Citric acid is one of the most important organic acids in the foods and pharmaceutical industries, citric acid had extracted from natural resources specially citrus fruits, then citric acid was begin to produced with using Aspergillus niger. This study aim to use Aspergillus niger to produce citric acid by screening some strains which can produce citric acid and screening some media which used for citric acid production, then to reach the maximum citric acid production were studied some factors affected on citric acid production. We study the effect of immobilized the spores with calsium algenate, and use this immobiliezd spores as an inoculum to produce citric acid. The possibility of citric acid production was 51.1 g/L in fermentor by the selected stain No, 2, meduim which containing (g/L): sugar cane molasses 272, ammonium phosphate 2, phosophoric acid 0.3 ml, magnesium sulfate 0.2, pH 5.5, at 30°C, after eight days of fermentation.

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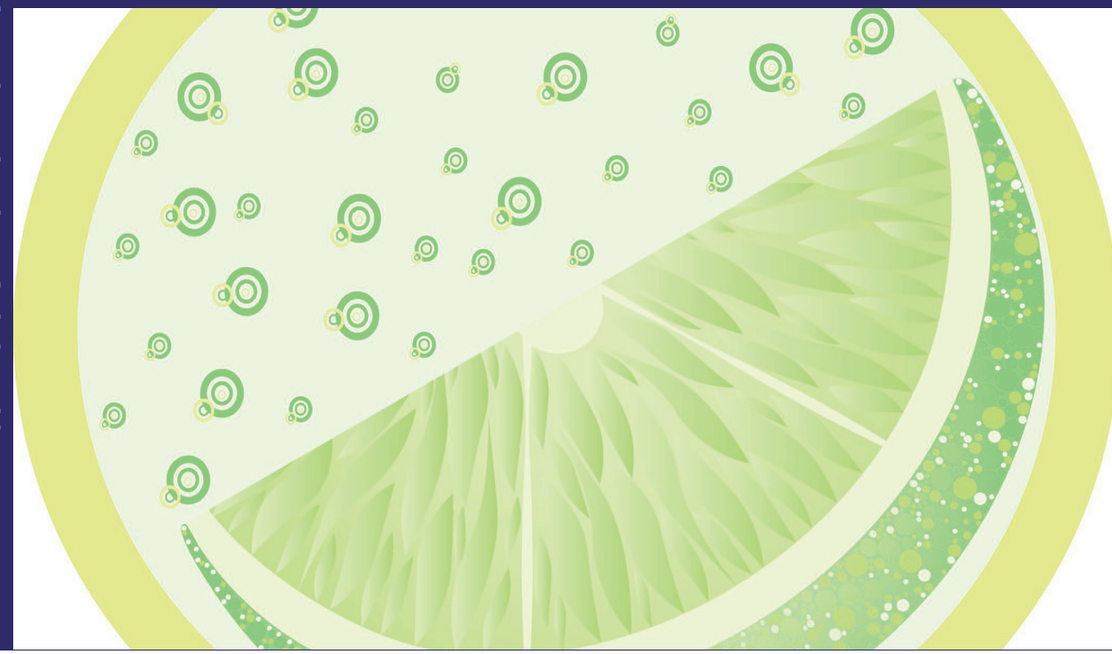
The fungal production of citric acid



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Studies on the microbial production of citric acid from cane molasses

By

SHERIF MOHAMED EL-KADI

B. Sc. Agric. (General section), Mansoura University, 1996

THESIS

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MASTER OF SCIENCE

IN

AGRICULTURAL MICROBIOLOGY

Supervised

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INTRODUCTION

Citric acid ($CH_2COOHCOHCOOHCH_2COOH$) is an important chemical used in medicines, 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 applied as condiment, preservative (as in beverage and sweets), antioxidant when acting with ascorbic acid (as in fruit freezing) and pH adjustor (as in preparation of sweets and fruit jelly). Sodium citrate, potassium citrate and calcium citrate are proved to be safe and dependable as chelating agents and food dyes. Several different species of molds have the ability to convert sugars to citric acid, but *Aspergillus niger* is most widely used for its commercial production. The development of citric acid industries in Egypt illustrates the values of applying new ideas in an old industry. From few years, the production of citric acid by mold fermentation was undertaken and the industry has grow until today the world annual production exceeds 350.000 tonnes. After production by this method became practicle. Egyption production of citric acid exceeds 5.000 tonnes.

Citric acid is usually produced in the monohydrate form ($C_6H_8O_7.H_2O$), crystals of which are colourless and odourless with a sour taste and readily efflorescent in dry air. It is more soluble in cold water (133 g/100 ml) than in hot water. The Molcular weight of citric acid monohydrate is 210.14, specific gravity 1.542, melting point loses water at 70-75 ˚C and boiling point decomposes at about 175˚C.

The development of processes for citric acid fermentation can be divided into 3 phases. In the first phase, citric acid production was confined to species of *Penicillium* and *Aspergillus* under surface culture. The second

phase, beginning in the 1930s, consisted of the development of submerged fermentation processes for citric acid production using *A. niger*. The third phase, which is of recent origin, involves the development of solid state culture, continuous culture and mutistage fermentation techniques for citric acid production. Thus, there are three principale methods for citric acid production.

Many sugars may serve as the substrate for the production of citric acid; however, molasses is generally used. The carbohydrate is incorporated into a medium containing an inorganic nitrogen compound as well as inorganic salts. The sterile medium is dispensed into shallow panes and inoculated with mold spores. This is an aerobic process; consequently a large surface area provides an adequate supply of oxygen. An alternative to this method of production is the submerged-culture technique, in which the inoculated medium is contained in large tanks through which a supply of sterile air is forced. The strain of mold employed, the composition of the medium, the degree of aeration, and the temperature of incubation all have an effect on the yield of citric acid.

The current study has been undertaken to examine the possibility of citric acid production by *A. niger* as well as to maximize the production level as follows:

1- Screening some strains which can produce citric acid.

2- Screening some media which used for citric acid production.

3- Studying some factors which affected on citric acid production .

2. REVIEW OF LITERATURE

2.1.Citric acid-producing microorganisms:

Thousands of microorganisms were investigated for their ability to produce citric acid. There are many microorganisms, including fungi, yeasts, and bacteria, that can produce citric acid by fermentation. However, only a few accumulate citric acid, when grown in solutions containing sugar, that quantitatively reach to commercial scale. *Aspergillus niger* is the most reliable producer for production of much larger quantities of citric acid, and to work out the conditions under which the citric acid yield could be maximized **(El-Kadi, 2003)**.

2.1.1.Fungi:

A. niger strians have proved to be an excellent provider of citric acid. It gives the best yield per time unit, strains of *A. niger* have usually given most successful production, both in the laboratory and on an industrial scales. Many of *A. niger* strains possess fairly uniform biochemical characteristics, are easily cultivated, and produce a negligible quantity of undesirable end products. *A. niger* can utilize many cheap raw materials to produce citric acid, thereby making the process economical **(Kapoor *et al*., 1987)**.

A. niger belong to class: Ascomycetes, subclass: Plectomycetes, order: Eurotiales, family: Eurotialaceae. *A. niger* colonies on Czapek agar is attaining a diameter of 25-30 mm in 10 days to two weeks, the color is black and the basal mycelium is white. The conidiophore is about 1.5-3.0 mm tall, composed of an unbranched stipe with a basal foot cell and a swollen apex (vesicle). The stipes wide are 15-20 µm and thick-walled. The conidial head color is black to black-brown composed of a vesicle,

metulae, phialides and conidia. The conidial diameter is 4-5 µm , radiate tending to splet into losse columns **(Hawksworth *et al*., 1995)**.

Many fungi can produce citric acid of these microorganisms are *Aspergillus niger, A. japonnicus, A. awamori, A. clavatus, A. fenicis, A. fonsecaeus, A. lunchensis, A. usami, A. wentii, A. flavus* **(El-Kadi, 2003 and Prescott and Dunn, 1959)**, *A. phoenicus, A. lanosus* **(Kapoor *et al*., 1987)**, *A. foetidus* **(Kristiansen and Sinclair, 1979)**, *Trichoderma viride* **(Prescott and Dunn, 1959)**, *Mucor pirifermis, Ustulina vulgaris, Ascochyta* sp., *Abisidia* sp., *Talaromyces* sp., *Acremonium* sp., *Eupenicillium* sp. **(Kapoor *et al*., 1987)**, *Penicillium janthinellum* var. *kuensanii* and *P. restrictum* var. *kuensanii,* **Prescott and Dunn, (1959)** *P. simplicissimum* **(Franz *et al*. 1993, Burgstaller *et al*., 1994 and Gallmetzer, *et al*., 1998)** and *Phanerochaete chrysosporium* **(Moreira *et al.,* 1996).**

2.1.2.Yeasts:

Many investigators focused their studies on the genus *Candida*. Other genera could produce citric acid such as *Hansenula*, *Debaryomyces, Saccharomycopsis, Pichia, Torulopsis, Torula, Trichosporon, Rhodotorula, Sporobolomyces, Endomyces, Nematospora, Saccharomyces* and *Zygosaccharomyces* **(Kapoor *et al*., 1987)**.

Candida subtropicalis, Pichia farinosa, Candida fibrae and *Torulopsis xylinum* produce citric acid when grown in solutions-containing various C12-16 alcohols, which can serve as carbon sources. *Candida oleophila* was used to produce citric acid by fermentation of waste glucose (32.6% glucose and 3% fructose). The fermentation yield, after 5 days, was 70.5% calcium citrate based on the initial carbon content **(Ishi *et al*., 1972)**. *Candida zelanoides* was used for citric acid production during cultivation

on acetic acid or calcium acetate as a sole carbon source **(Kapoor *et al*., 1987)**.

Many species of yeasts also accumulate citric acid in their growth media along with relatively large amounts of isocitric acid. Of these organisms are *Candida intermedia*, *Candida petrophilum*, *Candida hitachinica*, *Candida sucrosa*, *Torula* sp., *Trichosporon* sp., *Rhodotorula* sp., *Sporobolomyces* sp., *Endomyces* sp., *Nematospora* sp., *Saccharomyces* sp. and *Zygosaccharomyces* sp.**(Kapoor *et al*., 1987)**.

Candida tropicalis, Candida guilliermondii **(Miall and Parker, 1975)**, *Candida parapsilosis, Hansenula anomala* **(Oh *et al*., 1973)**, *Hansenula satyrans, Hansenula calirornica, Debaryomyces hesenii* and *Saccharomycopsis lipolytica* produce citric acid when grown in solutions containing glucose. The highest producer was *Candida lipolytica*, being 8.2 g/L **(El-Sawy *et al*., 1986)**.

A yeast strain of *Saccharomycopsis lipolytica* has been proven to be capable of producing citric acid either on a synthetic medium or on sugar cane molasses **(El-Sawy *et al*., 1986)**.

