SOME FACTORS AFFECTING CITRIC ACID PRODUCTION FROM SUGAR CANE MOLASSES BY *ASPERGILLUS NIGER*

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ABSTRACT

The production of citric acid from cane molasses by *Aspergillus niger* in submerged culture was investigated. Different factors were affected on the citric acid production and the results revealed that: The highest value of citric acid concentration 29.84 g/l was obtained with 1.5 g/l $K_4Fe(CN)_6$. Maximum productivity of citric acid was obtained with the treatment of cane molasses with 10 g/l ammonium oxalate. At 0.20 g/l $MgSO_4.7H_2O$, citric acid reached its maximum being 33.2 g/L. 5×10^6 spores/100 ml was the best inoculum volume and citric acid concentration was 32.88.

Certain metal ions are known to be inhibitory and some of it have stimulatory effect of citric acid production. Zn^{++} was have an inhibitory effect but Ca^{++} was have the stimulatory effect and citric acid production was 18.44, 32.51 g/l respectively. In the absence of ethanol, citric acid reached its maximum being 33.61 g/l.

The air supply resulted in improved citric acid concentration being 51.1 g/l after 8 days. Citric acid production with immobilized spores on sodium alginate was 35.71 g/l after 6 days and the maximum of citric acid was at pH 5.5.

Key Words: citric acid production, *Aspergillus niger*, sugar cane molasses, trace elements, inoculum volume, ethanol addition, fermentor, and immobilization

INTRODUCTION

Citric acid is used for the food and farmaceutical industries. In the last years, a considerable interest has been shown in using agricultural products and their wastes such as sugar cane molasses and sugar beet molasses for citric acid production by *A. niger*. **(Mashhoor *et al*., 1987; Gary and Sharma, 1991; Mellowes *et al*., 1991; Roukas and Alichanidis, 1991; El-Abyad et al., 1992; Hamissa *et al*., 1992; Esuoso, 1994; Mayilvahanan *et al*., 1996; Jianlong, 1998; Parvez *et al*., 1998; Pazouki *et al* ., 2000 and El-Kady, 2003)**. There are many factors affecting on citric aicd production, including $K_4Fe(CN)_6$, ammonium oxalate, $MgSO_4.7H_2O$, inoculum volume, metal ions , ethanol and immobilization. **(El-Kady, 2003)**

It was known that suger cane molasses has high amount of heavy metals **(Rasmy, 1999)**. Various techniques have been used to remove metallic inhibitory substances from the substrate. Pretreatment of molasses is carried out in order to eliminate the heavy metals. Defferent concentrations of $K_4Fe(CN)_6$ are used because of its role to precipitate the undesirable trace elements and the direct effect on the inhibition of the isocitric dehydrogenase **(Kapoor *et al*., 1987)**. Potassium ferrocyanide was stimulated the activity of some citric acid condensing enzyme by suppressing the poisonous effect of some ions such as iron, zinc, copper, magnesium, calcium, potassium, and sodium **(Chanda *et al*., 1990)**. The presence of the Fe and Mn in high concentrations is considered to be inhibitory to citric acid fermentation. Dilution of molasses to suitable sugar concentration and addition of $K_4Fe(CN)_6$ help in diluting the undesirable high concentration of trace elements **(Mashhoor *et al*., 1987; Roukas and Kotzekidou, 1997 and El-Kady, 2003)**.

It was reported that cane molasses has high concentration of calcium, therefore, ammonium oxalate was used to transform calcium to the unavailable form **(Mashhoor *et al*., 1987; Rasmy, 1999 and El-Kady, 2003)**

Magnesium is essential for the action of a variety of enzymes in the microbial cell and is required for both growth and citric acid production **(Prescott and Dunn, 1959)**. The incorporation of magnesium sulfate in the culture medium support citric acid production. Not only $(SO4)^{-2}$ ion is necessary for citric acid formation but also Mg^{+2} ion. The joint effect of both ions are necessary for citric acid formation **(Kristiansen and sicnclair, 1979)**.

The effect of inoculum size on citric acid production is determined by varying the number of spores added as inoculum to the culture medium.The optimum inoculum size may vary depending on the substrate and experimental conditions used **(Maddox and Brooks, 1995)**.

