MW	3.05	6.05	8.64	11.87	15.11	18.00	21.90
S8							
CAY	4.70	5.98	6.40	6.83	8.11	9.39	7.69
RSC	124.15	109.50	80.70	60.20	39.93	20.00	8.55

Table 1 Continue

Isolates				**Days**			
	1	**2**	**3**	**4**	**5**	**6**	**7**
MW	3.54	4.50	6.50	9.12	12.22	15.60	17.00
M9							
CAY	4.70	5.55	6.40	7.69	9.82	8.97	7.26
RSC	126.50	109.85	90.65	71.42	37.05	19.00	8.22
MW	4.06	6.55	8.98	10.23	11.09	12.11	13.55
B1O							
CAY	2.56	4.27	5.98	5.98	7.26	8.11	7.69
RSC	134.65	111.45	90.45	60.50	43.85	31.70	16.34
MW	3.11	4.88	6.00	7.90	9.89	11.77	13.57
B11							
CAY	3.42	3.42	5.55	6.40	7.26	3.42	2.56
RSC	131.20	119.25	97.00	81.00	62.85	49.10	20.50
MW	5.00	8.20	9.14	12.55	16.00	19.02	19.50
O12							
CAY	2.56	2.56	2.56	2.99	2.99	3.42	2.56
RSC	137.20	124.90	114.65	90.50	75.05	48.88	30.85
MW	2.22	4.44	6.01	8.08	11.11	13.78	14.84
O13							
CAY	4.27	4.70	6.83	7.69	9.39	8.11	8.11
RSC	132.90	118.85	101.75	82.50	54.35	30.80	15.45
MW	2.90	3.74	4.44	7.07	9.03	10.91	11.06
WB14							
CAY	4.70	6.40	6.83	7.69	8.11	8.54	7.69
RSC	131.10	102.30	78.40	50.50	38.65	25.80	10.00
MW	3.50	5.03	5.94	7.07	9.77	11.11	13.99

Key: CAY = Citric acid yield, RSC = Residual sugar concentration, MW = Mycelial weight.

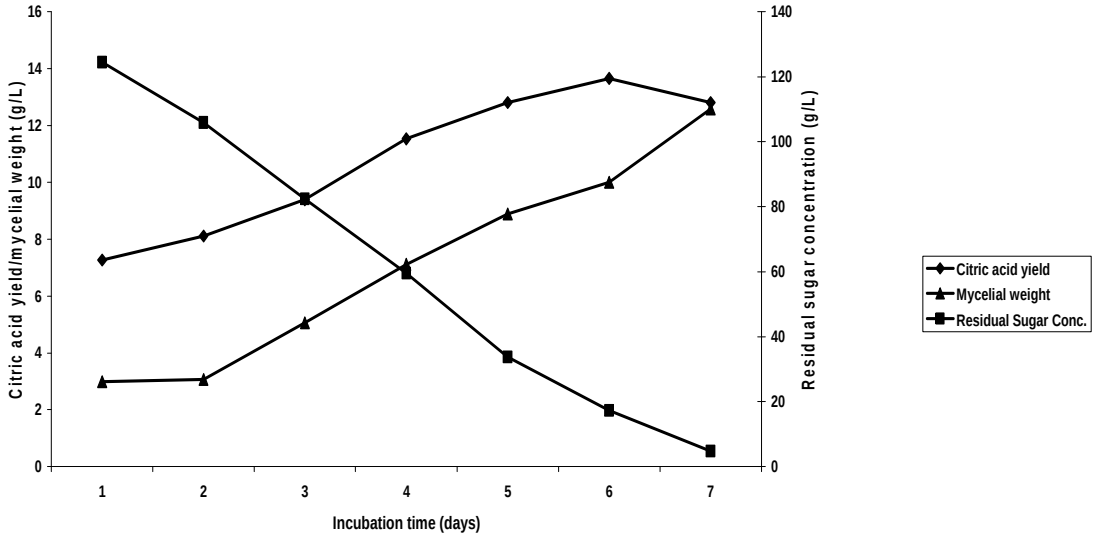

Figure 1: Citric acid yield, residual sugar concentration and mycelia weight of *A. niger* at 10 minutes exposure to UV radiation