Citric acid yield of the yeast strain *Yarrowia lipolytica* 704 grown on glucose ranged from 0.38-0.77 g/g. The yield was dependent on both biomass and nitrogen concentration. Increasing the biomass concentration by 3% (w/v) increased fermentation productivities from 0.6 to 1.22 g citric acid /L/h **(Sokolov *et al*., 1995)**. Citric acid production from another strains, *i.e*., *Y. lipolytica* ATCC 20346, was reported by **(Antonucci *et al*., 2001)**, *Y. lipolytica* VKM Y-2373 **(Arzumanov *et al*., 2000)** and *Y. lipolytica* N1 **(Kamzolova *et al*., 1996, Finogenova *et al*., 1996 and Kamzolova *et al*., 1997)**.

Citric acid production by *Candida lipolytica* NCIM 3472 was studied in shake culture using glucose and molasses as carbon sources. Methanol addition (3% v/v) at 40 h of fermentation, a reduction in citric acid production by *C. lipolytica* was observed with addition of methanol. Maximum citric acid concentration of 8.4 kg/m^3 was achieved when using glucose without methanol. Methanol addition changes the nature of product formation of *C. lipolytica* **(Pazouki *et al*., 2000)**.

Crolla and Kennedy, (2001) reported that, hydrocarbons have the potential of producing high concentrations of citric acid by *Candida lipolytica.* They fuond that, the maximum concentration of citric acid produced was 9.8 g/L and the optimum levels of each parameter for citric acid production were, 10-12% volume for initial biomass concentration, 10-15% volume for n-paraffin concentration, 10 mg/L for ferric nitrate concentration, and 26-30°C for temperature.

2.1.3. Bacteria:

Several attempts have been carried out to isolate the bacterial species capable of synthesising citric acid. *Corynebacterium* sp. was used to produce citric acid from n-paraffins. Also, *Arthrobacter paraffinens* was used to produce citric acid from dodecane or a mixture of C12-C14 paraffins in addition to salts **(Kyowa Ferment. Ind., 1970)**. *Bacillus licheniformis, Bacillus subtilis* and *Brevibacterium flavum* have been found to possess the ability to produce citric acid from glucose, isocitric acid or from hydrocarbons **(Kapoor *et al*., 1987)**.

Alternate method for producing citric acid by growing *Klebsiella* sp. *Aerobacter* sp., *Pseudomonas* sp. and *Micrococcus* sp. in isocitric acid containing media was reported by several investigators **(Ohmori and Ikeno, 1973 and Takayama and Adachi, 1974)**.

2.2. Biochemical mechanisms of citric acid production:

In the tricarboxylic acid cycle, (citric acid cycle or the Krebs cycle) the carbon entering the cycle is generally converted into biomass, energy and CO_2. Only a small amount would accumulate as citric acid under balanced growth. The main mechanism of accumulation is the manipulation of medium constituents and growth conditions. Under balanced growth conditions, if the TCA cycle was blocked to accumulate citric acid this would in turn result in no regeneration of oxaloacetate, and therefore, the cycle would stop. It is possible that the addition of Cu^{2+} and Fe deficiency could result in the inhibition of the enzyme that converts citrate to isocitrate (aconitase). This would result in citrate accumulation, however, would ultimately result in the blocking of the TCA cycle since no oxaloacetate is recycled. Accumulation of citrate results in the inhibition of the enzyme isocitrate dehydrogenase which converts isocitrate to a ketoglutarate. This would result in further citrate accumulation, however, it cannot initiate citrate accumulation. The precondition for sufficient citric acid production is a medium deficient in one or more essential elements which can be realized by limiting the concentration of one of the nutrition elements, phosphorus, manganese, iron or zinc. In the submerged fermentation processes, aeration was extremely critical and citric acid production was stimulated by increased aeration **(El-Kadi, 2003).**

2.3. The main theories related to citric acid:

The inhibition of the enzyme α ketoglutarate dehydrogenase which converts α ketoglutarate to succinyl Co A. This enzyme is inhibited by NH_4^+ ions, oxaloacetate and NADH. If any of these are overproduced, the enzyme is inhibited, α ketoglutarate accumulates and inhibits isocitrate dehydrogenase resulting in citrate accumulates. If citrate accumulates, it inhibits the enzyme phosphofructokinase which converts fructose 6P to

fructose1,6 diP in glycolysis. This shuts down glycolysis and the TCA cycle and there is no further citrate accumulation. The presence of NH_4^+ and PO_4^{3-} ions can reverse this inhibition and allow further citrate accumulation. Mn^{2+} deficiencies result in the presence within the cell of acid proteases. These proteases degrade cellular proteins releasing free $NH4^{2+}$ ions which can play a role in theories 1 and 2 and result in a citrate accumulation. This causes a decrease in intracellular proteins and nucleic acids, thus more C to go into TCA and citrate accumulation, less into cellular biomass. The enzyme citrate synthase which converts acetyl CoA+ oxaloacetate into citrate is stimulated by the presence of oxaloacetate **(El-Kadi, 2003).**

2.4. Citric Acid Extraction:

The traditional method is commonly used for the time being. The principle of calciums salt method is: Mix lime and calcium carbonate - neutralization reaction - derive calcium citrate deposition-filteration and separation-double decomposition reaction between calcium citrate and sulphur-changes into citric acid and calcium sulphate deposition after separating - citric acid liquid is derived. The next steps are to remove metal action by ion exchange, then, by means of concentration crystallization, separation, and drying, the citirc acid crystallized product is dereived at last. Citric acid crystallized can divide into citric acid monohydrate and citric acid nonhydrate. It can be separated into three classes/grades: medicine class, food class and industry class **(El-Kadi, 2003).**

Broth harvested and mycelium removed by filtration (rotary vacumn filter). Oxalic acid side product is removed by adding $Ca(OH)_2$ to the filtrate at pH 5.8. This causes the oxalic acid to precipitate out as calcium oxalate. The calcium citrate remains in the solution and is precipitated out

as monocalcium citrate by pH adjustment to pH 7.2 and 70-90°C. H_2SO_4 is then added to dissolve the calcium citrate and calcium sulphate precipitates out and is removed by centrifugation, filtration and dried. Citric acid is then purified by initially passing through activated charcoal and ion exchange resins to remove impurities. It is then crystallised by continuous centrifugation and evaporation. >40°C crystallises out as citric acid anhydrous, <36.5°C crystallises out as citric acid monohydrate. Crystals dried at 50-60°C. Food/pharmaceuticals high purity, industrial lower purity **(El-Kadi, 2003)**.

2.5. Factors affecting citric acid production:

Citric acid production is strongly affected by the composition of the culture medium. Thus, there are many factors which influence production of citric acid.

2.5.1. Effect of raw materials, pH and temperature on citric acid production:

2.5.1.1. Sugar cane molasses:

Maximum citric acid was obtained in cane sugar molasses (15% sugar) by *A. niger* I3 being 5.10 g/L. Addition of 0.3% ammonium carbonate gave the highest citric acid being 5.71 g/L. Increasing the concentration of monopotassium phosphate to 0.01% resulted in increasing the yield and conversion coefficient. Addition of $MgSO_4.7H_2O$ (0.05 – 0.2%) had a deleterious effect on citric acid production in molasses medium. Addition of 2% methyl alcohol just before fermentation in molasses medium stimulated citric acid production. Delaying the alcohol addition resulted in a corresponding decrease in citric acid yield. The highest yield was obtained when 0.12% of ferrocyanide was used in the absence of methanol being 59%. Complete decalcification of molasses

increased citric acid yield and conversion coefficient **(Mashhoor *et al*., 1987)**.

Cane molasses was diluted to sugar concentration 12%, and ions of heavy metals (Zn, Cu, Fe and Mn) were precipitated by adding hexacyanoferrate (final concentration 1 g/L, 15 min at 90°C, pH 4.5); after filtration and before autoclaving (15 min, 121°C), the medium was supplemented with 0.25% NH_4NO_3, 0.1% KH_2PO_4 and 0.025% $MgSO_4.7H_2O$. The medium providing extra surface for fungal growth but also by absorbing certain impurities. When the same bagasse bed with pseudo-immobilized mycelium was reused in the next fermentation cycle, the fermentation time decreased from 10 to 5 days, while conversion still reached 80%; indeed, 4 cycles were performed before the fermentation efficiency began to decrease. Evidently the usual process comprises a growth phase followed by a biosynthesis phase. Elimination of the growth phase increased the daily yield of citric acid to 19.2 g/L for 20-25 days of semicontinuous operation, with much less downtime in cleaning and charging the fermentor **(Garg and Sharma, 1991)**.

A method for laboratory-scale production of citric acid is described. Frozen dried spores of *A. niger* (NRRL 1996) were used to prepare a spore suspension as inoculum for various media: synthetic (control) medium; untreated molasses (74% sugar content); charcoal-treated molasses; $K_4Fe(CN)_6$-treated molasses; H_3PO_4 and methanol-treated molasses; treated and untreated sugarcane juice. A laboratory shaker and an aquarium pump were compared for aeration of the media. IR spectra of the citric acid products are reproduced. Although molasses was found to require extensive pretreatment it was a relatively cheap byproduct not requiring special storage conditions. Cane juice, however, required storage at low

temperature and heat treatment prior to inoculation. It is suggested that cane could be juiced and the juice conveyed directly to the fermenter for heat treatment to overcome these problems (**Mellowes *et al*., 1991**).

Treatment of *A. niger* 20 with UV radiation for 12, 18 or 24 min resulted in the development of 31 isolates, some of which differed in morphology and sporulation ability. All 10 of the mutants isolated after exposure for 12 min gave lower yields of citric acid than the parent strain. Higher citric acid yields were obtained from 6 of the 11 mutants isolated after 18 min and from 3 of the 10 isolated after 24 min. The most productive members of these groups were 5-UV-18 (>26% higher yield) and 19-UV-24 (>32% higher). When 0.04% $K_4Fe(CN)_6$ was added to the molasses immediately after autoclaving at 95-100°C [often done in industry to scavenge undesirable metals], the only higher-yielding isolates were 5-UV-18 and 21-UV-24, and the increase was only 3%. (**Hamissa *et al*., 1992**).

Citric acid production by *A. niger* was studied using media based on cane molasses providing 15-18% w/v sugars, and the effects of nitrilotriacetic acid (NTA) and 8- hydroxyquinoline on mycelial dry weight and citric acid yield were investigated. These complexing agents showed an effect only when added during inoculation of the fungus, and the improvements obtained were very concentration-dependent; addition 1 or 3 days after inoculation had negligible effect. Final citric acid concentration was approximately equal to 9 g/dm3 in a control culture, approximately equal to 20 g/dm^3 with 200 p.p.m. NTA added during inoculation, and 41.6 g/dm^3 with 500 p.p.m. 8- hydroxyquinoline added during inoculation. It is therefore suggested that 8-hydroxyquinoline be added during industrial production of citric acid from molasses using *A. niger* (**Esuoso, 1994**).

