J. Agric. Sci. Mansoura Univ., 34 (5): 4301 - 4313, 2009

MUTATION OF *Aspergillus niger* WITH GAMMA RADIATION FOR IMPROVING CITRIC ACID PRODUCTION

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ABSTRACT

Gamma radiation was used to induce citric acid overproduction mutations in some of the locally isolated strains of *Aspergillus niger*. Comparison between the parent and mutant strains was studied. The study revealed that, the most two isolates potent in the acid production were identified as *A. niger* F_1 and F_6. Modified Czapek's medium proved to be the best medium for citric acid production. As for the influence of irradiation of *A. niger* F_1 and F_6 strains using gamma rays in different doses (25 – 200 k rad) on the production of citric acid, one mutant obtained from 175 k rad treatment for *A. niger* F_1 and another mutant obtained from 100 k rad treatment for *A. niger* F_6. These two mutants (*A. niger* MF_1 and *A. niger* MF_6) increased the citric acid production as compared with the parent strains. The best sugar concentration of beet molasses was when the dilution of sugar reached 15% from the basal medium. Addition of potassium ferrocyanide to beet molasses, to precipitate excess heavy metals, at a concentration of 0.15% gave the highest conversion coefficient and yield of citric acid for parent and mutant strains. The addition of alcohols, namely methanol or ethanol (4%) to beet molasses medium greatly stimulated citric acid production. Methanol addition (4%) gave the best result of conversion coefficient and yield of citric acid for *A. niger* F_1 and F_6 as well as MF_1 and MF6, followed by ethanol treatment (4%) which gave an increase in citric acid as compared to control. The proper time for addition of methanol was found to be immediately at the beginning of fermentation.
Keywords: Citric acid, *Aspergillus niger*, mutation, γ-radiation beet molasses, alcohol.

INTRODUCTION

Citric acid is one of the few bulk chemicals produced by fermentation and is the most exploited biochemical product. Citric acid has a broad use in the household, in the preparation of numerous industrial products and in many industrial areas such as the food, pharmaceutical and chemical industries and as a cleaning agent. The supply of natural citric acid is limited and the demand can only be satisfied by biotechnological fermentation processes. (Anastassiadis *et al.*, 2002).

The optimization of fermentation conditions are of primary importance in the development of any fermentation process owing to their impact on the economy and practability of the process. (He *et al.*, 2004). The production of citric acid using cheap carbon source from agri-industrial byproducts provides considerable combined benefit of waste material management and decrease of citric acid production cost (John *et al.*, 2006 and Barrington & Kim, 2008).

Fermentation media for citric acid biosynthesis should be consist of substrates necessary for the growth of microorganism, primarily the carbon, nitrogen and phosphorus sources. Along with the carbon, nitrogen, phosphorous and potassium sources, the minerals such as Fe, Zn, Mn, Cu and Mg are also critical factors which need to be optimized for the production of citric acid (Lofty *et al.*, 2007 and Imandi *et al.*, 2008). Citric acid produced by *A. niger* is extremely sensitive to trace metals present in molasses such as

iron, zinc, copper and manganese, etc. especially during submerged fermentation. The concentration of these heavy metals should be decreased well below that required for optimal mycelial growth (Majolli and Aguirre, 1999 and El-Holi and Al-Delaimy, 2003).

Improvement of the microbial producer strain offers the greatest opportunity for cost reduction without significant capital outlay (Stanbury *et al.,* 1995). This is achieved when a selected strain can synthesize a higher proportion of the product using the same amount of raw materials. Since citric acid is an intermediate of energy metabolism, its concentration can rise to appreciable amounts under conditions of metabolic imbalances (Hutter, 1983). Strains with superior characters, such as enhanced citric acid production and increased rate of fermentation have been previously selected after subjecting the genetic material to physical or chemical mutagenic agents (Ikram-Ul *et al.*, 2003, 2004 and 2005). *A. niger* has the potential to produce a number of primary and secondary metabolites. The techniques of ultraviolet irradiations, gamma rays or N-methyl, N-nitro-N-nitroso-guanidine (MNNG) induced mutagenesis are useful to improve the yield of various secondary metabolites by *A. niger* (Gupta and Sharma, 1995 and Ikram-Ul *et al.*, 2004).