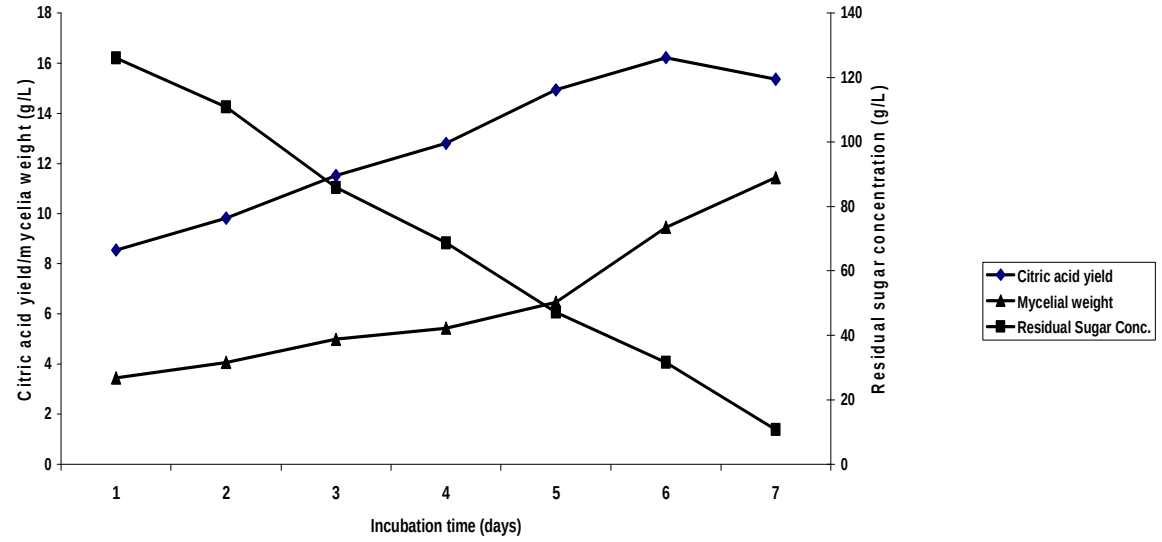

Figure 2: Citric acid yield, residual sugar concentration and mycelia weight of *A. niger* at 30 minutes exposure to UV radiation

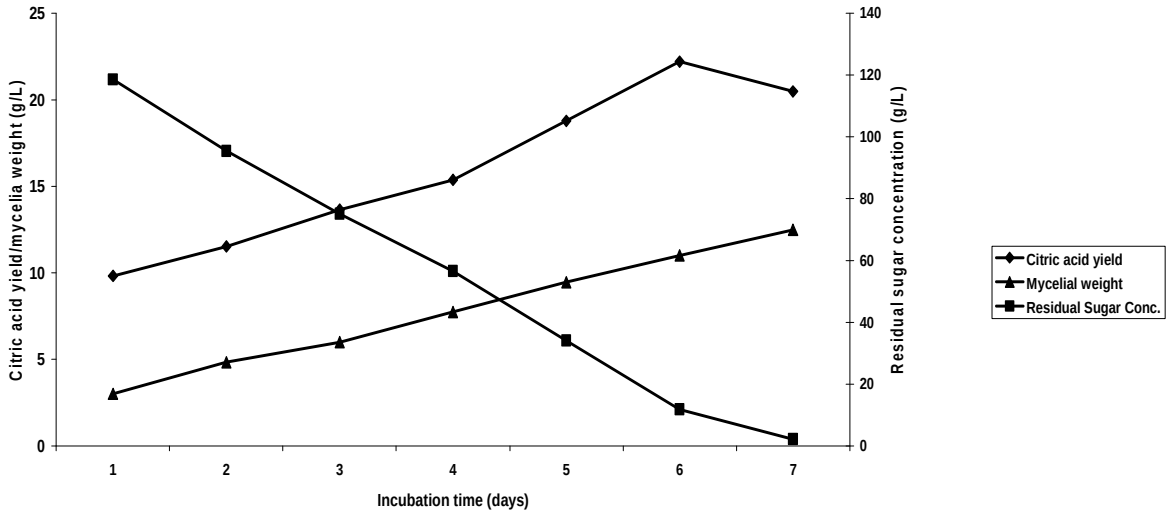

Figure 3: Citric acid yield, residual sugar concentration and mycelia weight of *A. niger* at 45 minutes exposure to UV radiation