Possible techniques for rapid production and higher output of citric acid from cane molasses were studied using *A. niger* 684 as producer organism by **(Mayilvahanan *et al*., 1996)**. Effect of pretreatment molasses at 10% total reducing sugar (TRS) with H_2SO_4, $K_4Fe(CN)_6$, $Ca_3(PO_4)_2$ (followed or not by HCl) or bentonite were compared. Contents of (TRS), NH_4NO_3 and phosphate were optimized and the long unproductive lag period normally associated with this type of fermentation was shortened. In the optimized process, a stage wise strategy was adopted, inoculum was produced at pH 6.5 on medium with 5% (TRS) supplied by molasses pretreated with H_2SO_4 and grown similarly until a suitable biomass had accumulated; during the exponential growth phase (TRS) was adjusted to 10% with concentrated molasses (16% TRS) pretreatment with $Ca_3(PO_4)_2$ and fermentation was performed at pH 3.0. The process gave a citric acid yield of 60.1 g/L in 165 h.

Citric acid production by *A. niger* NIAB280 and by the mutant strain RP7 in batch culture on cane molasses was studied. Strain RP7 produced 120 g/L, while the parental strain produced 80 g/L from 150g molasses/L and the fermentation period was decreased from 10 days for NIAB280 to 6-7 days for RP7. The mutant grew faster than its parent **(Parvez *et al*., 1998)**.

Citric acid production by *A. niger* NCIM 548 was studied in shake culture using glucose and molasses as carbon sources. Methanol addition (3% v/v) at 40 h of fermentation enhanced the production of citric acid by *A. niger*. Maximum citric acid concentration of 12 kg/m^3 was obtained using molasses in the presence of methanol. Product formation by *A. niger* is either nongrowth associated or partially growth associated depending on the substrate. **(Pazouki *et al*., 2000)**.

2.5.1.2. Sugar beet molasses:

Production of citric acid from beet molasses at a varying pH profile using cell recycle of *A. niger* was investigated. Best results in terms of citric acid concentration, yield, productivity and specific citric acid productivity were obtained with a substrate pH of 3.0 **(Roukas and Alichanidis, 1991)**.

The citric acid yield from Egyptian beet molasses was higher for *A. niger* strain 20 than for strains 10 and 599; optima were 30% v/v molasses (15% sugars), 11 days at 30°C and initial pH 8.35. Inclusion of 0.05% N as $(NH_4)_2SO_4$ increased the yield, but 0.1% N from several other N sources - or >0.1% as $(NH_4)_2SO_4$ - decreased it. No extra inorganic P was needed to supplement the 0.072% already present. Removal of suspended sludge by centrifugation increased the yield; addition of low-MW alcohols to the resulting molasses did not increase the yield, and ion- exchange treatment (particularly cation exchange) decreased it. Addition of 0.04% $K_4Fe(CN)_6$ to autoclaved medium at 95-100°C led to considerably higher yield than (optimum) addition of 0.6% before autoclaving at 121°C. The highest yield obtained was 53.4% on initial sugar content **(El-Abyad *et al*., 1992; El-Kadi, 2003; Hauka *et al*., 2005a and Hauka *et al*., 2005b**)

A medium based on untreated beet molasses providing 100 g total reducing sugar/L was used as to produce citric acid by *A. niger* W1-2. **(Jianlong, 1998)**.

2.5.1.3. Cassava waste:

The production of citric acid from cassava (*Manihot esculenta*) waste using solid state fermentation was evaluated by **(Kumalaningsih, 1994a)**. They found that, optimal pH and temperature during the fermentation process using *A. niger* were determined as pH 5.5 and 35°C. Pretreatment

of the waste with 6% CaO (6 hr) followed by dehydration inoculum was produced at 45°C for 5 hr had the greatest viability: spreading of this inoculum on trays of cassava solid waste resulted in a citric acid production of 8.79% (w/d.wt basis).

Kumalaningsih, (1994b) treated cassava solid waste with 0.8% CaO. They found that,the treatment reduce the bound cyanide content by 30%. The cyanide would otherwise have inhibited citric acid production through fermentation by *A. niger*. Optimal conditions for citric acid production from treated cassava solid waste were determined as pH 4.5 and a water content of 65 %(w/w): 12.85% after 5 d. at 35°C.

Vandenberghe *et al*., (2000) evaluate three different agro-industrial wastes, sugar cane bagasse, coffee husk and cassava bagasse for their efficiency in production of citric acid by a culture of *A. niger* by solid-state fermentation. They found that, cassava bagasse was the best supported fungal growth, giving the highest yield of citric acid among the tested substrates. The fungal strain had good adaptation to the cassava bagasse substrate and increased the protein content to 23 g/kg in the fermented matter. Citric acid production reached a maximum (88 g/kg dry matter) when fermentation was carried out with cassava bagasse having initial moisture of 62% at 26°C for 120h.

2.5.1.4. Cheese whey:

A medium containing deproteinized whey at pH 3.5, 10% salt and 4% methanol was used to produce citric acid production by strains of *A. niger* in shake culture by **(Khorshid *et al*., 1992)**. Citric acid produced was a 4-fold increase by *A. niger* CAIM167 than *A. niger* CAIM111. They showed that, using cheese whey as a substrate for citric acid production by

A. niger provided a possible means of beneficial purpose, as well as reducing COD and BOD.

A medium containing salt-whey obtained from the manufacture of Domiati cheese was used to produce citric acid by two stains of *A. niger* (CAIM111 and CAIM167) by **(El-Samragy *et al*., 1993a)** as a means of pollution control. They found that, the initial pH was 3.5 and 4% methanol was added. The COD of salt-whey reduced to 75.6 and BOD reduced to 77.8% of their original values. In shaking fermentation techniqueby **El-Samragy *et al*., (1993b)** found that *A. niger* CAIM111 and CAIM167 exhibited a high ability to convert lactose to citric acid, *i. e.* 10.6 and 8.2 g/L respectively, while *A. niger* CAIM132 produced only 5 g/L. **El-Samragy *et al*., (1996)** found that the maximum citric acid was (1.06 and 0.82 g/L) and the conversion coefficient was (5.58 and 7.45%) from the two strains respectively. PH was 3.5 and incubation period was 9 days and salt concentration (10% w/v).

2.5.1.5. Date syrup:

Roukas and Kotzekidou, (1997) produced citric acid from date syrup, and various pretreatments for removal of heavy metals were investigated, involving H_2SO_4, 1 or 2% tricalcium phosphate at pH 7, tricalcium phosphate + HCl to pH 3, potassium ferrocyanide, and EDTA. Of these, only neutral tricalcium phosphate increased the output of citric acid from subsequent fermentation. They found that, 2% addition increased the final citric acid concentration from 45 to 55 (±1.5) g/L; citric acid yield remained 50%, while sugars utilization increased from 60 to 73.3%. The optimum pH for citric acid production was 6.5. Addition of 4% (v/v) methanol to a solution of date syrup pretreated with 2% tricalcium

phosphate increased the output of citric acid from 55 to 90 g/L and the sugars utilization to approximately equal to 96.5%.

2.5.1.6. Carob pods:

The production of citric acid from carob pods extract by *A. niger* in surface fermentation was investigated. The amount of citric acid was 85.5 g/L. The initial sugar concentration in the fermentation medium was 200g/kg, pH 6.5 and the temperature was 30°C **(Roukas, 1998)**. On the other hand, in soled state fermentation **(Roukas, 1999)** found that the highest citric acid produced by *A. niger* from carob pods was (176±4g/kg dry pod), citric acid yield (55±2%), sugar utilization (64±2.5%) and biomass dry weight (30±0.7g/kg wet substrate) were obtained at a particle size to 0.5 mm., moisture level of 65%, pH of 6.5 and temperature of 30°C.

2.5.1.7. Fig fruit:

The production of citric acid from fig fruits by *A. niger* ATCC 10577 cultivated by solid state fermentation was investigated by **Roukas, (2000)**. The maximum amount of citric acid concentration 64 g/kg dry fig and citric acid yield 8% were obtained at a moisture level of 75%, initial pH 7.0, temperature 30°C and fermentation time 15 days. The addition of 6% (w/w) methanol into substrate increased the concentration of citric acid from 64 to 96 g/kg dry fig.

2.5.1.8. Glucose:

Citric acid production was performed by solid-state culture of *A. niger* CBS 733-88 on 10% glucose medium by **(Pallares *et al*., 1996a)**. Optimized submerged and solid-state cultures were compared. When solid state culture was used, the citric acid concentration reached its maximum of 21.24 g/L in 6 days and the citric acid productivity was twice that of submerged culture, which took 14 days.

Kirimura *et al*., (2000) were cultivated *A. niger* WU-2223L, a citric acid-producing strain, in a medium containing 120 g/L of glucose, and the effects of 2% (v/v) methanol was investigated. When *A. niger* WU-2223L was cultivated with methanol, both citric acid production and citric acid productivity, shown as the ratio of production per mycelial dry weight..

Pazouki *et al*., (2000) studied citric acid production by *A. niger* NCLM 458 and *Candida lipolytica* NCIM 3472 was studied in shake cultures using glucose and molasses as carbon source. The maximum citric acid concentration (12 kg/m^3) was obtained with *A. niger* in the presence of methanol, while maximum citric acid concentration (8.4 kg/m^3) was obtained with *Candida lipolytica* using glucose without methanol.

2.5.1.9. Inulin:

Inulin is a natural polyfructose consisting of β2-1 linked polyfructose chains with a terminal glucose unit. Inulin is commonly found as a reserve carbohydrate in tubers in plants such as chicory. Citric acid production by *A. niger* using inulin (14% w/v) as a carbon source at 30°C and pH 2.5 in surface fermentation was investigated by **Drysdale and McKay, (1995).** They found that, *A. niger* ATCC 9142 preduced 14 g/L citric acid after incubation as a surface culture in static flasks for 24 days. When air was passed over the surface of the culture it resulted in improved yield of citric acid of 29 g/L after 24 days.

2.5.1.10. Maize starch-hydrolysate:

Maize starch-hydrolysate was used as a substrate for citric acid production from *A. niger* strains by **Mourya and Jauhri, (2000)**. The culture conditions for citric acid production were optimized: concentration of starch-hydrolysate was 15%, (glucose equivalent); ammonium nitrate, 0.25%; KH_2PO_4, 0.15%; nicotinic acid, 0.0001% and initial pH of 2.0. The

citric acid was 490 g citric acid from kg of glucose consumed in 8 days of incubation at 30°C. The productivity of 341 mg/L/h corresponded to 49% substrate conversion to citric acid.

2.5.1.11. Mussel processing effluents:

Mussel processing effluents (generated from the molusc), the effluents is dumped in the coastal water, therby severely polluting the very medium that generates the resources. Its utilization for the recovery of proteins or as food seasoning. Recently, its use as substrate, with or without saccharification for various fermentation products such as citric acid. Pretreatment of mussel processing effluents for citric acid production by *A. niger* in submerged culture and initial levels of $K_4Fe(CN)_6$, methanol, phosphate, and carbon source were studied by **Pintado *et al*., (1997)**. The main effect of $K_4Fe(CN)_6$ is toxic while the presence of methanol seems to involve morphological changes that increase the superficial area of the mycelial pellets, therby promoting an unspecific metabolic activation. Therefore enhances the toxic effect of $K_4Fe(CN)_6$, but also favors citric acid accumulation. The net responser depends on the concentrations of the carbon source and phosphorus.