In the present investigation, challenges were made for raising potent of *A. niger* for citric acid production by random mutagenesis using gamma radiation. For further cost reduction, the ability of the selected mutaginized strain to utilize agro-industrial by-products as carbon and nitrogen sources was also examined.

MATERIALS AND METHODS

Microorganisms

Microorganisms were isolated from natural sources such as soil, orange, grapes and air contamination. Isolates of the discrete colonies were transferred to slants Czapek's agar medium respectively after further purification on the same medium. The obtained fungi were tested to their ability to produce citric acid. Fifty ml portions of modified Czapek's medium were dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized and pH was adjusted to 6.0. The flasks were inoculated by suitable spore suspension of any of the isolates, and then incubated at 30°C. The determinations of acidity were carried out at daily intervals. The strain which gave the highest acidity was selected and identified (Neweigy, 1972). Citric acid determination was carried out and the produced acid was found to be almost citric acid.

Effect of gamma radiation

Spores of each selected strain of *A. niger* were grown on a solid medium in Petri dishes and slants. After 10 days of incubation at 30°C, spore suspension was prepared from each slant, then the plate cultures and the spore suspensions were irradiated with different doses of gamma radiation namely 25, 50, 75, 100, 125, 150, 175 and 200 k rad. Serial dilutions in sterile water were made for all treatments as well as control. One ml of spore suspension of each of the final three dilutions was plated on Czapek's agar medium in Petri dishes. After 36 hours of incubation, ten discrete developing

colonies (chosen at random) of each treatment and control were transferred to 50 ml portions of modified Czapek's solution medium in 250 ml Erlenmeyer flasks. Inoculated flasks were incubated at 30°C for 15 days. During fermentation period, total acidity was determined at intervals by the titration of 1 ml aliquots against 0.1N sodium hydroxide solution using phenolphthalein as an indicator to select the strain which produced the highest percentage of citric acid (Neweigy, 1980). At the highest values of titratable acidity, citric acid was measured.

Standard inoculum

The molasses agar which consist of (g/L): molasses (300.0); KH_2PO_4 (0.50); NH_4NO_3 (2.0); distilled water (1000 ml), was used as a sporulation medium. This medium was inoculated with the required strain of *A. niger*. After 10 days of incubation at 30°C, spores were scraped using sterilized water. Two ml of spore suspension was used for inoculation of the fermentation media.

Pre-treatment of molasses

Beet molasses obtained from the sugar factory at El-Hamol, Kafr El-Sheikh gavernorate, was used in the present study. Beet molasses contains water 24%, sugar contents 51% and ash contents 3.1%. Ash contents include ions such as Mn, Al, Fe, Cu and Zn in variable ratio (Abo-Aly, 1996). Sugar content was diluted to about 50% sugar level. The molasses solution, after adding 35 ml of 1N H_2SO_4 per litre, was boiled for an hour, cooled, neutralized and was left to stand overnight for clarification (Panda *et al.*, 1984). The clear supernatant liquid was used.

Fermentation

Fifty ml portions of the fermentation medium were dispensed in 250 ml Erlenmeyer flasks. After sterilization, the pH was adjusted using suitable amount of sterilized acid. Suitable inoculum (2ml) was added to each flask then flasks were incubated at 30°C until the end of the fermentation period. Chemical analysis was done periodically along the fermentation period. Four replicates were made for each treatment. During the fermentation period, 1 ml of the fermented liquor was taken at intervals for determining the titratable acidity. At the end of the incubation period the biomass was pressed to force the liquid out. Biomass was dried at 70°C for 48 hours, and the residual of the fermented liquor was taken for chemical determination (residual sugar and citric acid determination).

Estimation of the total titratable acidity

The total acidity was determined by the titration of 1 ml aliquots against 0.1N NaOH solution using phenolphthalein as an indicator.

Estimation of sugars

Total and reducing sugar were determined spectrophotometrically according to the method described by Dubois *et al.* (1956) and Thomas and Dutcher (1924), respectively. The sugar content was calculated as glucose from standard curve prepared for glucose.

Estimation of citric acid

The estimation of citric acid was carried out colourimetrically according to the method described by Marrier and Boulets (1958).