The influence of metal ions on citric acid production can be explained by the following obsevations. Fe^{+2} and Zn^{+2} have a critical role in citric acid prodiction, it generally agreed that the concentration should be very low. A high concentration of these metals allows vegetative growth at the cost of acid extraction **(Prescott and Dunn, 1959)**. When the Krebs cycle is operating, citrate is formed from condensation of acety-CoA and oxaloacetate. In high yielding citric acid fermentation, oxaloacetate must be established. Two CO_2 fixation reactions in citric acid producing strains of *A. niger.* One originated with pyruvate and the other with phosphoenolpyruvate, both leading oxaloacetate. The latter reaction is inhibited by zinc and copper **(Kristiansen and Sinclair, 1979)**. Certain matal ions are known to be inhibitory for citric acid porduction by *A. niger* in submerged fermentation, even at concentrations as low as 1 mg/L. In contrast, other reports showed that, in this situation, these metal ions are much less inhibitory and may even have a stimulatory effect on citrate production **(Maddox and Brooks, 1995)**.The presence of $CuSO_4.5H_2O$ concentration higher than 100 mg/L affected acid production which remarkably decreased. Some authers believed that Cu^{++} may stimulate citric acid production by inhibiting aconitase (EC 4.2.1.3); others reject this possibility, finding no alterations in the citrate-ios-citrate ratio when adding Cu^{++} under the conditons of citric acid production. A possible form of inhibition would be competing with Fe^{++}, which is a structureal component of the aconitase molecule **(Benuzzi and Segovia, 1995)**. Tricarboxylic acid cycle enzyme are located in mitochondria. carnitine acetytransferase (CAT) was located both in the mitochondria and in the cytosol. (CAT) can be considered as an enzyme necessary for transport of acetyl groups through mitochondrial membrane in both directions. (CAT) catalyzes the reversible transfer of short chain acetyl groups between CoA and carnitine. Acetyl-CoA necessary for the synthyesis of citrate in mitochondria. Inhibition of (CAT) with Cu^{+2} could contribute to a greater citric acid biosynthesis **(Jernejc and Legisa, 1995)**. One of the key conditions for the occurrence of citric acid accumulation is a deficiency of manganese ions in the nutrient medium. *A niger* grown on manganese deficient medium exhibited an elevated pool of intracellular ammonium ions. The increase in intracellular ammonium ions may be the factor responsible for antagonization of the feedback inhibition of phosphofructokinase activity by citrate. It has been suggested that it is necessary to provide a mechanism by which the feedback inhibition of citrate biosynthesis by citrate at the phosphofructokinase step could be over-come in order to achieve an over production of citric acid **(Kim *et al.,* 1995)**.

Several researches have attempted to explain the biochemical role of alcohol on citric acid production by *A. niger*. **Rugsaseel *et al*. (1995a), Roukas and Kotzekidou (1997), Saha *et al*. (1999),** and **Pintado *et al*. (1997). Rugsaseel *et al*. (1995a)** found that the addition of methanol to the production medium remarkably depressed celular protein synthesis without inhibiting nitrogen uptake, thus causing an increase of amino acids, peptides and low-molecular-mass protein pooled in the mycelium especially at the early stage of cultivation. Also, it changed the activity of some enzymes in or related to TCA cycle, rendering them suitable for citric acid accumulation. The stimulation effect of methanol can be attributed to the inhibition of spore formation and it has an effect on the cell permeability level; it allows citrate to be excreted from the cell. The cell then responde by increasing its citrate production via repression of 2-oxoglutarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite **(Roukas and Kotzekidou, 1997; Rugsaseel *et al*. 1995a and El-Kady, 2003)**reported

that, the mycelial growth was inhibited by the increase of methanol concentration. In contrast, **Pintado *et al.* (1997)** reported that methanol seems to increase the biomass surface area resulted a higher number of pellets and thereby it promotes increased mass and substrate/product transfers.

Nutrient levels and environmental conditions are important factors that regulate the citric acid production. Another factor of critial importance is the oxygen supply to the culture because of the microbial production of citric acid is a high aerobic process **(Drysdale and McKay, 1995 and Kim *et al.*, 1995 and El-Kady, 2003)**.

