FACTORS CONTROLLING CITRIC ACID PRODUCTION BY SOME OF *ASPERGILLUS NIGER* STRAINS

Hauka, F. I. A.; M. M. A. El-Sawah; M. M. Kassem and Sh. M. El-Kady.
Microbiol. Dept., Fac. Agric., Mansoura Univ., Mansoura, Egypt
sherifelkadi@du.edu.eg

ABSTRACT

Different strains of *A. niger* were used to examine their ability to produce citric acid and the results revealed that most of the nine tested strains were able to produce citric acid. *A. niger* CA2 was the highest producer strain during the fermentation on medium No. 2, while highest biomass weight was obtained during the growth of *A. niger* CA2 on medium No.1. But, highest value of consumption of sugars was found during the growth of *A. niger* NRRL 2270 on medium No.5. Highest value of conversion coefficient and the citric acid yield were obtained during the growth of *A. niger* CA2 on medium No. 2. Maximum production of citric acid was obtained after 8 days of fermentation and pH 5.5. At the same time, highest value of biomass, conversion coefficient and total sugars consumed were obtained at these conditions. At 15% sugars concentration, citric acid reached its maximum being 29.3 g/L.

Maximum production (32.03 g/L) of citric acid was obtained with 2.0 g/L ammonium phosphate, also, give highest amount of biomass and citric acid yield. Highest value of citric acid concentration (32.88 g/L) and citric acid yield (21.92%) were obtained with 0.3 ml/L of phosphoric acid which was found as the best phosphorus source.

Key Words: *Aspergillus niger*, citric acid production and yield, biomass, conversion coefficient

INTRODUCTION

Citric acid is an important organic acid used in medicine, flavoring extracts, food and in candies, the manufacture of ink and dyeing. As an edible acidifier, citric acid is widely used for its advantages of high solubility, least toxicity, strong chelating power and pleasant taste. It is also applied as condiment, preservative in beverage and sweets, antioxidant when acting with ascorbic acid in fruit freezing and pH adjustor as in preparation of sweets and fruit jelly. Therefor, many fungi can used for citric acid production **(Franz *et al*., 1993, Burgstaller *et al*., 1994, Moreira *et al*., 1996; Gallmetzer *et al*., 1998 and El-Kady, 2003)**. There are many organisms, including fungi, yeast and bacteria, that can produce citric acid by fermentation. But, the molds have the ability to convert sugars to citric acid. *Aspergillus niger* was the most reliable producer for production of citric acid.

Aspergillus niger strains which have proved to be excellent producer of citric acid, it give the best yield per time unit and can be utilize many cheap raw materials to produce this organic acid. Thereby, making the process economic **(Hawksworth, *et al*., 1995; Gallmetzer *et al*., 1998 and El-Kady, 2003)**. Several factors were strongly affecting the production of citric acid, *i. e*., sugars, pH and temperature, so, many attempts have been carried out to study the effect of environmental and nutrional conditions on microorganisms producing citric acid **(Garg and Sharma, 1991; Esuoso, 1994 and Vandenberghe *et al*., 2000)**.

The current study has been undertaken to examine the possibility of citric acid production by different strains of *A. niger* as well as to maximize the production level by studying the effect of some nutrional and environmental factors.

MATERIALS AND METHODS

Microorganisms:

Six local fungal strains, namely *Aspergillus niger* CA1; *A. niger* CA2; *A. niger* CA3; *A. niger* CA4; *A. niger* CA5 and *A niger* CA6, used in the present work were obtained from Microbiol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt

A. niger NRRL 2270; *A. niger* NRRL 3 and *A. niger* NRRL 67 were obtained from Microbial Properties Research Unit, National Center for Agricultural

Utilization Research, Agricultural Research Service, USA. These cultures were maintained on Potato-Dextrose Agar (PDA) slants at 5°C and subcultured monthly.

Culture media:

Six cultural media were used as basal meda for citric acid production, these were: medium No.1 of **Currie, (1917)**; medium No.2 of **Mashhoor *et al.*, (1987)**; medium No.3 of **Saha *et al.*, (1999)**; Medium No.4 of **Karow, (1942)**; medium No.5 of **Doelger and Prescott, (1934)**; and medium No.6 of **Papagianni *et al.*, (1999)**.

Preparation of fungal spores suspension:

Spores appeared on PDA slant after 7 days were scraped by using 5 mL sterilized saline solution containing 8 g NaCl/L and suspended in 50 mL of the same solution. Spores count was performed in a Hematocytometer (model Buerker MOM buda pest) direct hemocytometer counting **(Pintado *et al.*, 1997)**.