2.5.1.12. Okara:

Okara (soy-residue), a cellulosic byproduct of the soya milk and tofu (soy paneer) industry, was used for the production of citric acid by solid-state fermentation using the cellulolytic fungus *A. terreus* and citric acid producing *A. niger*. Okara supplemented with ammonium sulfate (0.1 % N) when fermented by *A. niger* with simultaneous saccharification using A. terreus at pH 8.3 and incubation temperature of 30°C resulted in production of 5.10 g citric acid/100 grams dry solids by the eleventh day (**Khare *et al*., 1995**).

2.5.1.13. Potato:

An external-loop airlift bioreactor was used for the production of citric acid from the mash of dried sweet potato with its dregs by *A. niger*. The effects of air flow rate and liquid volume on citric acid production were investigated. A comparison of citric acid fermentation was made between the external-loop airlift bioreactor and the mechanically stirred tank bioreactor. After a total fermentation time of 65 h, an average of 10.6 mg/ml citric acid concentration was obtained in the external-loop airlift bioreactor under an air flow rate of 1.3 vvm, liquid volume of 8.5 L as compared to 9.6 mg/ml citric acid concentration in the 10 L mechanically stirred tank bioreactor under optimised operation conditions: agitation rate 200 rpm, air flow rate 1.0 vvm and liquid volume 6.5 L.**(Yuguo *et al*.,1999)**.

White sugar, potato starch and both untreated and decationized dextrose syrups were used as substrates for submerged citric acid biosynthesis using a mutant of *A. niger*. The same yield of product (80%) was achieved with both syrups and the starch despite the substrates having different trace metal contents. On sugar, the mutant was more sensitive than the parent to Cd^{2+}, Mo^{2+}, and As^{3+}, with decreasing yields of citric acid at 10 mg of ions/L, but Fe^{2+}, Mn^{2+} and V^{2+} below 50 mg/L, and Cr^{3+}, Ni^{2+}, Cu^{2+} up to 100 mg/L ,did not significantly inhibit citric acid production **(Lesniak *et al*.,2002)**.

2.5.1.14. Soya whey:

Soya whey, a byproduct generated during the tofu-making process was supplemented with 10% sucrose and was used for citric acid production by *A. niger* cells immobilized in agarose beads in comparison with the free cells by **Khare *et al*., (1994)**, inoculated with 10% (w/v) free

or immobilized cells, and incubated at 30°C with agitation at 200-220 r.p.m. Maximum citric acid yields of 21 g/L with free cells and 27 g/L with immobilized (compared with an average yield of 22.5% for cane molasses) were recorded on the 10th day under repeated batch conditions.

2.5.1.15. Sugar syrup from the Palmyra palm:

Palmyra jaggery, sugar syrup from the palmyra palm (*Borassus flabelliformis*) [*B. flabellifer*] is a suitable substrate for increasing the yield of citric acid using *A. niger* MTCC 281 by submerged fermentation **Ambati and Ayyanna, (2001)**. They found that, higher yield was obtained after optimizing media components and conditions of fermentation. Maximum citric acid production was obtained at pH 5.35, 29.76°C, 5.7 days of fermentation with 221.66 g of substrate/L, 0.479 g of ammonium nitrate/L and 2.33 g of potassium ferrocyanide/L.

2.5.1.16. Sweet sorghum:

The production of citric acid from sweet sorghum juice by *A. niger* ITCC was studied by **Nain, (2000)**. The amount of citric acid produced by the strain was 2.28% (w/v) during growth on sorghum juice containing 5.5% (w/v) sugar.

2.5.1.17. Turnip whey:

Turnip whey and beet whey from production of leaf protein, which pose serious disposal problems, were tested by **Chanda *et al.*, (1990)** as possible substrates for citric acid production by *A. niger* NCIM 595, each being fortified with sucrose to give 10% total sugars in the medium. They stated that, citric acid outputs (% w/v) after 9 days' fermentation reached 2.75% from beet whey and 3.5% from turnip whey. In further tests, when turnip whey was supplemented with molasses to give 15% w/v

carbohydrates, + various concentrations of $K_4Fe(CN)_6$, the maximum citric acid output (5.0%) was obtained with 0.06% $K_4Fe(CN)_6$.

2.5.1.18. Waste water from a distillery using barley or tapioca:

Waste water from a distillery using barley or tapioca as the raw material for ethanol fermentation was treated to produce citric acid and to reduce the amount of waste water for further treatment by **Myung *et al*., (1992)**. An attempt was made to enhance the efficiency of process under various conditions using *A. niger* in a batch bioreactor. The concentration of citric acid were increased to 9.6 and 16.9 g/L in a batch bioreactor by *A. niger* ATCC 9142 using 50 g/L and 100 g/L of reducing sugar in tapioca derived waste water, respectively. The barley derived waste water with 50 g/litre reducing sugar gave 2.4 g/L citric acid using *A. niger* ATCC 9142. The concentration of citric acid and mycelial dry weight were shown to be 4.38, 50.8 and 9.6, 12.6 g/L in a batch bioreactor using *A. niger* KCTC 1231 and ATCC 9142 with 50 g/L reducing sugar in tapioca derived waste water. *A. niger* ATCC 9142 gave good citric acid production, while *A. niger* KCTC 1231 gave enhanced C.O.D. removal. The fermentation process of citric acid formation reduced the C.O.D. value of 70.8%, which reduced the organic loading rate for subsequent activated sludge treatment.

2.5.1.19. Yam bean:

Sarangbin and Watanapokasin, (1999) selected of mutant strains of *A. niger* in semi-solid culture for enhancing citric acid production from yam bean (Pachyrhizus erosus). Citric acid productivity by the selected mutant strains was tested on a modified starch-methyl red agar plate. The best mutant strain YW-112 was obtained and produced 106 g/L of citric acid, whereas the parental strain Yang no. 2 produced 58 g/L, from 140 g/L of soluble starch in semi-solid culture at 5 days of cultivation time.

2.5.1.20. Other raw materials:

Hang and Woodams, (1989) used apple pomace to produce citric acid by *A. niger* in solid-state culture and banana extract was utilized as carbon source to produce citric acid by **(Sassi *et al*., 1991).**

Garg and Hang, (1995) used carrot processing solid waste as carbohydrate source for production of citric acid in solid state fermentation using *A. niger*. Citric acid yield was 36 g/100g fermentable sugar consumed.

A parental strain of *A. niger* Yang no. 2 and mutant strains showing resistance to 2-deoxy-D-glucose (DG) were used for citric acid production by in a semi-solid culture containing 100 g cellobiose/L, using sugarcane bagasse as carrier by **Sarangbin *et al*., (1993)**. They found that, the strain C192 produced 49.6 g/L of citric acid, 1.6 times as much citric acid as Yang no. 2 produced, from 100 g cellobiose/L and showed enhanced β-glucosidase production.

Coffee husk was used as a substrate for citric acid production from *A. niger* CFRTI 30 by **(Shankaranand and Lonsane, 1994).** They found that, *A. niger* CFRTI 30 produced 1.3 g citric acid/10 g of dry coffee husk fermented in over 72 hr in a solid-state system moistened with 75 mM NaOH(aq). Citric acid production increased after the addition of Fe^{2+}, Cu^{2+}, and Zn^{2+} whereas addition of $(NH_4)_2SO_4$ or sucrose had no effect. Under standardized conditions the commercial potential was realized when citric acid production reached 1.5 g citric acid/10 g coffee husk

2.5.2 The effect of Oxygen supply on citric acid production:

The addition of H_2O_2 was used to stimulate citric acid production by improving the concentration of dissolved oxygen, and it had an effect on

the inhibition of aconitate hydratase- that enzyme starting citrate breakdown in the TCA cycle (**Röhr *et al*., 1983**).

It is known from nitrogen-limited batch cultures of *A. niger* that intracellular ammonium concentration is closely related to the specific oxygen uptake rate, especially during the idiophase of the culture. The responses of specific oxygen uptake rate to ammonium pulse feeding were examined at different intracellular ammonium concentrations during culture for 8 days at 30°C on synthetic media initially containing 10% sucrose and 0.1% NH_4NO_3 (both w/v) at pH 3.8 (not controlled). A quantitative relationship between the specific oxygen uptake rate and the intracellular ammonium content was obtained and applied to promote overproduction of citric acid. Maximum yield was obtained when the intracellular ammonium content was maintained at 3.0-4.5 mM/g cells throughout the feeding of supplementary ammonium (*e.g*. from 60 to 108 h); a higher value led to extra biomass production and lower product yield. Using 4 mM/g, the final citric acid concentration was 68 g/litre, or 76% on sucrose utilized (**Kim *et al*., 1995**).

Chemostat cultures of *A. niger* A60 were aerated with mixtures containing elevated CO_2 (2, 4, 7.5, 10 and 18% v/v), modelling possible conditions near the base of a bioreactor. The initial sucrose concentration in culture medium was 14% (w/v) during inoculum development and batch culture, and 5% in the medium fed during continuous fermentation (the feed being started after 72 h in batch mode) to citrate and gluconate. The effects of higher CO_2 content upon biomass, citrate, and gluconate steady-state values were quantified and the effects on key morphological indices (hyphal growth unit, mean main hyphal length, and mean branch length) were assessed using computerized image analysis techniques. These were

compared with steady-state values from a control gassed solely with air. Elevated CO_2 inhibited growth and organic acid formation and generally led to an increase in the value of the morphological parameters, but these effects were more modest than might have been expected from results of previous batch studies on filamentous fungi **(McIntyre and McNeil, 1997)**.

Obtaining high citric acid productivity in *A. niger* fermentation on beet molasses substrate, a certain redox potential profile with two maxima (260 and 280 mV) and two minima (180 and 80 mV) must be maintained. The most effective regulation of redox potential is by regulation of aeration and agitation. It has been shown that control of redox potential by aeration and agitation is a most successful method for scale-up from 10 L laboratory scale to the 100 and 1000 L pilot-plant scale, even in geometrically dissimilar stirred- tank reactors **(Berovic, 1999)**.

Saha *et al*., (1999) studied the used of methanol or ethanol in citric acid production with *A. niger* AJ 117173 strain. Continuous and repeated-batch fermentations were conducted with the addition of 2% ethanol (plus 100 g sucrose/litre in the medium). Continuous fermentation for 50 days gave a better citric acid yield (85%) and average productivity (3.8 g/L/day) than repeated-batch fermentation over 60 days (65% , 2.3 g/L/day).

In a laboratory study, high yield of citric acid production by *A. niger* on beet molasses substrate was contingent on redox potential levlels 260 and 280 mV (maxima) and 180 and 80 mV (minima) during the biosynthesis stages. Redox potential was regulated by chemical and physical methods. Chemical methods comprising addition of oxidants and reductants did not block microbial growth, but evidently inhibited citric acid biosynthesis. The physical method of regulation, by variation of

agitation and aeration, was the most effective and appropriate for producing high citric acid yield **(Berovic *et al*., 2000)**.