Citric acid has been produced by conventional submerged culture in which the biomass is suspended in the medium. Therefore, its separation from the medium and the bimass is difficult. The fermentation method using the immobilized biomass, on the other hand, provides ease of separation for the product, and thus continuous production of citric acid can be readily achieved. By using immobilized cells, the process can be controlled more easily than with a batch system of free cells. In addition, immobilized cells are more suitable than free mass. Recenty, various investigations concerning citric acid production with immobilized *A. niger* **(Bayraktar and Mahmetoglu, 2000 and El-Kady, 2003)**.

MATERIALS AND METHODS

Microorganisms:

A local fungal strains, namely *Aspergillus niger* CA2 was used in the present work were obtained from Microbiol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt

Maintainance of stock cultures:

The original culture was maintained on Potato-Dextrose Agar (PDA) slants at 5°C and subcultured monthly.

Media:

Potato dextrose agar:

This was supplied as a dry powder preparation from Merck Co. Peagents, Diagnostica, Chemical, D-6100 Darmstadt, Federal Republic of Germany.

Culture media:

One litre of clear supernatant diluted molasses; Urea, 1.2 g/L; $MgSO_4.7H_2O$, 0.4 g/L; H_3PO_4, 0.4 ml/L and pH was 7.0.

For preparing sugare cane molasses for addition to the above medium, cane molasses 273 g/L was diluted with distilled water to give a total sugars concentration 15%.

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)**.

Culture conditions:

Cultivation was made in 250 mL Erlenmeyer flasks, each containing 100 mL of sterile medium. Inoculum containing 5×10^6 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 was dried. Values of pH were determined in the culture filtrate using a pH meter, model CG 710. The cultures filtrate were centrifuged, and the clear supernatants were used for citric acid and residual sugars determinations.

Determinations:

Determination of citric acid using the reference titration method:

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

Conversion coefficient and yield of citric acid:

Conversion coefficient and yield of citric acid were calculated according to **(El-Sawy, 1986)**.

Determination of total carbohydrate:

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

Biomass production:

Mycelial dry weight was determined by drying the filtered cake or pellets at 70°C. until constant weight was attained (after about 48 hrs.) **(Mashhoor *et al*., 1987)**.

Microorganism and immobilization:

The spore suspension of *A. niger* CA 2 was prepared to gave 5.0×10^6 /mL. 50 mL of 3% of sodium alginate was prepared and mixed to this spore suspension with agitation until the medium become homogenous then passed through narrow tube to drop into 0.05M $CaCl_2$ solution at pH 6.5 and 35°C to prepare the beads with diameter of approximately 3mm. The beads was left 1h at 20°C, washed with distilled water and stored in 0.05M $CaCl_2$ solution at 4°C. The wieght of the beads was 100g. The immobilized spores were washed with distilled sterile water, and 4g of spherical immobilized spores were suspended in a 500ml Erlenmeyer flask containing 100mL medium **(Bayraktar and Mehmetoglu, 2000)**.

The fermentor experiment:

The fermentor used in this experiment was a MULTIGEN (new Brunswick sciemtific Co. INC. Made in New Jersey U.S.A.) with a working volume of 1L. The internal diameter of the culture vessel was 12 cm and the height was 23 cm. (2.6 L.). The agitation system (stirrer) speed was 200 rpm. The process temperature was maintained at 30°C and the air flow rate was 1vvm (volume of air / volume of medium. min). During the reactor operation, a small portion (10 ml) of the fermentation broth was sampled. The sample was filtered through a filter paper. PH, citric acid and residual total sugars were determined **(McIntyre and McNeil, 1997)**.

RESULTS AND DISCUSSION

1- Some factors affecting of citric acid prodoction.

1- 1- Effect of $K_4Fe(CN)_6$ concentrations on citric acid production:

The results on the effect of $K_4Fe(CN)_6$ on citric acid production are presented in Table (1). The citric acid concentration was increased with the increasing of $K_4Fe(CN)_6$ from 0.30 to 1.50 g/L. The citric acid production from untreated molasses was low in comparison to $K_4Fe(CN)_6$ treated medium. The highest value of citric acid concentration 29.84 g/L was obtained with 1.50 g/l $K_4Fe(CN)_6$. The obtained results are in line with **Kapoor *et al*., 1987; Chanda *et al*., 1990; Mashhoor *et al*., 1987and Roukas and Kotzekidou, 1997**.