Calculations

Substrate utilization efficiency (S.U.E.) was calculated according to Herbert *et al.* (1956) by the following equation: S.U.E. = [Consumed sugar(g)/Original sugar (g)] x 100.

Conversion coefficient or conversion percentage was calculated according to Foster (1949) as follows: [Amount of citric acid produced (g)/Consumed sugar (g)] x 100.

Citric acid yield (%) was calculated as follows: [Amount of citric acid produced(g)/Original sugar (g)] x 100.

RESULTS AND DISCUSSION

Effect of different incubation periods

In a screening experiment, twelve isolates of fungi were examined for, total titrable acidity production at different incubation periods. As shown in Table (1), most of the isolates produce highly amounts of titrable acidity after 13 days of growth. Generally, the isolates F1 and F6 recorded high values in the acidity production at different incubation periods. Therefore, the two isolates were selected and identified as *A. niger*.

Table 1. Total titratable acidity produced by selected isolates of *Aspergillus* sp. (NaOH ml equivalent).

Incubation Period (Days)	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}
5	3.5	2.5	2.5	3.1	3.0	2.7	2.7	2.7	1.0	3.0	1.5	3.2
7	4.0	3.0	2.8	3.2	3.3	4.2	2.9	3.0	1.5	3.7	1.7	3.5
9	4.5	3.1	3.2	3.2	3.8	5.2	3.0	3.5	2.0	3.9	1.9	4.0
11	5.0	3.3	3.5	3.5	4.3	5.9	3.5	4.0	2.8	4.3	2.0	4.5
13	6.2	3.3	4.7	4.0	5.1	6.5	3.8	5.0	3.0	5.0	2.5	5.0
15	5.8	3.0	5.3	4.7	4.6	6.0	4.0	4.5	2.7	5.2	2.0	4.7
17	4.7	2.8	4.6	4.3	4.5	5.0	3.7	4.3	2.4	4.2	1.8	4.5

F_1-F_{12} = Isolates of fungi isolated from different natural sources.

Effect of different media

Data in Table (2) give a comparison between four media namely, Bernhauer (Neweigy, 1972), Modified Czapek's, Chmiel (1975) and Uchio *et al.* (1976) media, in order to improve the biomass formation, consumed sugar, substrate utilization efficiency, conversion coefficient and citric acid yield for the both strains *A. niger* F1 and F6, the observation indicated that Modified Czapek's medium was the best medium for the two strains. In this medium, conversion coefficient reached 50.42% and 52.25% for *A. niger* F_1 and F_6, respectively, also, the yield of citric acid reached 40.0% and 43.33% for F_1 and F_6, respectively after 13 days incubation. Upon further incubation citric acid production showed gradual slow decrease. This medium was followed by Bernhauer medium (M_1) for both *A. niger* strains. However, media M_3 and M_4 were unsuitable for citric acid production by the working strains. Therefore, modified Czapek's medium was used for further studies as a basal medium.

Effect of gamma radiation on citric acid production

Gamma rays were used for induction of mutation. In this experiment different γ-doses were used in both spore suspension and plate cultures for two strains *A. niger* F1 and F6. The best dose was determined as its effect on citric acid production. Tables (3 & 4) indicate that the mutants which resulted from plate cultures treatment were better than those obtained from spore suspension treatment.

Table 2. Effect of different media on the production of citric acid by the two strains of *A. niger* (F_1& F_6).

Media*	Biomass (g/100 ml)	CS (g/100 ml)	S.U.E. (%)	CC (%)	Citric acid yield (%)
A. niger F_1					
M_1	2.32	10.03	66.87	47.85	32.0
M_2	2.87	11.9	79.33	50.42	40.0
M_3	0.47	7.95	52.98	7.55	4.0
M_4	0.26	8.64	57.64	3.47	2.0
A. niger F_6					
M_1	2.91	10.57	70.47	49.19	34.66
M_2	2.27	12.44	82.93	52.25	43.33
M_3	0.85	9.45	63.02	25.39	16.0
M_4	0.53	8.32	55.48	14.42	8.0

Media used: M1 = Bernhauer (Neweigy, 1972), M_2 = Modified Czapek's medium, M_3: Chmiel (1975) M_4 = Uchio *et al.* (1976). *S.U.E. = Substrate utilization efficiency, CS, consumed Sugar and CC, Conversion Coefficient.