Due to the significant oxygen requirement during citric acid production and the relatively low solubility of oxygen in water, aeration is critical. The potential use of n-dodecane as an oxygen-vector for improvement of citric acid production by *A. niger* was studied. The volumetric fraction of oxygen-vector has a great influence on the volumetric oxygen transfer coefficient kLa. With the addition of an oxygen-vector to the fermentation medium with a final concentration of 5%, the kL a value reached a maximum value (130/h), which is twice that of the control experiment. The addition of 5% (v/v) n-dodecane enhanced citric acid accumulation, reduced residual sugar concentration and stimulated mycelial growth. Adding n- dodecane had no adverse effects on the cells of *A. niger*. The results of enzyme assays indicated that no significant differences were observed between the activity of citrate synthase of two kinds of mycelial cell-free extracts **(Jianlong, 2000)**.

2.5.3 Effect of the trace elements (metal ions) on citric acid production:

Kapoor *et al*., (1987) reported that, *A. niger* needs a vraiety of divalent trace elements such as Fe^{+2}, Cu^{+2}, Zn^{+2} and Mg^{+2}, etc., for growth and citric acid production. However, citric acid production is very sensitive to the concentration of these metals in the fermentation media. In fact, successful citric acid production depends to a great extent on the control of the concentration of trace elements. Other trace elements such as Mn^{+2}, Ba^{+2}, Al^{+3}, etc., have been reported to have an effect on fungal morfology and citric acid production, at concentrations that generally do not inhibit growth.

Jernejc *et al*., (1990) were grown *A. niger* strain A60 (NRRL 2270) in media based on 140 g sucrose/L, with the inclusion of (a) $CuSO_4.5H_2O$ at 40 mg/L to favour citric acid accumulation or (b) $MnSO_4.H_2O$ at 3.38 mg/L to hinder it. Studies on mycelial lipids, sterols and fatty acids after 1, 3 and 5 days are reported. Ergosterol was the only sterol identified in a mycelia; b mycelia contained at least 5 other sterols and a larger amount of ergosterol (indicating differences in membrane-bound enzymes), more oleic and stearic acids, more neutral lipid and phospholipid, and less glycolipid.

A. niger NRRL 595 was grown statically for 6 days at 30°C on synthetic media by **Abd El-Naby, (1997)**, pH 5.4 unamended or amended with different concentrations of Mn^{2+}, Fe^{2+} or Cu^{2+}. Citric acid yield was reduced by approximately 78, 86.6 and 81.7% in the presence of Mn^{2+}, Fe^{2+} and Cu^{2+}, respectively. Fungal growth was also reduced by the inclusion of the metals by approximately 26, 35 and 59.7% for Mn^{2+}, Fe^{2+} and Cu^{2+}, respectively. The fungus was grown on solid media containing these metal ions to isolate tolerant strains. After 30 days the tolerant fungi were able to grow in liquid culture with high concentrations of the metal ions. Citric acid yield was also increased in tolerant cultures grown on media with the metal ions added. Tolerant and non- tolerant strains of *A. niger* were grown on cane molasses and the production of citric acid was studied. Fungi adapted to growth on media containing the metal ions gave higher yields of citric acid when grown on crude molasses compared with non- adapted fungi.

Pera and Callieri, (1997) studied the addition of 0.5 g/L $CaCl_2$ to the fermentation medium and they found that, this addition lowered the final dry biomass by 35% and increased the uptake of phosphate and

sucrose, and the production of citric acid by 15, 35 and 50%, respectively. In a medium deprived of Ca^{2+} the microorganism displayed both a pelleted and a filamentous form of growth, the hyphae being scarcely branched, without bulbous cells. 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-β-D-glucosaminidase, a lytic enzyme, might be involved in these morphological changes.

Ca phytate was used by **Jianlong, (1998)** as an additive to enhance citric acid production by *A. niger* W1-2 from a medium based on untreated beet molasses providing 100 g total reducing sugar/L. The addition of phytate increased the utilization of reducing sugar and enhanced citric acid accumulation. The mycelial growth was slightly inhibited. The effect of phytate addition was depend on the dose and the state of fermentation when it was added at the begining of incubation, the optimum concentration, of phytate (10 g/L) resulted in about a 3.1 fold increase in citric acid accumulation (from approximately equal to 20 to > 60 g/L). During the fermentation, phytate addition at 16 g/L after 3 days' incubation gave the maximum citric acid production which was about 2.8 fold higher than in the control experiment.

2.5.4 Effect of the immobilization on citric acid production:

Citric acid is a broadly used chemical with extensive industrial importance. However, it has limited production by *A. niger* owing to severe technological difficulties. Citric acid has produced by conventional

submerged culture in which the biomass is suspended in the medium. Therefore, its separation from the medium and the biomass 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 easisly than with a batch system of free cells. In addition, immobilized cells are more stable than free mass **(El-Kadi, 2003)**

Horitsu *et al*., (1988) reported three processes for the production of organic acids by fermentation: (1) citric acid from beet molasses, diluted to 10% sucrose and adjusted to pH 5.8, by *A. niger* G-011 immobilized in polyacrylamide gel or calcium alginate gel; (2) itaconic acid from 6% glucose solution by *A. terreus* G-026 immobilized in polyacrylamide gel; (3) lactic acid from 5% glucose solution by *Rhizopus oryzae* AHU 6536 immobilized in calcium alginate. Repeated batch fermentations were carried out on a laboratory scale; citric acid was also produced by a continuous process in a 2-stage reactor. Of additives tested, $MgSO_4$, NH_4NO_3 and $CaCO_3$ enhanced the outputs of citric acid, itaconic acid and lactic acid respectively. Citric acid output increased with amount of aeration up to 1.4 L/min in 100 ml substrate; it was greater when slices of gel rather than cubes were used. Maximum productivities achieved (in mg/h per 10 g gel) were: citric acid, 25; itaconic acid, 28; lactic acid, 52. Corresponding half-lives of the enzymes were 105, 30 and 17 days.

Roukas, (1991) was immobilized spores of *A. niger* ATCC 9142 in alginate gel beads and grown for 4 days at 30°C on molasses medium at pH 6.5 containing 20 g sucrose and 0.6 g N compounds/L, in flasks shaken at 250 r.p.m. The beads were then washed and incubated at 30°C in

production medium containing 14% total sugars, either in shake-flasks or in a glass bioreactor (60 g beads and 300 ml medium in 600 ml capacity) aerated at 0.5, 1.5 or 2.5 L/min. Maximum yield of citric acid was observed after 28 days, being 35 g/L in shake-flasks and <28 g/L (lower with less aeration) in the bioreactor; when the beads were reused in shake-flasks, the citric acid concentration in successive batches reached 40, 37.5 and 30 g/L.

Pallares *et al*., (1996b) grown *A. niger* initially on a medium containing 100 g sucrose/L and they immobilized it in polyurethane foam cylinders and used to ferment a medium containing 50 g sucrose + 400 mg NH_4NO_3/L, continuously fed at 0.1 vvd to a fluidized bed bioreactor of capacity 125 ml at 30°C, with initial pH 4.0 and aeration at 2 vvm. The effect of using a pulsing device on citric acid production was studied by evaluating the subsequent productivity and stability of the process. Pulsation and the limited nitrogen availability controlled bioparticle morphology sufficiently to avoid bed compaction. An optimum pulsation frequency of 0.3 s-1 enabled bioreactor stability to be maintained for >30 d; citric acid output increased by >52%, reaching approximately equal to 35 g in 24 days.

Kahlon *et al*., (1992) used sugar cane molasses as sole carbon source for citric acid production by the fungus *A. niger* NCIM-683 entrapped in sodium alginate gel. Maximum citric acid yield was obtained from a medium containing 15% (w/v) sugars, with 67.33% of sugars utilized, after 6 days at 30°C and pH 5.5. This yield was higher than that obtained using free fungal mycelium in similar conditions (6.37 vs. 5.62 g/100 ml molasses medium).

Khare *et al*., (1994) used *A. niger* cells immobilized in agarose beads for the production of citric acid from soya whey, a byproduct

generated during the tofu-making process. Soya whey samples were supplemented with 10% sucrose, inoculated with 10% (w/v) free or immobilized cells, and incubated at 30°C with agitation at 200-220 r.p.m. They found that, maximum citric acid yields of 21 g/L with free cells and 27 g/L with immobilized cells (compared with an average yield of 22.5% for cane molasses) were recorded on the 10th day under repeated batch conditions.

Sanroman *et al*., (1994) studied the entrapment, 7 ml spore suspension (2 x 10^6 spores/ml) was immobilized into 1 g polyurethane foam (as regular cylinders of dimension 2 mm and density 40 kg/m^3, total internal capacity approximately equal to 14 ml) by repeated compression and expansion within a syringe, and static incubation (with manual agitation once a day) was carried out on medium containing 5% w/v glucose + salts (initial pH 4) for 3 days at 30°C and they studied the adsorption, 1 g foam was mixed with 50 ml of the same medium containing 5 x 10^5 spores/ml and shaken at 200 r.p.m. for 3 days at 30°C. In this 1st phase, citric acid production was 9.4 g/L during entrapment and 2.8 g/L during adsorption. However, in the subsequent production phase (7-8 days in an orbital shaker or a fluidized bed reactor), the entrapped fungus typically yielded only 3.1 g citric acid/L, compared with 14.7 g/L for the adsorbed.

Rugsaseel *et al.*, (1995a) used gucose as a carbon source to produce citric acid by *A. niger*. They recorded that, a markedly low citric acid productivity in shake culture, but a high productivity in semi-solid. Since the viscosity of the medium was assumed to be one of the important factors for citric acid productivity in shake culture, the effect of the addition of viscous substances on citric acid productivity were examined. Gelatin,

agar, carageenan, carboxymethylcellulose and polyethylene glycol were used as a viscous additions to the medium. The viscous substances functioned as protectants for the mycelium from physiological stresses and resulted in remarkably increased citric acid productivity in shake culture.

Kirimura *et al*., (1999) reported that the addition of 2.0 mg carboxymethylcellulose (CMC)/ml as a viscous additive to the medium reduced drastically the amount of extracellular polysaccharide accumulated to 1.5 mg/ml, but increased the citric acid produced to 52.0 mg/ml.

Conidia of *A. niger* were immobilized by **Bayraktar and Mehmetoglu, (2000)** in calcium alginate gel for the production of citric acid. *A. niger* requires a 2-day preactivation period at a 0.05 g/L NH_4NO_3 concentration. They found that, maximum citric acid production was attained with medium containing 0.01 g/L of NH_4NO_3. The rate of citric acid production in the nitrogenous medium was 33% higher when oxygen was used instead of air during the production phase. This corresponds to an increase of 85% when compared to production when neither oxygen nor air was fed into the system. In the nonnitrogenous medium citric acid concentration remained similar regardless of the use of air or oxygen. The advantage of using immobilized cells is that production is achieved easily in the continuous system. Therefore, citric acid production was also tested using a packed-bed bioreactor, and an increase in productivity by a factor of 22 was achieved compared to the batch system.