Also at 1.50 g/l $K_4Fe(CN)_6$ biomass was 25.5 g/L, consumed total sugars concentration was 83.0 g/L, conversion coefficient was 35.952% and citric acid yield was 19.893%.

potasium ferrocyanide $K_4Fe(CN)_6$, 1.2 g/L was added to the hot diluted molasses to precipitate heavy metals, the clear supernatant was used for citric acid production.

1- 2- Effect of decalcification of molasses with ammonium oxalate on citric acid production.

The results on the effect of ammonium oxalate on citric acid production are presented in Table (2).The citric acid concentration was increased with the increasing of ammonium oxalate then declined. The citric acid production from untreated molasses was low in comparison to ammonium oxalate treated meduim. The highest value of citric acid concentration 29.88 g/L was obtained with 10.0 g/L ammonium oxalate.

Table (1): Effect of $K_4Fe(CN)_6$ concentrations on citric acid production by *A. niger* CA2.

$K_4Fe(CN)_6$ g/L	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	4.75	03.36	18.2	39.0	8.615	2.240
0.30	4.65	04.96	15.8	25.0	19.840	3.307
0.60	4.60	05.12	14.0	30.0	17.067	3.413
0.90	4.15	14.00	16.8	41.0	34.146	9.333
1.20	3.90	29.13	24.8	85.5	34.070	19.420
1.50	3.75	29.84	25.5	83.0	35.952	19.893
2.00	3.80	26.00	30.3	74.0	35.135	17.333

Also at 10.0 g/L ammonium oxalate, biomass was 25.0 g/L, consumed total sugars concentration was 82.75 g/L, conversion coefficient was 36.109% and citric acid yield was 19.920%. The obtained results are in line with **Mashhoor *et al.*, 1987**.

potasium ferrocyanide $K_4Fe(CN)_6$, 1.2 g/L was added to the hot diluted molasses to precipitate heavy metals; Ammonium oxalate 10 g/L was added also to the diluted molasses and left overnight in the refrigerator at 5°C for complete precipitation of calcium oxalate, the clear supernatant was used for citric acid production.

Table (2): Effect of ammonium oxalate concentrations on citric acid production by *A. niger* CA2.

Ammonium oxalate g/L	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	3.95	19.50	27.5	66.00	29.545	13.000
5.00	3.90	21.84	29.0	62.00	35.226	14.560
10.00	3.75	29.88	25.0	82.75	36.109	19.920
15.00	3.95	13.28	16.1	77.00	17.247	8.853
20.00	4.15	12.64	11.9	55.50	22.775	8.427
25.00	4.20	09.76	11.4	60.50	16.132	6.507

1- 3- Effect of $MgSO_4.7H_2O$ concentrations on citric acid production:

The present experiment was conducted to investigate the effect of incorporation different concentrations of magnesium sulfate on the productivity of citric acid. Magnesium sulfate was added in fourteen different concentrations ranging from 0.10 to 0.7 g/L. The results on the effect of magnesium sulfate on citric acid production are presented in Table (3). The citric acid concentration was increased with increasing the amount of magnesium sulfate then declined. The citric acid production from control was low in comparison to magnesium sulfate supplemented medium.

The amount of the citric acid increased with increasing magnesium sulfate concentration from 0.00 to 0.20 g/L, the variation of citric acid was slightly change under magnesium sulfate concentration range from 0.25 to 0.40 g/L, then the amount of the citric acid declined. These results are in harmony with those of **El-Sawy *et al.* (1986) and Sakurai *et al.* (1999)**.

The highest value of citric acid concentration (33.20 g/L) was obtained with 0.20 g/L magnesium sulfate. Also at 0.20 g/L magnesium sulfate biomass was 19.4 g/L, consumed total sugars concentration was 73.5 g/L, conversion coefficient was 45.170% and citric acid yield was 22.133%. Therefore, this concentration of magnesium sulfate (0.20 g/L) which proved to be the optimum, was used in the following experiments.