Cultural conditions:

Cultivation was made in 250 mL Erlenmeyer flasks, each containing 100 mL of sterile medium. Inoculum containing $5x10^6$ spore was transferred to the culture medium. The flasks were incubated at 30°C on a rotary shaker at 160 r.p.m. After incubation period (6 days) the culture broth from each flask was filtered off to separate the mycelium from the culture filtrate. Mycelium was washed twice with 50 ml. of distilled water and dried. Values of pH were determined in the culture filtrate using a pH meter, model CG 710. The cultural filtrates were centrifuged, and the clear supernatants were used for citric acid and residual sugars determinations.

Determination of citric acid by Pyridine-acetic anhydride method:

Produced citric acid was colorimetrically determined by pyridine-acetic anhydride method according to **Marier and Boulet, (1958)**.

Determination of citric acid using the reference titration method:

Citric acid was determined using the reference titration method according to **Rugsaseel *et al.*, (1995)**.

Conversion coefficient and yield of citric acid:

Conversion coefficient and yield of citric acid were calculated according to **Foster, (1949)**.

Determination of total carbohydrate:

Total carbohydrates were determined as glucose according to the method of **Dubois *et al.*, (1956)**.

Determination of reducing sugars:

The residual reducing sugars were determined as glucose by the method of **Nelson, (1944)**.

Fangal biomass determination:

Mycelial dry weight was determined by drying the filtered cake or pellets at 70°C. until constant weight was attained.

RESULTS AND DISCUSSION

I- Selection of the most active citric acid producer and suitable medium.

Data presented in Tables (1-7) showed that the most tested strains were able to produce citric acid during their growth in submerged fermentation system in the used media. The highest production of citric acid being 22.90 g citric acid/L was obtained by *A. nigre* CA2 on medium No. 2 which containing sugare cane molasses. **Röhr *et al.*, (1983)** reported that sugar cane molasses is widely used as carbon source although it's problem in matal ions content. Thus, this strain and this medium was chosen for further studies. Many authors were reported different concentrations of citric acid *i. e.* 25 g/L **(Chanda *et al.*, 1990)**, 41 g/L **(Kim *et al.*,**

1995), 179 g/kg dry pod **(Roukas, 1998)** and 490 g citric acid from kg of glucose consumed **(Mourya and Jauhri, 2000)**.

Dried mycellium weight (D M W) of *A. niger* strains ranged from 0.00 to 48.5 g/L. The highest weight was obtained by *A. niger* CA2 on medium No. 1. Different amounts of dry weight were obtained by **Kim *et al.*, (1995), Roukas, (1998)** and **Papagianni *et al.*, (1999)**.

Consumation of sugars (C.S) by the tested strains ranged from 2.50 % to 68.50 %. The highest value was consumed during growth *A. niger* NRRL 2270 on medium No. 5. Similar observations were reported by **Chanda *et al.*, (1990)** and **Roukas, (1998)**.

Conversion coefficient (C.C) by the tested strains ranged from 0.00 % to 30.946 %. The highest value was found during the growth of *A. niger* CA2 on medium No. 2. **Jianlong *et al.* (2000)** achieved sugar conversion up to 82.2% and **Mourya and Jauhri (2000)** reported that 49% of substrate converted to citric acid.

Citric acid yield by the tested strains ranged from 0.00 % to 15.267 %. The highest value was obtained by *A. niger* CA during the growth on medium No. 2. Many authors were obtained a different citric acid yield, *i. e.* 55% **(Roukas, 1998)** and 80 % **(Lesniak *et al.*, 2002)**.

Table (1): Screening of some citric acid producing strains cultivated on medium No.(1).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	3.45	00.92	06.0	56.0	1.640	0.613
CA2	2.55	17.30	48.5	86.0	20.120	11.530
CA3	3.50	00.68	08.8	30.0	2.267	0.453
CA4	3.50	00.73	03.5	71.0	1.028	0.487
CA5	3.50	00.84	05.2	37.0	2.270	0.560
CA6	3.50	00.90	07.2	10.0	9.000	0.600
NRRL3	3.30	03.00	37.4	42.5	7.059	2.000
NRRL67	3.15	05.50	21.2	52.5	10.476	3.667
NRRL2270	4.10	00.00	24.6	10.0	0.000	0.000