Continuous and batch production of citric acid from sucrose was investigated by **Sankpal *et al*., (2001)** using *A. niger* NCIM 588. Mycelia of *A. niger* grown on cellulose microfibrils formed a uniform and thin mycelial proliferation under controlled conditions of cultivation rich in oxygen. In the fed batch mode using a recycle reactor, the dissolved

oxygen (DO) of the system was maintained at 20 mg/L using oxygen enriched air. This improved volumetric productivity to 1.85 g/L/h of citric acid, representing an increase of at least 15-fold over results obtained simultaneously using shake-flasks and 1.6-fold over a conventional aerated batch reactor. It was possible to substitute sucrose with sugarcane juice as a carbon source in a fed batch recycle system. An overall specific production rate of citric acid of 0.147 and 0.208 g/g/h was achieved using cane juice and sucrose, respectively. In continuous fermentation, a medium containing 50 g/L of sucrose was allowed to drip through the fabric support at a residence time of 20 h. As a result of interface interaction, a citric acid volumetric productivity of 2.08 g/L/h was achieved for 26 days without any significant loss in productivity.

2.5.5 Effect of the use of Fermentor:

The citric acid excretion of Ca-alginate-immobilized cells of *A. niger* in batch culture decreased with a half-time of approximately 19 days. Reactivation of the biocatalysts by regeneration in growth medium was possible, but it was followed by a submerged sporulation of the fungus, and medium was highly contaminated with free cells. Citric acid production could better be prolonged by semicontinuous cultivation with medium exchange every 7 or 14 days. After 32 days the remaining activity in semicontinuous culture was 1.4-fold higher than in comparable batch experiments. Similar improvements were obtained with a continuous process at a dilution rate of 0.125 v/v X d, whereby medium efflux kept completely free of detaching mycelia **(Eikmeier and Rehm,1987)**.

Beet molasses media were subjected to 9 days' submerged fermentation by *A. niger* strain EU-119 in typical industrial conditions, using normal air or air containing 0.2-2 million negative ions/cm^3,

generated by passing through a partitioned cylinder of dielectric material, fitted with a perforated-plate earth and a set of corona-discharging needle electrodes. This 'electroaeration' increased the citric acid yield by 10-18%, presumably via metabolic stimulation of the organism. The concentrations of the 9 main amino acids (aspartic and glutamic acids, alanine, glycine, isoleucine, leucine, lysine, serine and threonine) were monitored. With treated air, changes in these concentrations occurred in the same directions as with normal air but were usually somewhat more pronounced. It is recommended that electroaeration be applied either continuously, generating 90-100 ions/cm3, or for 20 min in each 80 min, generating 3 x 105 ions/cm^3 in the first 24 h, 900/cm^3 in the next 13 h and none thereafter **(Glushchenko and Glushchenko, 1988)**

Beet molasses substrates, containing 12.5% total reducing sugars and optimally dosed with $K_4Fe(CN)_6$, were fermented by *A. niger* in a 7 L stirred tank reactor at 30°C, aerated at 1 vol./min for >150 h. Mycelial growth from conidia to pellets was monitored by microscope, and rheological data were obtained by Rheotest 3 viscometer. The behaviour was nearly Newtonian for the first 29 h, after which pseudoplasticity arose due to the presence of mycelial pellets. The greatest changes in rheological properties occurred during the period of maximum production of citric acid and exponential increase of biomass **(Berovic *et al*., 1991)**.

Beet molasses media were fermented by *A. niger* in an 'Ultroferm LKB 1601 Broma' laboratory fermenter of capacity 5 L; effects of pH (2.5-6.0), temperature (23-35°C) and substrate concentration (100-180 g/L) on specific rate of fermentation and citric acid yield were studied. Optima were found to be: pH >4 (4.5); 27.5-31.5 (28) °C; 135-150 (140) g/L; aeration 0.3 vvm, and agitation 500 r.p.m. Biomass reached approximately

equal to 14 g DM/L, and citric acid output 53.26 g/L (yield approximately equal to 58.5% on sugar consumed) **(Beggah and Ait-Amar, 1992)**.

Medium based on 14% w/v sucrose was fermented for 160 h at 30°C by pellets of *A. niger* strain CBS 246-65 in a continuous stirred tank reactor of working capacity 2 L, with constant aeration at 1 vvm; oxygen saturation was initially 100% and pH initially 3. The influence of pellet size (diameter 0.5-1 or 1.5-2.0 mm) on the course of citric acid production was studied. Best results were obtained with small pellets; overall hourly productivity exceeded many reported values, reaching 205 mg/L without full optimization of the process **(Cameselle *et al*., 1994)**.

A higher sucrose concentration was used for the germination of spores, which caused a higher intracellular level of the osmoregulator, glycerol, to be present. When citric acid started to be excreted into the medium, the substrate was suddenly diluted. Optimization of this procedure resulted in a nearly tripled volumetric rate (g/L/h) of acid production, while the overall fermentation time was halved compared with the usual batch process. Yet, a characteristic delay was observed at the start of the acid excretion after the dilution. Hypo-osmotic shock caused a prominent elevation of intracellular cyclic AMP levels. Simultaneously, the specific activity of 6-phosphofructo-1-kinase increased significantly, probably due to phosphorylation of the protein molecule by cyclic AMP-dependent protein kinase. Specific 6-phosphofructo-1-kinase activity was much higher in the treated than in the normally growing mycelium. The metabolic flow through glycolysis was expected to be higher, which should contribute to a higher volumetric rate of acid production **(Legisa and Gradisnik-Grapulin, 1995)**.

A mathematical model is presented to describe citric acid production from sucrose + ethanol via continuous and semicontinuous methods of fermentation by *A. niger* (strain F722) in a membrane bioreactor of working volume 20 L. Its adequacy was demonstrated using experimental data. Citric acid productivity in the membrane reactor was approximately equal to 3 times that in a traditional batch reactor **(Vinarov *et al*., 1998)**.

A continuous 4-stage process for citric acid production via fermentation of sucrose by *A. niger* with recirculation of the broth was designed and tested. The process consists of the fermentation stage, extraction stage, re-extraction stage and product stage. The fermentations were conducted in a reciprocating- jet bioreactor of capacity 20 dm^3 with *A. niger* ATCC 9142 previously grown on medium based on 40 kg sucrose + 30 L of methanol/m^3; the medium initially contained 150 kg sucrose/m^3 and was eventually fed a stream containing 100 kg sucrose/m^3 at up to 3 dm^3/day, with constant aeration at 0.4 m3/h. The influence of the nitrogen concentration in the influent, the age of the fungus and strong dilution of the biosuspension are discussed. Though continuous operation improved citric fermentation, the rate of citric acid production decreased with increasing fermentation time and could not be restored by slow growth or adding fresh biomass; this may indicate accumulation of unidentified inhibitors **(Wieczorek and Brauer, 1998)**.

Use of methanol or ethanol in the magneto-biotechnological technique was effective in enhancing citric acid production in a magnetic drum contactor operation with *A. niger* AJ 117173 strain. Continuous and repeated-batch fermentations were conducted with the addition of 2% ethanol (plus 100 g sucrose/L in the medium). Continuous fermentation for 50 days gave a better citric acid yield (85%) and average productivity (3.8

g/L/day) than repeated-batch fermentation over 60 days (65% , 2.3 g/L/day) **(Saha *et al*., 1999)**.

Citric acid fermentation using biofilm such as surface culture and cultivation by rotating disk contactor (RDC) was examined. A simple model was presented to describe the time courses of citric acid production by surface culture using *A. niger*. The model is expressed by Monod type cell growth and Luedeking-Piret type citric acid production rate equations. The experimental time course in citric acid production period by surface culture was well simulated with this model. The time course of citric acid production by rotating disk contactor was simulated well with this model taking into consideration the oxygen distribution in the biofilm. The relation between the specific biofilm surface area and the rate of citric acid production was also explained by the simulation. Overall productivity of RDC for a 3-times-repeated batch operation was 1.7 times higher than that for the non-repeated batch culture which is comparable to that of the submerged culture **(Sakurai *et al*., 1999)**.

3.MATERIALS AND METHODS

3.1.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.

3.2.Maintainance of stock cultures:

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

3.3.Media:

3.3.1.Potato dextrose agar:

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

3.3.2.Culture media:

Six cultural media were used as basal medium for citric acid production, these media were:

3.3.2.1.Medium No.1:(Currie, 1917)

Sucrose 15%; NH_4NO_3 0.2%; $Ba(OH)_2.H_2O$ 0.1%; Lecithin (Lecithin, egg crude; Made in England; BDH.) 0.1%; $MnSO_4.H_2O$ traces; $ZnSO_4.7H_2O$ trace and pH was 3.5.

3.3.2.2.Medium No.2:(Mashhoor *et al.*, 1987)

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%; 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 **(Mashhoor, *et al*., 1987)**.

3.3.2.3.Medium No.3:(Saha *et al.*, 1999)

Glucose, 14.0%; $(NH_4)_2CO_3$, 0.2%; KH_2PO_4, 0.01%; $MnSO_4.H_2O$, 0.01%; Methanol, 2.0% and pH was 3.5.

3.3.2.4.Medium No.4:(Karow, 1942)

Sucrose, 15.0%; Urea, 0.1%; $MgSO_4.7H_2O$, 0.05%; KH_2PO_4, 0.008%; $MnSO_4.H_2O$, 0.002%; KCl, 0.1%; $ZnSO_4.7H_2O$, 0.001% and pH was 2.00.

3.3.2.5.Medium No.5:(Doelger and Prescott, 1934)

Sucrose, 10.0%; NH_4NO_3, 0.05%; $MgSO_4.7H_2O$, 0.01%; and pH was 2.20.

3.3.2.6.Medium No.6:(Papagianni *et al.*, 1999c)

Glucose, 14.0%; NH_4NO_3, 0.2%; KH_2PO_4, 0.2%; $MgSO_4.7H_2O$, 0.025%; $ZnSO_4.7H_2O$, 0.002%; $FeCl_2$, 0.002% and pH was 2.60.

3.4.Raw materials:

Sugar cane molasses was obtained from The Sugar Refinery Factory at El-Hawamdia, Giza, Egypt. Sugar cane molasses contained 55% total sugars.

3.5.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)**.

3.6.Inoculum:

The inoculum was counted by the equation:

$$\text{Inoculum volume} = \frac{5x10^6}{\overline{X} \text{ x d x c}}$$

Where $\overline{X}$ = the mean of spores; d = dilution rate, c = constant (the factor of slide $2.5x10^5$).

3.7.Culture conditions:

Unless otherwise stated, 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 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.

3.8.Determinations:

3.8.1.Citric acid determinations:

3.8.1.1.Pyridine-acetic anhydride method:

Citric acid estimation was carried out colorimetrically with pyridine-acetic anhydride method according to **(Marier and Boulet, 1958)** as follows:

Reagents:

- Reagent-grade acetic anhydride and pyridine.

- Anhydrous citric acid.