1- 4- Effect of the inoculum volume (size) on citric acid production:

This experiment was conducted to study the effect of different amounts of inoculum volume on the production of citric acid. Inoculum volume was added in eight different amounts ranging from 1×10^6 to 20×10^6 spores/ 100 ml. The results on the effect of inoculum size on citric acid production are presented in Table (4). The results demonstrate a marked

effect on citric acid concentration in the biganning of fermentation time then declined, perhaps reflecting the rate of total biomass production. Similar results were observed with **Maddox and Brooks (1995)**.

Table (3): Effect of MgSO$_4$.7H$_2$O concentrations on citric acid production by *A. niger* CA2.

MgSO$_4$.7H$_2$O g/L	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	3.75	28.80	14.8	62.5	46.080	19.200
0.01	3.55	29.57	16.8	64.0	46.203	19.713
0.02	3.50	29.82	15.9	66.0	45.182	19.880
0.03	3.50	29.92	15.9	64.5	46.388	19.947
0.05	3.55	29.60	18.3	69.0	42.899	19.733
0.10	3.60	29.31	17.6	71.0	41.282	19.540
0.15	3.45	32.88	18.8	75.5	43.550	21.920
0.20	3.35	33.20	19.4	73.5	45.170	22.133
0.25	3.40	32.29	18.8	76.5	42.209	21.527
0.30	3.55	34.2	18.8	76.0	42.658	21.613
0.35	3.50	32.89	18.9	75.0	43.853	21.927
0.40	3.50	32.4	19.2	71.5	45.091	21.493
0.50	3.60	31.12	22.1	74.5	41.772	20.747
0.60	3.60	36.4	23.9	74.0	41.405	20.427
0.70	3.80	27.20	21.6	76.5	35.556	18.133

The amount of the citric acid increased with increasing inoculum volume amount from 1x10^6 to 5x10^6 spores/ 100 ml, then the amount of the citric acid declined.

The highest value of citric acid concentration 32.88 g/L was obtained with 5x10^6 spores/ 100 ml.

At 5x10^6 spores/ 100 ml citric acid reached its maximum being 32.88 g/L, dried biomass was 21.1 g/L, consumed total sugars concentration was 74.5 g/L, conversion coefficient was 44.134% and citric acid yield was 21.920%. Therefore, this amount of Inoculum volume 5x10^6 Spores/ 100 ml which proved to be the optimum, was used in the following experiments.

Table (4): Effect of the inoculum volume (size) on citric acid production by *A. niger* CA2.

Spores/100 ml	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
1x10^6	4.50	12.80	09.6	50.0	25.600	8.533
3x10^6	4.30	17.80	10.5	67.5	26.370	11.867
5x10^6	3.40	32.88	21.1	74.5	44.134	21.920
7x10^6	3.80	27.28	12.5	75.0	36.373	18.187
9x10^6	4.10	25.68	12.9	73.5	34.939	17.120
11x10^6	4.20	20.08	12.5	71.0	28.282	13.387
13x10^6	4.50	12.32	10.9	61.0	20.197	8.213
20x10^6	4.80	09.28	12.2	38.5	24.104	6.187

1- 5- Effect of the trace elements (metal ions) on citric acid production:

Ten different trace elements were used to investigate their effect on citric acid production. Tested trace elements were added separately to basal medium to give trace elements concentration of (100 μg/L).

The results on the effect of trace elements on citric acid production are presented in Table (5).The results revaled that there were slightly inhibitory or stimulatory effects when these metal ions were added, the results agree with **Maddox and Brooks (1995)** who studied the effect of Fe^{+2}, Cu^{+2}, Zn^{+2} and Mn^{+2}, either alone or in combination on citric acid production.

In this experiments, highest amount of citric acid was obtained with Ca^{+2} being 32.51 g/L, dried biomass was 26.2 g/L, consumed total sugars concentration was 80.5 g/L, conversion coefficient was 40.385% an d citric acid yield was 21.673%.The other tested sources gave lower citric acid concentrations. Therefore, Ca^{+2} was the best ion for citric acid production. Simelar results were obtained by **Kumalaningsih, (1994), Pera and Callieri, (1997), Jianlong, (1998) and Lesniak et al., (2002)**.

Table (5): Effect of trace elements (metal ions) on citric acid production by A. niger CA2.