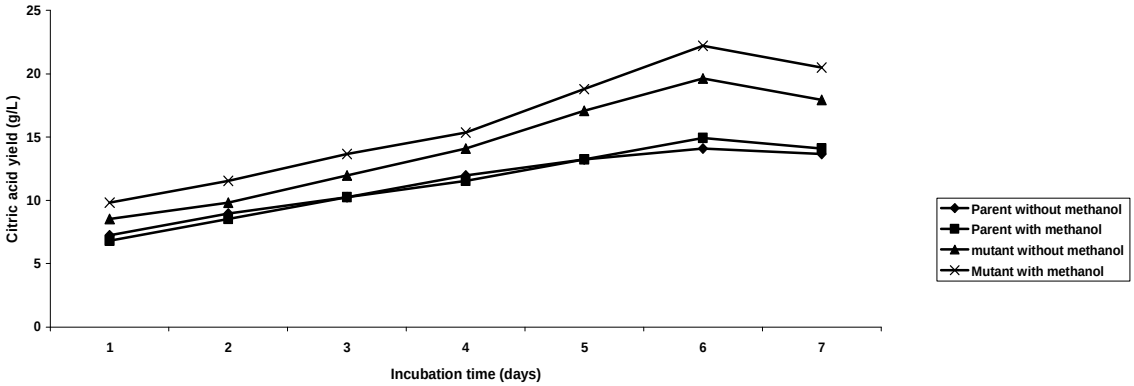

Figure 4: Comparism of Citric acid yield between parent and mutant *A. niger* with and without methanol as additive

DISCUSSION

Screening of *Aspergillus niger* Isolates for Citric Acid Production

Fourteen isolates of *Aspergillus niger* code named CP1- 4 (Cellulase producing), OP5 and OP6 (from orange pulp), S7 and S8 (from soil), M9 (from mango), B10 and B11 (from bread), O12 and O13 (from onions) and WB14 (from wheat bran) were screened for citric acid production. Of these isolates, CP3 produced the highest amount of citric acid (12.81g/l) after six days. The citric acid yield was much lower than those of the 12 isolates of *Aspergillus niger* studied by Ali *et al*, 2002 in which the best isolate *A. niger* GCBT7 produced 84.95g/l citric acid. The low yield obtained from the isolates studied in this work compared to those studied by Ali *et al.*, 2002 may be due to differences in carbon source (glucose was used in this work while molasses was used by Ali *et al.*, 2002), molasses consists predominantly of sucrose, the low yield may also be due to difference in strain of the *A. niger* used in both studies. Variation in temperature may have also contributed to the low yield obtained in this study, the shaker used in this study did not have temperature control mechanism, and fermentation was therefore based on ambient temperature which was between 26^{o}C-29.5^{o}C during the studies. Ali *et al.*, 2002 used a constant temperature of 30^{o}C.

Preliminary Strain Improvement Studies

Highest citric acid yield (22.20g/l) was obtained from the 45 minutes mutants (M45) after 6 days of fermentation. The *A. niger* isolates exposed to ultraviolet radiation for 10 minutes yielded less citric acid (13.66g/l) compared to the parent (14.95g/l) after 144h (six days) of fermentation. This may be because exposure of the parent *A. niger* (CP3) to ultraviolet irradiation for ten minutes was too short to have caused any positive mutation.

The 45 minutes mutant (M45) gave 1.49 fold increase in citric acid yield while the 30 minutes mutant (M30) gave 1.08 fold increase in citric acid yield compared to the parent strain (CP3). The higher citric acid yield by these mutants may be because phosphofructokinase regarded as a key regulatory enzyme for citric acid formation is able to overcome feedback inhibition by citrate (Ward *et al.,* 2006). The yield obtained in this study by all the three mutants was much higher than the yield of 12.81g/l reported by Abdullah-Al-Mahin *et al.* (2012). Several workers in this field reported higher citric acid yield from mutant *A. niger* than the parent strain. (Ledezma *et al*., 1970; Haq *et al.*, 2002; Zhang *et al.*, 2002; Ikram-Ul *et al.*, 2003; Marin *et al.* 2003; Iqbal *et al*., 2004).

CONCLUSION

The results of the isolation and screening tests showed that all the *A. niger* isolates studied have the potential for citric acid production. Citric acid productivity further increased after strain improvement studies. The mutant (M45) obtained was also able to produce more citric acid without methanol as additive than the parent (CP3) strain with methanol.

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