Concerning the effect of different gamma rays doses on citric acid produced by irradiated mutant *A. niger* F_1 and F_6 strains, results in Tables (3 & 4) showed that citric acid obtained by *A. niger* F_1 remarkably increased in one out of ten isolates exposed to 175 k rad compared with untreated strains (control). For *A. niger* F_6, exposure of this strain to 100 k rad increased the citric acid production as compared to control. This may be due to that gamma rays caused certain effects (direct or indirect) which caused breaks in the DNA of these isolates and as a result, activation took place (Ward, 1985).

Table (3) showed that among all mutants, maximum product yield of citric acid was achieved by the mutant M4 of *A. niger* F1 (52.3%) with approximately (12%) increase when compared to the wild type. In the same trend, data in Table (4) indicate that the mutant M7 of *A. niger* F6 produce (66.6%) citric acid approximately (14.6%) increase when compared to the wild type. These mutants increased the citric acid production as compared with the control treatment. So the two mutants (*A. niger* MF_1 and *A. niger* MF_6) were selected to complete this study. These results are in agreement with Neweigy (1980) who found that gamma radiation treatments affected the citric acid production and other physiological characteristics of the fungus namely, sugar consumption, conversion coefficient and mycelial dry weight. Also, Ikram-Ul *et al.* (2004) exposed the suspension of *A. niger* GCB-75 to UV-induced mutagenesis. The mutant GCMC-7 has greater commercial potential than the parental strain with regard to citrate synthase activity.

Table 3. Citric acid produced by gamma irradiated mutant *Aspergillus niger* strain F_1 incubated for 13 days.

Treatment	γ-dose (k rad)	Citric acid (%) of mutant F_1 strains											Parent strain
		1	2	3	4	5	6	7	8	9	10	Mean	
Spore Suspension	25	20.0	9.3	22.6	10.6	13.3	20.0	16.0	20.0	17.3	13.3	16.2	41.3
	50	16.6	12.6	10.0	11.3	12.0	13.3	12.0	12.6	17.3	18	13.6	
	75	20.0	14.0	20.0	13.3	15.3	18.0	12.0	18.6	11.3	12.0	15.4	
	100	12.6	11.3	18.6	16.0	12.0	13.3	12.6	14.6	13.3	9.3	13.3	
	125	16.6	20.6	12.0	16.6	13.3	11.3	6.6	14.0	13.3	18.0	14.2	
	150	20.0	10.0	13.3	16.0	12.6	16.0	13.3	16.6	20.0	22.0	16.0	
	175	11.3	20.6	15.3	9.3	8.6	18.6	8.6	10.6	8.6	16.0	12.8	
	200	8.6	18.0	21.3	16.6	8.6	10.6	10.6	12.0	11.3	14.6	13.2	
Plate Cultures	25	18.6	22.0	21.3	24.6	20.6	26.6	18.6	10.6	23.3	18.6	20.5	
	50	14.6	21.3	15.3	19.3	22.0	22.6	28.0	30.0	31.3	16.0	22.0	
	75	18.6	20.0	25.3	11.3	19.3	18.0	22.6	22.0	22.0	18.0	19.7	
	100	25.3	30.0	18.0	28.0	20.0	28.6	30.6	21.3	16.6	26.0	24.4	
	125	18.6	14.0	10.0	12.0	22.0	13.3	16.6	21.3	21.3	16.6	16.6	
	150	8.0	11.3	13.3	8.6	8.6	9.3	14.6	10.0	12.6	13.3	10.9	
	175	38.0	34.6	32.0	53.3	40.6	36.6	38.0	34.6	33.3	30.0	37.1	
	200	36.6	36.0	34.6	32.6	33.3	36.6	39.3	35.3	34.6	38.0	35.7	

Effect of different dilutions of molasses on citric acid production

The presence of some elements in high concentrations is considered to be inhibitory to citric acid fermentation. Dilution of molasses to a suitable sugar concentration may also help in diluting the undesirable high concentrations of trace elements. To investigate this point, an experiment was constructed using different dilutions of the experimental molasses in which the sugar content varied from 5% to 20%. Every dilution was supplemented with the inorganic nutrients of the modified Czapek's medium and inoculated with *A. niger* F_1, F_6 and mutant strains MF_1 and MF_6.