Procedure:

To a test tube, 1 mL of culture filtrate (containing from 25 to 200 µg of citric acid), or citric acid standard, or water (for the reagent blank) was added, followed by 1.30 mL of pyridine, and swirl the briskly. After similar additions have been made to all tubes in a series, add 5.70 mL of acetic anhydride, swirl the tube again, and immediately place it in the constant-temperature water bath at 32°C. Color development was completed after 30 min., and the color was stable for at least another 30 min., either in the 32°C bath or in air at a temperature of 22-29°C. The color intensity was read at 420 mµ with the blank set at 100% transmission and the citric acid content of the samples were estimate by reference to the standards.

3.8.1.2. Determination of citric acid using the reference titration method:

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

50 mL of the samples of fermented molasses was titrated with 0.5 N NaOH to end point. To know the end point the fermented molasses was titrated until pH 8.1 (the iso-electric point) because it was impossible to use an indicator in the dark molasses medium. The resulting citric acid was calculated as grams per 100 mL as follow:

$$\frac{\text{mL NaOH} \times \text{N} \times 6.4}{\text{mL sample}} = \text{g citric acid per 100 mL}$$

3.8.2.Conversion coefficient and yield of citric acid:

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

$$\text{Conversion coefficient \%} = \frac{\text{Amount of citric acid produced (g/L)} \times 100}{\text{Sugar consumed (g/L)}}$$

$$\text{Yield \%} = \frac{\text{Amount of citric acid produced (g/L)} \times 100}{\text{Initial sugar concentration (g/L)}}$$

3.8.3.Determination of total carbohydrate:

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

Reagents:

- 5% (w/v) solution of phenol in water.

- Concentrated sulfuric acid (sp. gr. 1.84).

Procedure:

Into thick walled tubes, 1 mL of sample was piptted containing the equivalent of 20-100 µg glucose.

A reagent blank containing 1 mL of water and a set of glucose standards (20-100 µg glucose, in a volume of 1 mL) were prepared at the same time. To all tubes 1 mL of 5% phenol solution was added. Then 5 mL of concentrated sulfuric acid were added, directing the stream of acid on the surface of liquid with shaking. The tubes were allowed to stand in water bath at 25°C. for 10 to 20 min. before reading at 490 µm.

3.8.4.Determination of reducing sugars:

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

Reagents:

Copper reagent

24.0 g sodium tartrate and 48.0 g sodium carbonate was dissolved in 500 mL (Solution1). 32.0 g sodium bicarbonate was dissolved in 80 mL of

copper sulfate 10% (w/v) (solution2). Solution2 was added to Solution1 with shaking and the volume was completed to 1000 mL and that was solution 3. 360 g sodium sulfate was dissolved in 1000 mL of distilled water, boiling to remove the air, then completed to 1000 mL (solution4). Solution 4 was added to solution3 to make the volume 2000 mL and that was the copper reagent.

Arsenomolybdate reagent:

50 g ammonium molydbate was dissolved in 900 mL of distilled water, 42 mL sulfuric acid was added and the solution was mixed well solution1. 6 g disodium hydrogen arsenate was dissolved in 50 mL of distilled water (solution2). Solution2 was added to solution1 and the volume was completed to 2000 mL and that was the arsenomolybdate reagent.

Procedure:

1 mL of sample containing the equivalent of (20-100 µg) glucose was piptted. A reagent blank containing 1 mL of water, and a set of glucose standards (20-100 µg glucose), in a volume of 1 mL were prepared at the same time. To all tubes 1 mL of copper reagent was added, boiled at 100°C. for 10 min., cooled to 30°C., 1 mL of arsenomolybdate reagent was added with shaking and stand for 20 min. at room temperature, 7.0 mL of distilled water was added to make the end total volume 10.0 mL and reading at 620 µm.

3.8.5.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)**.

3.8.6.Microorganism and immobilization:

The spore suspension of *A. niger* NRRL-2270 was prepared to gave $5.0x10^6$ /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)**.

3.8.7.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)**.

4.RESULTS AND DISCSSION

The first section

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

Citric acid is a metabolic product of molds of the genera *Penicillium, Mucor, Ustulina, Botrytis, Ascochyta, Absidia, Talaromyces, Acremonium, Eupenicillium* and *Aspergillus.* Even today, strains of *Aspergillus niger* have dominated other both in laboratory and industrial scale production of citric acid. The major advantages in using *A. niger* for producing citric acid are the ease with which it can be handled, the cheap raw materials that it can utilize for citric acid production, and high and consistent yields, therby making the process economical **(Kapoor *et al*., 1987)**. The present part of thesis deals with evaluation the production of citric acid by nine isolates of *Aspergillus niger* during growth on different media. The results obtained are presented in Tables (1a-f). Results showed that most tested isolates were able to produce citric acid during growth in submerged fermentation system in the used media. The highest production of citric acid being 22.90 g citric acid /L was obtained during growth *A. nigre* CA2 on medium No. 2. 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)**, 15.4 g/L **(Rugsaseel *et al*., 1995a)**, 19.9 g/L **(Rugsaseel *et al*., 1995b)**, 41 g/L **(Kim *et al*., 1995)**, 69 g/kg substrate **(Maddox and Brooks, 1995)**, 18 g/L **(Drysdale and MaKay, 1995)**, 78 kg/m^3 **(McIntyre and McNeil, 1997)**, 36.5±1.5 g/L **(Roukas and Kotzekidow, 1997)**, 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.) ranged from 0.00 to 48.5 g/L. The highest weight was obtained during growth of *A. niger* CA2 on medium No. 1. Many authors were found different amounts of dry weight *i. e.* 34 g/L **(Chanda *et al*., 1990)**, 16.9 g/L **(Rugsaseel *et al*., 1995a)**, 23.4 g/L **(Rugsaseel *et al*., 1995b)**, 14 g/L **(Kim *et al*., 1995)**, 32 g/kg substrate **(Maddox and Brooks, 1995)**, 19.78 g/L **(Drysdale and McKay, 1995)**, 18.2 kg/m^3 **(McIntyre and McNeil, 1997)**, 25.0 ± 1.0 g/L **(Roukas and Kotzekidou, 1997)**, 30 g/kg wet substrate **(Roukas, 1998)** and 12 kg/m^3 **(Papagianni *et al*., 1999c)**.

Consumation of sugars (C. S.) by tested strains ranged from 2.50 % to 68.50 %. The highest value was obtained during growth *A. niger* NRRL 2270 on medium No. 5. Many authors were found different values of consumed sugars *i. e.* 92 g/L **(Chanda *et al*., 1990)**, 119 g starch was used **(Maddox and Brooks, 1995)**, 113.5 g/L **(Drysdale and McKay, 1995)**, 93.1 g/L **(Rugsaseel *et al*., 1995a)**, 125 kg/m^3 **(McIntyre and McNeil, 1997)** 56.5 ± 1.2 g/L, **(Roukas and Kotzekidow, 1997)**, and the total amount of utiized sugars was 64 % **(Roukas, 1998)**. Conversion coefficient (C. C.) by tested strains ranged from 0.00 % to 30.946 %.

The highest value was obtained during growth *A. niger* CA2 on medium No. 2. **Mashhoor *et al*. (1987)** found that conversion coefficeint was 66.9 %, while **Garg and Sharma (1991)** obtained 80%, but **El-Samragy *et al*. (1996)** revealed it 5.58 %, **Jianlong *et al*. (2000)** achieved sugar conversion 82.2% and **Mourya and Jauhri (2000)** reported that 49 % substrate convert to citric acid. Citric acid yield by tested strains ranged from 0.00 % to 15.267 %. The highest value was obtained during growth *A. niger* CA on medium No. 2. Many authors were obtained a different citric acid yield, *i. e.* 58 % **(Maddox and Brooks, 1995)**, 50 % **(Kim *et al*.,**

1995), 21.4 % (**Rugsaseel *et al*., 1995a**), 62.4 % (**McIntyre and McNeil, 1997**), 43.0 ± 1.7 % (**Roukas and Kotzekidow, 1997**), 55% (**Roukas, 1998**) and 80 % (**Lesniak *et al*., 2002**).

Table (1-a): 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

Where D. M. W. = dried mycelum wieght, C.S. = consumed sugar and C.C.= conversion coefficient. Medium No.1 (**Currie, 1917**): Sucrose 15%; NH_4NO_3 0.2%; $Ba(OH)_2.H_2O$ 0.1%; Lecithin (Lecithin, egg crude; Made in England; BDH.) 0.1%; $MnSO_4.H_2O$ trace; $ZnSO_4.7H_2O$ trace and pH was 3.5.

Table (1-b): 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

Medium No. 2 (**Mashhoor *et al.*, 1987**): One litre of clear supernatant diluted molasses; Urea 1.2 g/L; $MgSO_4.7H_2O$ 04 g/L; H_3PO_4 0.4 ml/L and pH was 7.0.

Table (1-c): 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

Medium No. 3 (**Saha *et al.*, 1999**): Glucose 14.0%; $(NH_4)_2CO_3$ 0.2%; KH_2PO_4 0.01%; $MnSO_4.H_2O$ 0.01%; Methanol 2.0% and pH was 3.5.

Table (1-d): 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

Medium No.4 (**Karow, 1942**): Sucrose 15.0%; Urea 0.1%; $MgSO_4.7H_2O$ 0.05%; KH_2PO_4 0.008%; $MnSO_4.H_2O$ 0.002%; KCl 0.1%; $ZnSO_4.7H_2O$ 0.001% and pH was 2.00.

Table (1-e): 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

Medium No.5 (**Doelger and Prescott, 1934**): Sucrose 10.0%; NH_4NO_3 0.05%; $MgSO_4.7H_2O$ 0.01%; and pH was 2.20.

Table (1-f): 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

Medium No.6 (**Papgianni *et al.*, 1999c**): Glucose 14.0%; NH_4NO_3 0.2%; KH_2PO_4 0.2%; $MgSO_4.7H_2O$ 0.025%; $ZnSO_4.7H_2O$ 0.002%; $FeCl_2$ 0.002% and pH was 2.60.

It could observe from Table No.(1-b) and Fig. (2) that the best strain of citric acid producer was *A. niger* CA2 on medium No.2, and the results agree with **Drysdale and McKay (1995)** who reported that molasses is the traditional substrate for citric acid production by microorganismes, most commonly *A. niger*.

The second section

4.2.The nutritional and environmental conditions for citric acid production:

The purpose of this experiment is to investigate the optimal conditions for citric acid production by *Aspergillus niger* CA 2 using medium No. 2. Thus cultural condition such as time course, initial pH, the treatment of molasses with potassium ferrocyanide and ammonium oxalate, nitrogen sources, phosphorus sources, the addition of magnesium sulfate, inoculum volume, trace elements, and the addition of ethanol were investigated.

4.2.1.Effect of time course on citric acid production:

The results on the effect of time course on citric acid production are presented in Table (2). The results showed that the concentration of citric acid increased with the increasing the fermentation time. The maximum citric acid concentration (23.20 g citric acid/L) was obtained after 8 days of fermentation and then decline, at this time the citric acid yield was 15.467 % and the conversion coefficient was 28.293%. These resultes were in harmony with **Rugsaseel *et al.* (1995a and b)** and **Maddox and Brooks (1995)** who reported that the maximum citrate concentration was achieved after 8 days of fermentation time. The biomass dry weight followed a pattern similar to citric acid production. The highest biomass dry weight (25.5 g dried biomass/L culture) was obtained after 9 days. These resultes agree with **Roukas (1999)** and **Drysdale and McKay (1995)** who reported that the biomass was 19.87 g/L when sucrose was used to preduce citric acid by *A. niger* and it was 21.55 g/L using air flow passed over the surface of the medium.