Trace elements (100µg/L)	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
Without	3.35	33.10	20.7	73.0	45.342	22.067
Fe⁺⁺	3.50	31.60	14.7	73.5	42.667	20.907
Cu⁺⁺	3.55	30.94	19.6	77.0	40.172	20.627
Zn⁺⁺	4.10	18.44	31.1	92.5	19.935	12.293
Mn⁺⁺	3.55	30.47	22.8	94.0	32.415	20.313
Ca⁺⁺	3.45	32.51	26.2	80.5	40.385	21.673
Ba⁺⁺	3.40	31.46	23.7	64.5	48.775	20.983
K⁺	3.55	30.91	21.2	72.0	42.931	20.607
Mo⁺⁺	3.65	28.67	18.9	74.0	38.743	19.113
Co⁺⁺	3.75	26.14	16.8	60.5	43.207	17.427
Ni⁺⁺	3.95	20.95	20.7	77.0	40.195	20.633

1- 6- Effect of $CaCl_2.2H_2O$ concentrations on citric acid production:

This experiment was conducted to study the effect of different concentrations of calcium chloride on the production of citric acid. Calcium chloride was added in five different concentrations ranging from 20 to 100 µg/L.

The results on the effect of calcium chloride on citric acid production are presented in Table (6).The citric acid concentration did not increased with the increasing of calcium chloride. The citric acid production from control was higher in comparison to calcium chloride supplemented medium. The highest yield of citric acid concentration 33.05 g/L was obtained in the absence of calcium chloride.

In the absence of calcium chloride, citric acid reached its maximum being 33.05 g/L, dried biomass was 20.4 g/L, consumed total sugars concentration was 73.5 g/L, conversion coefficient was 44.960% and citric acid yield was 22.033%.

Papagianni et al. (1999a and b) and **Bayraktar and Mehmetogu (2000)** were used $CaCl_2$ in the fermentatiom media to preduce citric acid.

Calcium chloride did not prove to be increasing for citric acid, this resules disagree with **Pera and Callieri (1997)** who reported that the addition of 0.5 g/L $CaCl_2$ to the fermentation medium increased the production of citric acid. An addition of Ca^{+2} induced a pelleted form of growth, highly branched hyphae and numerous bulbous cells. Bulbous cells growing in the presence of Ca^{+2} exhibited cell walls composed of laminated layers, and featured vesicles associated with the wall and/or the cell membrane, containing numerous inclusions. The cytotoxic effect of high concentrations of citric acid in the medium as well as an increase of the activity of N-acetyl-beta-D-glucosaminidase, a lytic enzyme, might be involved in these morphological changes.

1- 7- Effect of ethanol addition on citric acid production:

This experiment was conducted to study the effect of different concentrations of ethanol on the production of citric acid. Ethanol was added in seven different concentrations ranging from 1 to 7 ml/lL. The results on the effect of ethanol on citric acid production are presented in Table (7). The citric

acid production from control was higher in comparison to ethanol supplemented medium. These results agree with **Rugsaseel *et al.* (1995a)** who studied the influence of methanol on the ability to accumulate citric acid, and they found that there were slightly reduced citric acid accumulation obsarved for the strains with methanol.

Table (6): Effect of CaCl$_2$.2H$_2$O concentrations on citric acid production by *A. niger* CA2.

CaCl$_2$.2H$_2$O (µg/L)	Final pH	Citric acid g/L	D.M.W g/L	C.S. g/L	C.C. %	Yield %
0.00	3.35	33.05	20.4	73.5	44.960	22.033
20.00	3.45	32.68	32.7	106.0	30.830	21.787
40.00	3.40	31.36	24.6	90.5	34.652	20.907
60.00	3.70	28.96	31.7	69.5	41.669	19.307
80.00	3.75	27.10	30.7	79.5	34.088	18.067
100.00	3.45	32.02	24.6	81.5	39.288	21.347

The highest value of citric acid concentration 33.61 g/L was obtained without ethanol.This disagree with **Saha *et al.* (1999)** who reported that the use of alcohol was promoted citric acid production. In the continuous fermentation, methanol and ethanol had similar effects in increasing the citric acid yield.

In the absence of ethanol, citric acid reached its maximum being 33.61 g/L, dried biomass was 21.5 g/L, consumed total sugars concentration was 72.5 g/L, conversion coefficient was 46.359% and citric acid yield was 22.407%.