Data in Table (5) showed that the maximum yield of citric acid (58.0% and 61.33%) for parent strains F_1 and F_6, respectively and (74.0% and 81.33%) for mutant strains $MF_1$and MF_6, respectively, was obtained in the medium containing diluted molasses (15% sugar). In respect of conversion coefficient of citric acid, the mutant strain MF_1 gave higher values (87.13%) than the parent strain F1 (67.03) at 15% sugar. The same trend was obtained by *A. niger* MF_6 and F_6 which gave conversion coefficient 87.52% and 67.10% respectively. These results are in line with those obtained by Ul-Haq *et al.* (2002b). They found that maximum amount of citric acid (92.50 g/l) was obtained in the medium containing 159 g/l sugar. Also, Ul-Haq *et al.* (2002a) observed reduction in citric acid formation when the sugar concentration of molasses was increased. Also, Darani and Zoghi (2008) showed that increasing in yield and productivity was obtained by using higher initial sugar concentration (18% w/w).

Table 4. Citric acid produced by gamma irradiated mutant *Aspergillus niger* strains F_6 incubated for 13 days.

Treatment	γ- dose (k rad)	Citric acid (%) of mutant F_6 strains											Parent strain
		1	2	3	4	5	6	7	8	9	10	Mean	
Spore Suspension	25	12.0	16.6	16.6	10.6	11.3	15.3	18.6	14.6	18.0	18.0	15.2	52.0
	50	22.6	12.6	16.6	20.0	22.0	9.3	25.3	18.0	22.6	24.0	19.3	
	75	20.0	22.0	16.6	10.6	20.6	14.6	14.0	20.6	18.6	19.3	17.7	
	100	10.6	23.3	13.3	11.3	18.6	19.3	22.0	12.0	11.3	9.3	15.1	
	125	10.0	15.3	11.3	15.3	16.0	8.0	20.6	12.6	16.0	20.6	17.7	
	150	18.6	20.6	12.0	14.6	13.3	16.0	16.6	11.3	21.3	10.0	15.6	
	175	10.6	10.6	8.6	19.3	11.3	20.6	13.3	12.0	13.3	10.0	13.0	
	200	8.6	13.3	12.6	21.3	16.6	12.0	10.6	14.6	15.3	11.3	13.6	
Plate Cultures	25	22.6	36.0	26.6	36.0	34.6	36.0	46.0	25.3	28.0	30.0	32.1	
	50	36.0	30.6	46.6	36.6	25.3	24.0	38.6	27.3	29.3	32.0	32.6	
	75	32.6	20.6	25.3	20.6	33.3	39.3	22.0	26.6	22.6	30.0	27.3	
	100	46.6	45.3	26.6	40.0	22.6	24.6	66.6	29.3	22.6	9.0	35.4	
	125	26.6	33.3	33.3	32.0	28.0	24.6	24.6	26.0	26.6	24.0	27.9	
	150	22.6	28.6	25.3	26.6	26.6	25.3	20.0	23.3	16.0	28.0	22.6	
	175	20.0	30.6	23.3	20.0	30.0	13.3	20.6	20.0	20.0	16.6	21.4	
	200	20.0	26.6	17.3	22.0	23.3	19.3	16.6	21.3	16.0	15.3	19.8	

Table 5. Effect of different dilution of beet molasses on citric acid production by the parent and mutant strains of *A. niger*.

Molasses (%)	CS (g/100 ml)	S.U.E. (%)	CC (%)	Citric acid (%)	CS (g/100 ml)	S.U.E. (%)	CC (%)	Citric acid (%)
	A. niger F_1				*A. niger* MF_1			
5	3.52	70.39	56.82	40.00	4.32	86.41	64.81	56.00
10	8.79	87.90	62.57	55.00	8.44	84.40	86.49	73.00
15	12.98	86.53	67.03	58.00	12.74	84.93	87.13	74.00
20	16.85	84.25	17.21	14.50	16.70	83.39	31.74	26.50
	A. niger F_6				*A. niger* MF_6			
5	4.16	83.20	62.50	52.00	4.02	80.39	72.14	58.00
10	9.21	92.10	66.23	61.00	9.26	92.60	86.39	80.00
15	13.71	91.40	67.10	61.33	13.94	92.93	87.52	81.33
20	17.35	86.76	28.24	24.50	16.88	84.39	43.25	36.50

CS, consumed Sugar and CC, Conversion Coefficient.