Table (2): Screening of some citric acid producing strains cultivated on medium No.(2).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	6.20	03.62	16.3	50.0	7.240	2.413
CA2	3.90	22.90	16.4	74.0	30.946	15.267
CA3	6.25	03.01	16.7	56.0	5.375	2.007
CA4	6.40	01.63	15.7	58.0	2.810	1.087
CA5	6.40	01.60	15.5	54.0	2.963	1.067
CA6	6.55	00.48	03.5	50.0	0.960	0.320
NRRL3	4.10	20.16	18.9	99.0	20.364	13.440
NRRL67	3.95	21.22	18.1	82.5	25.721	14.147
NRRL2270	4.50	16.48	17.6	73.0	22.575	10.987

II-The nutritional and environmental conditions for citric acid production:

1- Effect of time course:

The results in Table (7) showed that the concentration of citric acid increased with the increasing the fermentation time and reached its maximum (23.20 g citric acid/L) after 8 days of incubation.During this Psriod, citric acid yield was 15.467 % and the conversion coefficient was 28.293%. **Maddox and Brooks (1995)** reported similar results. The highest biomass (25.5 g dried biomass/L culture) was obtained after 9 days. The results also shown that pH of cultivation medium were decreased during fermentation due to the release of citric acid. The lowest value of pH (3.90) was accompanied with the greatest concentration of citric acid.

Table (3): Screening of some citric acid producing strains cultivated on medium No.(3).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	3.40	01.28	02.0	55.0	2.327	0.914
CA2	2.80	09.50	15.3	35.0	6.786	6.786
CA3	3.45	00.21	00.2	48.5	0.433	0.150
CA4	3.50	00.00	00.2	35.0	0.000	0.000
CA5	3.45	00.00	00.2	26.5	0.000	0.000
CA6	3.50	00.00	00.0	03.5	0.000	0.000
NRRL3	3.35	03.75	08.0	32.5	11.538	2.679
NRRL67	3.20	05.45	08.5	48.5	11.340	3.929
NRRL2270	3.40	03.75	10.5	26.5	14.151	2.679

Table (4): Screening of some citric acid producer strains cultivated on medium No.(4).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	2.30	00.00	00.2	18.5	0.000	0.000
CA2	2.20	05.00	32.5	45.0	11.111	3.333
CA3	2.30	00.00	00.2	20.0	0.000	0.000
CA4	2.30	00.00	00.2	17.5	0.000	0.000
CA5	2.30	00.00	00.2	29.0	0.000	0.000
CA6	3.20	00.00	00.1	05.0	0.000	0.000
NRRL3	2.20	05.00	30.0	53.0	9.434	3.333
NRRL67	2.25	03.75	29.0	50.0	7.500	2.500
NRRL2270	2.20	06.00	31.0	66.5	9.023	4.000

Table (5): Screening of some citric acid producing strains cultivated on medium No.(5).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	2.40	00.00	00.2	27.0	0.000	0.000
CA2	2.35	00.00	03.3	33.5	0.000	0.000
CA3	2.35	00.00	00.2	33.0	0.000	0.000
CA4	2.35	00.00	00.2	05.0	0.000	0.000
CA5	2.35	00.00	00.2	39.0	0.000	0.000
CA6	2.40	00.00	00.2	36.0	0.000	0.000
NRRL3	2.30	03.00	05.6	66.5	4.511	6.000
NRRL67	2.40	00.00	06.6	37.5	0.000	0.000
NRRL2270	2.35	02.00	04.2	68.5	2.920	2.000

Table (6): Screening of some citric acid producing strains cultivated on medium No.(6).

Isolates	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
CA1	2.65	02.00	16.0	36.5	5.480	1.429
CA2	2.65	03.10	12.1	43.0	7.209	2.214
CA3	2.70	00.00	03.0	15.0	0.000	0.000
CA4	2.70	00.00	01.0	10.0	0.000	0.000
CA5	2.70	00.00	02.0	17.0	0.000	0.000
CA6	2.70	00.00	01.5	12.5	0.000	0.000
NRRL3	2.40	06.50	21.6	56.5	11.504	4.643
NRRL67	2.35	08.75	24.3	63.0	13.889	6.250
NRRL2270	2.45	05.10	16.3	45.0	11.333	3.643

The concentration of consumed sugars increased with the increase of the fermentation Period. The highest concentration (85.0 g consumed sugars/L medium) was observed after 10 days of cultivation. These results are in agreement with those reported by **Roukas (1999)**.