However ethanol did not prove to be the optimum, therefore, it was not incorporated in culture medium employed for citric acid production in the following experiments.

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

Ethanol (v/v)%	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
0.00	3.40	33.61	21.5	72.5	46.359	22.407
1.00	4.05	19.71	37.7	54.0	36.500	13.140
2.00	4.00	20.10	29.3	67.0	30.000	13.400
3.00	4.25	27.18	16.7	76.0	22.605	11.453
4.00	3.65	28.03	16.3	78.5	35.707	18.687
5.00	4.35	16.16	14.7	71.0	22.761	10.773
6.00	4.30	18.14	12.5	68.0	26.676	12.093
7.00	4.30	18.50	11.3	79.5	23.270	12.333

2- The Fermentor Experiment :

This experiment was conducted to study effect of the air supply on citric acid producton using the fementor.

2-1- Effect of the time course on citric acid production by *A. niger* CA2 using the fermentor.

The results on the effect of using fementor on citric acid production are presented in Table (8).The concentration of citric acid increased with air feeding and the increase in fermentation time. The air supply resulted in improved citric acid concentration. The maximum citric acid concentration (51.10 g citric acid/L) was obtained after 8 days of fermentation and then decline, at this time citric acid yield was 34.067 and conversion coefficient was 51.357%. Similar results were obtianed from **Kristiansen and Sinclair (1979), Drysdale and McKay (1995)** and **Bayraktar and Mehmetoglu (2000)**.

The pH decreased during fermentation was due to the citric acid production during fermentation of sugars, the lowest value of pH (3.15) was

accompanied with the greatest concentration of citric acid and then remained constant. The dried biomass was 31.5 g/L,

The concentration of consumed total sugars decreased during the fermentation, (85.0g total sugars/L) was observed after 8 days of incubation

Table (8): Effect of the time course on citric acid production by *A. niger* CA2 using the fermentor.

Days	Final pH	Citric acid g/L	C.S. g/L	C.C. %	Yield %
1	4.60	06.62	22.0	30.091	4.413
2	3.60	17.44	34.5	50.551	11.627
3	3.45	28.29	57.5	49.200	18.860
4	3.40	37.22	62.5	59.552	24.813
5	3.35	41.44	79.5	52.126	27.627
6	3.30	45.31	87.0	52.080	30.207
7	3.20	49.50	91.5	54.098	33.000
8	3.15	51.10	99.5	51.357	34.067
9	3.15	50.53	108.0	46.780	33.687
10	3.15	50.67	109.0	46.486	33.780

3- Citric acid production using immobilized spores:

3-1- Effect of the time course on citric acid production using immobilized spores:

The results on the effect of time course on citric acid production using immobilized spores are presented in Table (9).The concentration of citric acid increased with the increase in fermentation time. The maximum citric acid concentration (35.71 g citric acid/L) was obtained after 6 days of fermentation and then decline, at this time citric acid yield was 23.807 and conversion coefficient was 35.001%.

This citric acid concentration was higher than that obtained using free fungal mycelium in similar conditions. Similar results was obtained from **Kahlon *et al*. (1992)** and **Khare *et al*., (1994)**.

The pH decreased during fermentation was due to the citric acid production during fermentation of sugars, the lowest value of pH (3.35) was accompanied with the greatest concentration of citric acid and then remained constant. The dried biomass was 19.8 g/L,

The concentration of consumed total sugars decreased during the fermentation, (102.0g total sugars/L medium) was observed after 6 days of incubation.

Table (9): Effect of the time course on citric acid production using immobilized spores.

Days	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
1	5.70	05.22	08.0	28.0	18.643	3.480
2	5.40	08.10	03.2	31.0	26.129	5.400
3	4.35	15.62	12.0	58.5	26.701	10.413
4	3.95	24.26	15.2	87.5	27.726	16.173
5	3.65	29.12	19.2	91.5	31.825	19.413
6	3.35	35.71	19.8	102.0	35.001	23.807
7	3.35	34.50	21.6	105.0	23.857	23.000
8	3.35	34.59	36.8	118.0	29.314	23.060
9	3.40	33.76	36.2	129.0	26.171	22.507
10	3.40	33.54	34.8	131.5	25.506	22.360

3-2- Effect of initial pH on citric acid production using immobilized spores:

The initial of the pH was an important factor that affect the citric acid production. The purpose of this experiment was to determine the optimum initial pH that would result in the highest citric acid concentration.