Effect of potassium ferrocyanide addition to molasses medium on citric acid production

The effect of different concentrations of ferrocyanide (0.00-0.20%) on the production of citric acid by both wild type and their mutants was evaluated. As shown in Table (6), increasing in substrate utilization efficiency, conversion coefficient and yield of citric acid were obtained using mutant

strains of *A. niger* (MF1 and MF6) than the parent strains (F1 and F6). Accumulation of citric acid showed positive response with the increase of ferrocyanide concentration. The highest value of citric acid yield was obtained when 0.15% of ferrocyanide was used being to the parent strains F_1 and F_6 (72.0 and 76.67%) and to the mutant strains MF_1 and MF_6 (87.33 and 91.33%), respectively. Further increase in the concentration of ferrocyanide than 0.15%, citric acid yield was sharply decreased by the mutant strains than the parent strains. The consumed sugar and substrate utilization efficiency were also continuously decreased by increasing the concentration of ferrocyanide beyond 0.15%.

The purpose of ferrocyanide addition was first thought to precipitate the undesirable trace elements. Martin (1955) found that beside trace elements precipitation, ferrocyanide had also a direct effect on the inhibition of the isocitric dehydrogenase. Ferrocyanide may also inhibit the aconitase enzyme beside its inhibitions to the isocitric dehydrogenase. The obtained results are in harmony with the observations of Ali *et al.* (2002) and Saad (2006). They found that the addition of potassium ferrocyanide (0.02-0.15%) to autoclaved medium led to considerable higher yield of citric acid.

Table 6. Effect of different concentrations of potassium ferrocyanide on the production of citric acid by the parent and mutant strains of *A. niger*.

K ferro-cyanide (%)	CS (g/100ml)	S.U.E. (%)	CC (%)	Citric acid (%)	CS (g/100 ml)	S.U.E. (%)	CC (%)	Citric acid (%)
	A. niger F_1				*A. niger* MF_1			
0.00	13.90	92.66	59.71	55.33	13.30	88.67	68.42	60.67
0.05	14.15	94.32	69.26	65.33	13.42	89.47	76.75	68.67
0.10	14.29	95.27	72.08	68.67	13.75	71.67	77.09	70.67
0.15	14.61	97.40	73.92	72.00	13.97	93.13	93.77	87.33
0.20	13.48	89.87	70.47	63.33	13.21	88.06	42.39	37.33
	A. niger F_6				*A. niger* MF_6			
0.00	13.74	91.59	63.32	58.00	13.99	93.26	67.91	63.33
0.05	13.57	90.46	72.22	65.33	13.28	88.54	75.30	66.67
0.10	13.93	92.88	76.09	70.67	13.77	91.79	81.34	81.34
0.15	14.08	93.87	81.68	76.67	14.20	94.66	96.48	91.33
0.20	11.58	77.21	73.40	56.67	12.84	85.60	70.09	60.00

CS, consumed Sugar and CC, Conversion Coefficient.

Effect of addition of different alcohols to molasses medium on citric acid production

The most suitable type and concentration of lower alcohol (ethanol and methanol) to be added to the experimental molasses used for citric acid production by *A. niger* (F_1 & F_6) and there mutants (MF1 & MF6). From data presented in Tables (7 and 8) it can be observed that addition of 4% alcohol markedly stimulated citric acid yield. Increasing or decreasing alcohol concentration above or fewer than 4% resulted in sharp drop of citric acid yield. El-Holi and Al-Delaimy (2003) and Ul-Haq *et al.* (2003) found that higher methanol concentration (up to 5%) caused drastic decrease in citric acid production reaching its minimum with the addition of 5%. Mutant strains

of *A. niger* (MF_1 and MF_6) could tolerate the presence of low concentrations of methanol and ethanol alcohols added to beet molasses medium used in citric acid fermentation. The addition of methanol or ethanol (4%) to beet molasses medium greatly stimulated citric acid production. Methanol addition (4%) gave the best results of conversion coefficient and citric acid yield by the parent and mutant strains as compared to ethanol addition.