Table (7): Effect of time course on citric acid production by *A. niger* CA2

Days	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
1	6.20	01.58	02.0	05.0	9.600	0.320
2	5.45	02.88	04.0	29.0	9.931	1.920
3	4.70	05.47	07.3	41.5	13.181	3.647
4	4.40	10.86	08.7	55.0	19.745	7.240
5	4.15	16.13	10.9	68.5	23.547	10.753
6	4.05	21.50	15.6	72.5	29.656	14.333
7	3.90	23.00	19.7	80.5	28.571	15.333
8	3.90	23.20	23.6	82.0	28.293	15.467
9	3.90	23.00	25.5	82.5	27.879	15.333
10	3.90	22.80	25.0	85.0	26.824	15.200

2- <u>Effect of the initial culture pH:</u>

The resultes in Table (8) showed that citric acid concentration increased with the increasing of the initial pH from 4.0 to 5.5 and then decreased in the range of 6.0-8.0. The maximum amount of this organic acid was at pH 5.5. At this pH, the biomass was 29.0 g /L. The concentration of the total sugars consumed was 89.0 g /L. The conversion coefficient was 32.584% and citric acid yield was 19.333%. These results are in harmony with those reported by **Roukas (1999)**.

Table (8): Effect of the initial culture pH on citric acid production by *A. niger* CA2.

Initial pH	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
4.00	3.90	06.00	05.7	38.0	15.789	4.000
4.50	4.35	10.00	06.5	40.0	25.000	6.667
5.00	4.00	27.50	23.5	50.0	46.610	18.333
5.50	3.70	29.00	23.9	89.0	32.584	19.333
6.0	3.75	28.80	21.1	82.0	35.122	19.200
6.50	3.80	27.30	21.8	85.0	32.118	18.200
7.00	3.90	22.64	21.9	82.5	27.442	15.093
7.50	4.45	17.76	22.6	79.5	22.340	11.840
8.00	4.60	11.20	22.5	71.0	15.775	7.467

3- <u>Effect of the total sugars concentrations:</u>

The results presented in Table (9) illustrated that citric acid level was increased with the increasing of molasses from 5 to 15% total sugars. At 15% sugar concentration, citric acid reached its maximum being 29.30 g/L, biomass was 25.6 g/L, consumed total sugars concentration was 84.5 g/L, conversion coefficient was 34.675% and citric acid yield was 19.533%.The obtained results are in line with **Papagianni *et al.* (1999)** who reported that the benefit of high initial sucrose concentration indicated that free cells need a high osmotic pressure for citric acid production.

4- <u>Effect of the nitrogen sources:</u>

Eleven inorganic and organic nitrogen sources were investigated their effect on citric acid production. These sources were added separately to basal medium No.2 to give final nitrogen concentration of 0.0559 g N/100 ml. Data presented in Table (10) showed that, citric acid reached its maximum being 29.30 g/L with ammonium phosphate being 31.40 g/L, biomass was 20.0 g/L, consumed total sugars concentration was 73.0 g/L, conversion coefficient was 43.014% and citric acid yield was 20.933%. The other organic and inorganic sources gave lower citric acid production. Similar results were reportd by **Kim *et al.*, (1995)**.

Table (9): Effect of total sugars concentration on citric acid production by *A. niger* CA2.

Total sugars	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
5%	4.55	01.03	06.6	20.0	5.150	2.060
10%	4.20	15.06	09.7	29.5	51.051	15.060
15%	3.65	29.30	25.6	84.5	34.675	19.533
20%	4.10	19.73	24.8	107.0	18.439	9.865
25%	4.30	07.11	14.3	70.5	10.085	2.844

Table (10): Effect of the nitrogen sources on citric acid production by *A. niger* CA2.

Nitrogen sources	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
Without	4.25	10.70	11.5	61.0	17.541	7.133
Urea	3.70	29.70	25.5	83.0	35.783	19.800
NH_4NO_3	4.65	04.60	10.7	77.0	5.974	3.067
NH_4Cl	4.60	05.90	08.9	59.0	10.000	3.933
$(NH_4)_2SO_4$	4.60	05.80	10.3	57.0	10.175	3.867
$NH_4H_2PO_4$	3.55	31.40	20.0	73.0	43.014	20.933
KNO_3	4.15	08.20	15.7	56.0	1.464	5.467
$NaNO_3$	4.25	10.70	09.8	58.5	18.291	7.133
Peptone	4.55	07.04	18.7	55.0	12.800	4.693
Beef extract	4.55	07.10	16.4	62.0	11.452	4.733
Yeast extract	4.20	10.90	15.7	58.0	18.793	7.267
Malt	4.15	08.00	05.3	63.0	12.698	5.333

5- Effect of $NH_4H_2PO_4$ concentrations:

Since ammonium phosphate was the best nitrogen source for citric acid production, this experiment was conducted to study the effect of different concentrations of ammonium phosphate on the production of citric acid. The results in Table (11) showed that citric acid production was gradually increased with the increasing of ammonium phosphate up to 2 g/L culrture medium. This can be explained by **Maddox and Brooks (1995)** who reported that the relatively high nitrogen content may be the reason for poor citrate production. The resultes also revealed that high nitrogrn content in the fermentation medium repressed citric acid production. Maximum productivity of this organic acid was obtained with 2.0 g/L ammonium phosphate. At 2.0 g/L ammonium phosphate, biomass, consumed total sugars concentration, conversion coefficient and citric acid yield were 20.0 g/L, 74.0 g/L, 43.284%, 21.353%, respectively. Similar results were reported with **Bayraktar and Mehmetoglu (2000)**. The influence of nitrogen on the production of citric acid can be explained by the observation of **Kristiansen and Sinclair (1979)** who found that the supply of nitrogen was exhausted early and the subsequent increased in dry weight was due to an accumulation of carbon by the cells. Citric acid was produced during this phase of carbon storage and nitrogen limitation.

6- Effect of the phosphorus sources:

Six inorganic phosphorus sources were added separately to the basal medium No.2 to give final phosphorus concentration of 0.01043 g P/100 ml. equivalent 0.04 ml. H_3PO_4 85% (sp. gr. 1.685). The results in Table (12) showed the highest amount of citric acid obtained with phosphoric acid being 31.78 g/L, biomass was 19.3 g/L, consumed total sugars concentration was 75.0 g/L, conversion coefficient was 42.373% and citric acid yield was 21.187%.The other tested sources repressed the citric acid production. **Röhr *et al.*, (1983)** reported similar results.

Table (11): Effect of $NH_4H_2PO_4$ concentrations on citric acid production by *A. niger* CA2.

$NH_4H_2PO_4$ g/L	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	4.25	10.55	11.9	60.0	17.583	7.033
0.50	3.80	26.82	12.3	67.0	40.030	17.880
1.00	3.75	27.58	15.6	72.0	38.306	18.387
2.00	3.65	32.03	20.0	74.0	43.284	21.353
3.00	3.70	31.72	14.6	72.5	43.752	21.147
4.00	3.75	31.64	18.8	73.5	43.048	21.093
5.00	3.90	30.21	19.8	75.0	40.280	20.140
6.00	3.75	28.72	19.6	76.5	37.542	19.147
7.00	3.90	20.73	14.8	65.0	31.892	13.820

Table (12): Effect of the phosphorus sources on citric acid production by *A. niger* CA2.

Phosphorus sources	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
Without	3.85	23.04	15.0	99.0	23.273	15.360
H_3OP_4	3.50	31.78	19.3	75.0	42.373	21.187
NaH_2PO_4	3.85	24.64	17.3	91.0	27.077	16.427
Na_2HPO_4	3.95	20.26	17.9	84.0	24.119	13.507
KH_2OP_4	3.85	24.12	11.4	96.0	25.125	16.080
K_2HPO_4	3.85	23.72	13.7	95.0	24.968	15.813
$NH_4H_2PO_4$	3.80	25.84	15.5	71.0	36.394	17.227

7- Effect of H_3PO_4 concentrations:

As shown in Table (13), the increasing of the concentration of phosphoric acid resulted in increasing citric acid production, but increasing the phosphoric acid more than 0.3 ml resulted in a marked decrease in citric acid production. **Kapoor *et al.*, (1987)** reported that the concentration of phosphorus was low than regnired for optimal growth. The citric acid production in control was low in comparison to phosphoric acid supplemented medium. The highest value of citric acid concentration (32.88 g/L) was obtained with 0.30 ml/L phosphoric acid. Also at this concentration of phosphoric acid, biomass was 21.3 g/L, consumed total sugars concentration was 71.5 g/L, conversion coefficient was 45.986% and citric acid yield was 21.920%. Similar result was achieved by **Kapoor *et al.*, (1987)**.

Table (13): Effect of H_3PO_4 concentrations on citric acid production by *A. niger* CA2.

H_3PO_4 ml/L	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	4.15	23.54	15.7	89.0	26.449	15.693
0.10	3.80	25.73	14.8	86.0	29.919	17.153
0.20	3.75	27.08	20.7	81.0	33.432	18.053
0.30	3.45	32.88	21.3	71.5	45.986	21.920
0.40	3.75	31.83	20.4	76.5	41.608	21.220
0.50	3.85	26.75	17.8	78.5	34.076	17.833
0.60	3.80	24.51	15.7	81.0	30.259	16.340
0.70	3.90	20.51	14.4	86.5	23.849	13.673

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