The results on the effect of initial pH on citric acid production by immobilized spores are presented in Table (10).The pH range was 4.0 - 8.0, the citric acid concentration increased with the increasing of the initial pH from 4.0 to 5.5 and then decreased in the range 6.0 - 8.0. The maximum amount of citric acid was at pH 5.5, at this time, the biomass dry weight was (24.6 g dried biomass/L), consumed total sugars concentration was (86.5g total sugars/L), conversion coefficient was 40.994% and citric acid yield was 23.640%.

Table (10): Effect of initial pH on citric acid production by _A. niger_ CA2 using immobilized spores.

Initial pH	Final pH	Citric acid g/L	D.M.W. g/L	C.S. g/L	C.C. %	Yield %
4.00	3.85	03.26	00.6	25.0	13.040	2.1730
4.50	4.65	07.68	02.2	39.5	19.440	5.120
5.00	3.25	25.25	42.8	62.5	40.400	16.833
5.50	3.45	35.46	24.6	86.5	40.994	23.640
6.0	3.40	34.50	33.0	91.5	35.122	23.000
6.50	3.80	29.09	23.0	104.0	27.971	19.393
7.00	4.05	17.41	30.6	80.0	21.763	11.607
7.50	4.15	15.68	26.0	65.5	23.939	10.453
8.00	4.65	05.76	12.3	29.5	19.525	3.840

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بعض العوامل المؤثرة على إنتاج حامض الستريك من مولاس القصــب بواســطة فطر الأسبرجيللس نيجر

فتحي إسماعيل على حوقه – محمود محمد عوض الله السواح – محمد منصور قاسم – شريف محمد لطفي القاضي

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تمت دراسة إنتاج حامض الستريك بواسطة فطر الأسبرجيللس نيجر بإستخدام المزرعة المغمورة ، حيث تشير الدراسة إلى أن عملية إنتاج حامض الستريك تتأثر بعوامل مختلفة من أمثلتها

١– تأثير $K_4Fe(CN)_6$ على عملية الإنتاج حيث وصل أقصى تركيز لحامض الستريك ٢٩،٨٤ جرام/لتــر وذلك عند إضافة $K_4Fe(CN)_6$ بمعدل ١،٥ جرام/لتر.

٢– وبإضافة أكسلات الأمونيوم بمعدل ١٠ جرام/لتر وصل حامض الستريك إلى أعلى قيمه له.

٣– كما وصل الإنتاج إلى ٣٣،٢ جرام/لتر عند إسـتخدام كبريتـات الماغنسـيوم المائيـة بتركيـز ٠،٢٠ جرام/لتر.

٤- وعند إستخدام ٥×١٠ ٦ جرثومة كلقاح وصل إنتاج حامض الستريك إلى ٣٢٫٨٨ جرام/لتر.

٥- وعند دراسة التأثيرات السامة والمنشطة لبعض المعادن على إنتاج حامض الستريك وجـد أن أشـدها سمية هو أيون الزنك بينما كان لأيون الكالسيوم تأثير منشط على إنتاج الحامض وكانت الكمية المنتجة هـي ١٨٫٤٤ و ٣٢٫٥١ جرام/لتر على الترتيب.

٦- وبدراسة تأثير إضافة الإيثانول على عملية الإنتاج وجد أن له تأثير مثبط وكانت التجربة الكنترول هـي أفضل المعاملات حيث وصل إنتاج الحامض إلى ٣٣٫٦١ جرام/لتر.

٧- وبدراسة تأثير الإمداد بالهواء بإستخدام جهاز مخمر وجد أن تركيز حامض الستريك وصل إلــى ٥١٫١ جرام/لتر بعد ٨ أيام.

٨- وعند مقارنة إستخدام الجراثيم الحرة والجراثيم المسكنة بإستخدام مادة ألجينات الصـوديوم علـى إنتـاج الحامض وجد أن الجراثيم المسكنة كانت أفضل حيث وصل إنتاج حامض الستريك إلى ٣٥٫٧١ جــرام/لتــر وذلك عند درجة حموضة ٥٫٥ في ٦ أيام فقط.