Table 7. Effect of ethanol addition to beet molasses medium on citric acid production by the parent and mutant strains of *A. niger*.

Ethanol (%)	CS (g/100ml)	S.U.E (%)	CC (%)	Citric acid (%)	CS (g/100ml)	S.U.E. (%)	CC (%)	Citric acid (%)
	A. niger F_1				*A. niger* MF_1			
0	13.90	92.66	59.71	55.33	13.30	88.67	68.42	60.67
1	14.35	95.67	35.54	34.00	13.98	93.19	47.93	44.67
2	13.67	91.14	53.40	48.67	14.42	96.13	59.64	57.33
3	13.83	92.21	57.12	52.67	13.51	60.06	59.96	54.00
4	13.99	93.27	60.04	56.00	14.02	93.46	67.76	63.33
5	14.11	94.05	46.07	43.33	13.79	91.94	65.99	60.67
	A. niger F_6				*A. niger* MF_6			
0	13.74	91.59	63.32	58.00	13.99	93.26	67.91	63.33
1	14.03	94.52	46.33	34.33	14.07	93.80	52.59	49.33
2	13.60	90.66	56.62	51.33	14.14	64.26	67.19	63.33
3	14.04	93.60	62.68	58.67	13.86	92.40	73.59	68.00
4	13.38	89.19	66.52	59.33	14.15	94.34	83.39	78.67
5	14.03	93.54	56.31	52.67	13.99	93.26	74.34	69.33

CS, consumed Sugar and CC, Conversion Coefficient.

The observed increases in citric acid concentration showed that methanol and ethanol have a profound effect on the metabolism of sugars by *A. niger*. The mechanism by which methanol and ethanol stimulate citric acid production from sugars is not clear. Maddox *et al.* (1986) reported that the effect of methanol and ethanol are at the cell permeability level, allowing metabolites to be excreted from the cell. The results are in agreement with the findings of many investigators (Yaykasli *et al.*, 2005 and Darani and Zqghi, 2008) who found that alcohols addition to media used in citric acid fermentation increased the tolerance of *A. niger* to the presence of the high mineral content and decreased the harmful effect of these minerals on citric acid production from molasses.

Effect of the proper time of addition of methanol to molasses medium on citric acid production

The effect of addition time of methanol on production of citric acid is shown in Table (9). The time interval was ranged from 0 to 72 hour, after incubation. Production of citric acid gradually decreased somewhat when the time for addition of methanol was increased.

Table 8. Effect of methanol addition to beet molasses medium on citric acid production by the parent and mutant strains of *A. niger*.

Methanol (%)	CS (g/100ml)	S.U.E. (%)	CC (%)	Citric acid (%)	CS (g/100ml)	S.U.E (%)	CC (%)	Citric acid (%)
	A. niger F_1				*A. niger* MF_1			
0	13.90	92.65	57.56	53.33	13.30	88.67	67.67	60.00
1	14.40	96.01	36.11	34.67	14.19	94.59	52.15	49.33
2	13.94	92.92	46.63	43.33	14.49	96.60	62.11	60.00
3	13.83	92.19	54.23	50.00	13.27	88.46	85.91	76.00
4	13.65	91.01	67.39	61.33	14.42	96.13	97.09	93.33
5	14.17	94.47	50.81	48.00	14.14	94.27	79.21	74.67
	A. niger F_6				*A. niger* MF_6			
0	13.74	91.59	63.32	58.00	13.99	93.26	67.91	63.33
1	14.35	95.67	50.17	48.00	14.24	94.29	70.93	67.33
2	13.87	92.46	57.68	53.33	13.82	92.14	81.04	74.67
3	14.11	94.07	63.78	60.00	13.69	91.27	84.73	77.33
4	13.50	90.00	91.11	82.00	14.63	97.54	99.11	96.67
5	13.63	90.87	74.83	68.00	14.19	94.61	89.49	84.67

CS, consumed Sugar and CC, Conversion Coefficient.

Table 9. Effect of time of methanol addition to beet molasses medium on citric acid production by the parent and mutant strains of *A. niger*.

Incubation Period (Days)	Imme d-iately	After 24h	After 48h	After 72h	Imme d-iately	After 24h	After 48h	After 72h
	A. niger F_1				*A. niger* MF_1			
CS (g/100 ml)	14.01	3.87	12.29	12.75	14.87	14.75	13.87	13.36
S.U.E. (%)	93.41	92.46	82.84	85.01	99.14	98.33	92.47	89.06
CC (%)	67.09	64.17	65.91	58.82	80.69	78.64	77.14	66.62
Citric acid (%)	62.67	59.33	54.00	50.00	80.00	77.33	71.33	59.33
	A. niger F_6				*A. niger* MF_6			
CS (g/100 ml)	14.29	13.75	13.94	13.73	14.56	14.96	14.71	14.65
S.U.E. (%)	95.26	91.67	92.93	91.54	97.06	99.73	98.07	97.68
CC (%)	76.98	76.36	70.30	59.72	85.85	81.55	80.89	72.35
Citric acid (%)	73.33	70.00	65.33	54.67	83.33	81.33	79.33	70.67

CS, consumed Sugar and CC, Conversion Coefficient.

The highest yield of citric acid (80.00% and 83.33%) was obtained when methanol was added immediately to the production medium of the mutant strains MF1 and MF6, respectively. Further increase in the time of addition, did not enhance citric acid accumulation. The addition of methanol (4%) after 72 hours gave the poorest result in citric acid production (59.33 and 70.67%) by the mutant strains. Methanol markedly depressed the synthesis of the cell protein in the early stages of the cultivation and also increased the metabolic activity of enzyme (Hang and Woodams, 1986). From these facts, it was concluded that methanol induced enzyme activities

to become suitable for citric acid fermentation in the early stage of the cultivation. Addition of methanol after 24 hours or later has not been found to be beneficial. Earlier it was suggested that methanol increases the tolerance of fungi to trace elements such as Fe, Mn, Zn, etc (Ali *et al.*, 2002).

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تطفير فطر الأسبرجلس نيجر بأشعة جاما لتحسين إنتاجية حمض الستريك

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فى هذه الدراسة تم استخدام أشعة جاما لإحداث طفرات فى فطر الأسبرجلس نيجر تتميز بزيادة فى إنتاج حمض الستريك عن السلالات الأم. ولقد تمت دراسة مقارنة بين السلالات الأم والسلالات المطفرة. وبدأت الدراسة بعزل الفطر وكانت أفضل العزلات فى إنتاج حمض الستريك هما العزلتين F_1, F_6 وتم التعرف عليهما على أنهما اسبرجلس نيجر . كما درست أفضل بيئة فى الإنتاج والتى كانت بيئة تشابك المعدلة. وعند تعريض هاتين السلالتين للتشعيع بأشعة جاما على جرعات مختلفة (٢٥ – ٢٠٠ ك راد) التقطت سلالة مطفرة من تعريض السلالة F_1 لجرعة ١٧٥ ك راد، كما كانت هناك سلالة اخرى عند تعريض السلالة F_6 لجرعة ١٠٠ ك راد. وأطلق عليهما MF_1, MF_6 . هاتين السلالتين المطفرتين زادتا من إنتاج حمض الستريك عن السلالتين الأم. وعند استخدام مولاس بنجر السكر كبديل لمصدر الكربون كان أفضل تركيز للسكر فى المولاس عند تخفيفه الى ١٥% سكر. أما بالنسبة لإضافة فروسيانيد البوتاسيوم للمولاس بغرض ترسيب المعادن الثقيلة فكان تركيز ٠،١٥ % الأفضل فى معامل تحويل وإنتاج حمض الستريك لكل السلالات الأم والمطفرة. وبإضافة الإيثانول أو الميثانول لبيئة الإنتاج بتركيزات مختلفة أدت الى زيادة تدريجية فى إنتاج حمض الستريك إلا أن أفضل تركيز كان ٤% لكلا الكحولين. وإن كان كحول الميثانول تفوق على الإيثانول فى معامل التحويل وكذلك فى إنتاجية حمض الستريك. وكان أفضل وقت لإضافة كحول الميثانول هو عند بداية عملية التخمر مباشرة .