The pH of culture employed for citric acid decreased during fermentation due to the release of production citric acid during fermentation of sugars. The lowest value of pH (3.90) was accompanied with the greatest concentration of citric acid. The concentration of consumed sugars increased with the progress in the fermentation time. The highest concentration (85.0 g consumed total sugars/L medium) was observed after 10 days of cultivation.

Table (2): 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

The medium employed for citric acid production contained one liter of the clear supernatant diluted cane molasses " supplemented with" (g/L) [1.20 (w/v) urea; 4.0 (w/v) $MgSO_4.7H_2O$; 0.40 H_3PO4 (v/v)] Initial pH (7.0). Incubation temperature (30°C at 150 rpm)

4.2.2.Effect of the initial culture pH on citric acid production:

The addition of $CaCO_3$, as a neutralizing agent, to the culture media employed for citric acid production decreases consumption, mycelial growth and citric acid accumulation whether methanol was added or not **(Rugsaseel *et al.*, 1995a)**. The pH had no effect on citric acid production through any direct influence on the Krebs cycle. It is possible that pH affects the enzymes that are active in degrading the substrate and /or the permability of the cell membrane to sucrose and citric acid **(Kristiansen and Sinclair, 1979)**. Since the initial pH of the culture is an important

factor that affect the citric acid production, this experiment was undertaken to determine the optimum initial pH that would result in high citric acid level. The results on the effect of pH on citric acid production are presented in Table (3).

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 pH 5.5, the biomass was (29.0 g /L) and the biomass remained constant almost over the pH range 5.0 - 8.0. The concentration total sugars consumed was 89.0 g /L. The conversion coefficient was 32.584% and citric acid yield was 19.333%. These results agree with those of **Kahlon *et al*. (1992), Roukas (1999)** and **Roukas and Kotzekidou (1997)**.

Table (3): 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

The medium employed for citric acid production contained one liter of the clear supernatant diluted cane molasses "supplemented with" (g/L) [1.20 (w/v) urea; 4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

4.2.3.Effect of total sugars concentrations on citric acid production:

The concentration of sugars in the culture medium had been revaled to be important for citric acid synthesis. Sugar cane molasses is widely used as carbon source although it problem in metal ions content, on the

other hand, stimulators had also been found, this was special problems but cheap carbon source (**Röhr *et al*., 1983**). To investigate the affect of sugar concentration, an experiment was designed using different amount of molasses which the sugar content varied from 5 to 25%. Every one was supplemented with the inorganic nutrients of the basal medium.

The results on the effect of sugar concentration on citric acid production are presented in Table (4). The citric acid level was increased with the increasing of molasses from 5 to 15% total sugars, and then decline. These results are inharmony with **Papagianni *et al*. (1999c).** 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%.

Table (4): 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

The medium employed for citric acid production contained different concentrations of molasses calculated as total sugars "supplemented with" (g/L) [1.20 (w/v) urea; 4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

The obtained results are in line with **Papagianni *et al.* (1999c)** who reported that the benefit of high initial sucrose concentration indicates that free cells need a high osmotic pressure for optimal citric acid production. The concentration of sugars in culture during fermentation was important. No citric acid was preduced on media containing sugar less than 2.5%. A relatively low glucose level led to a less productive mycelium, with growth

being enhanced in expense of the citric acid production. Initial glucose concentration affected the specific rate of citric acid formation. The specific rate of citric acid formation decreases with a decrease in the initial glucose concentration. The maximum citric acid procution was at initial glucose concentration 150 kg/m^3 **(Papagianni *et al.,* 1999c)**.

4.2.4.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 (5) and graphically illustrated in Fig. (10). 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 $K_4Fe(CN)_6$.

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%.

Table (5): 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

The medium employed for citric acid production contained different concentrations of $K_4Fe(CN)_6$ and ammonium oxalate 10.0 g/l was added to the diluted molasses and was left overnight in the refrigerator at 5°C for complete precipitation of calcium oxalate "supplemented with" (g/L) [1.20 (w/v) urea; 4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

It was reported that molasses has high amount of heavy metals **(Rasmy, 1999)**. 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)**.

It is well know that the citric acid fermentation is greatly affected by the presence of some trace metals, so various techniques have been used to remove metallic inhibitory substances from the substrate. Heavy metals such as iron, zinc and cupper can cause a critical problem during citric acid fermentation, they inhibit the growth of microorganism and inactivation of the enzymes associated with citric acid metabolism in the TCA cycle **(Roukas and Kotzekidou, 1997)**.

4.2.5.Effect of decalcification of molasses with ammonium oxalate on citric acid production.

It was reported that cane molasses has high concentration of calcium **(Rasmy, 1999)**, therefore, different concentrations of ammonium oxalate are used to transform calcium to the unavailable form **(Mashhoor *et al*., 1987)**. The results on the effect of ammonium oxalate on citric acid

production are presented in Table (6). 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. 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%.

Table (6): 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

The medium employed for citric acid production contained 1.50 g/l. $K_4Fe(CN)_6$,and different concentrations of ammonium oxalate and was left overnight in the refrigerator at 5°C for complete precipitation of calcium oxalate. "supplemented with" (g/L) [1.20 (w/v) urea; 4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

4.2.6.Effect of the nitrogen sources on citric acid production:

It is known that nitrogen affects both mycelium formation and citric acid production **(Bayraktar and Mehmetoglu, 2000)**.

Therfore, the effect of nitrogen sources on citric acid production was studied. Eleven inorganic and organic nitrogen sources were investigated. Tested nitrogen sources were added separately to basal medium No.2 to give final nitrogen concentration of 0.0559 g N/100 ml, thus the amount of nitrogen was fixed. The results on the effect of nitrogen sources on citric acid production are presented in Table (7). The highest amount of citric

acid was obtained 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 tested organic and inorganic sources gave lower citric acid concentration Therefore, ammonium phosphate was the best nitrogen source for citric acid production, replaced for urea of medium No.2 in the following experiments.

Table (7): 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

The medium employed for citric acid production contained different sources of nitrogen (0.0559 g N/100 ml. equivalent 0.12g urea/100 ml.) "supplemented with" (g/L) [4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

Several researches have attempted to explain the biochemical role of intracellular ammonium ions in the regulation of citric acid production by *A. niger*. One of the key conditions for the citric acid accumulation is a deficiency of manganese ions in the nutrient medium. *A. niger* grewn 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, that is necessary to provide a mechanism by which the feedback inhibition of citrate biosynthesis by citrate at the step could be overcome in order to achieve an overproduction of citric acid.**(Kim *et al.*, 1995).**

4.2.7.Effect of $NH_4H_2PO_4$ concentrations on citric acid production:

Physiologically ammonium compounds are generally preferred, since during their consumption the pH decreases, which is a prerequisite of citric acid fermentation **(Röhr *et al.*, 1983)**. 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. Ammonium phosphate was added in eight different concentrations ranging from 0.50 to 7.0 g/L.

The results on the effect of ammonium phosphate on citric acid production are presented in Table (8). The citric acid concentration was increased with the increasing of ammonium phosphate then declined. The relatively high nitrogen content may be the reason for poor citrate production, and the knowledge that, *A. niger* produces citrate from carbohydrate only under nitrogen deficient conditions **(Maddox and Brooks, 1995)**.

The highest value of citric acid concentration 32.03 g/L was obtained with 2.0 g/L ammonium phosphate. Also at 2.0 g/L ammonium phosphate biomass was 20.0 g/L, consumed total sugars concentration was 74.0 g/L, conversion coefficient was 43.284% and citric acid yield was 21.353%. Therefore, this concentration of ammonium phosphate (2.00 g/L) which proved to be the optimum, was used in the following experiments

Table (8): 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

The medium employed for citric acid production contained different concentrations of $NH_4H_2PO_4$ "supplemented with" (g/L) [4.0 (w/v) $MgSO_4.7H_2O$;0.40 H_3PO4 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

Nitrogen affects both the growth of microorganisms and the production of citric acid. The variation in citric acid production was slitly changed under $NH_4H_2PO_4$ concentrations of 2-5 g/L. On the other hand, citric acid production decreased remarkably under a nitrogen concentration of 7 g/L. Similar resultes 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)**. In batch fermentation, it was found that the biomass increased throughout the fermentation. The supply of nitrogen was exhausted early and the subsequent increase 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, apparently by the carbon-storing cells. At high nitrogen levels, the number of cells formed will increase with the nitrogen concentrations and cytoplasm in the hyphae was streaming forward into the non producing tip of hyphae, which is not suffering nitrogen limitation, and the citric acid production will drop. Metabolic studies on citric acid

producing *A. niger* have revealed that intracellular ammonium ions play potent regulatory role at the phosphofructokinase step of the glycolytic pathway in citric acid biosynthesis **(Kim *et al.,* 1995)**.

4.2.8.Effect of the phosphorus sources on citric acid production:

Phosphorus serves as a macronutrient satisfying the demand of growing cells for synthesizing nucleotides and other phosphorus compounds **(Kapoor *et al*., 1987)**. In the present experiment, six inorganic phosphorus sources were investigated (Table 9). Tested phosphorus sources were added separately to 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) in the basal medium, thus the amount of phosphorus was fixed. The highest amount of citric acid was 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 gave lower citric acid concentrations. Therefore, phosphoric acid was the best phosphorus source for citric acid production.

The present of $(PO4)^{-2}$ causes increased growth and increased production of side byproducts. Helps stimulate phosphofructokinase if it becomes inhibited by citric acid. The addition of phosphorus in the fermentation medium led to a complete consumption of the present sugar and maximum citric acid concentration **(Röhr *et al*., 1983)**. Phosphorus is a nutrient whose effect on the production of citric acid has been related to metal content such as Mn, Fe and Zn of the media. In media with phosphorus limitation, the effect of trace metals become less acute **Pintado *et al.* (1997)**.

Table (9): 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

The medium employed for citric acid production contained different sources of phosphorus (0.01043 g P/100 ml. equivalent 0.04 ml. H_3PO4 85% (sp. gr. 1.685) "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$; 4.0 (w/v) $MgSO_4.7H_2O$] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

4.2.9.Effect of H_3PO_4 concentrations on citric acid production:

Since phosphoric acid was the best phosphorus source for citric acid production, an experiment was conducted to study the effect of different concentrations of phosphoric acid on the production of citric acid. Phosphoric acid was added in seven different concentrations ranging from 0.10 to 0.7 ml/L.

As shown in Table (10), the increasing of the concentration of phosphoric acid resulted in increasing citric acid production, but increasing the phosphoric acid more than level (0.3 ml) resulted in a marked decrease in citric acid production. The citric acid production from control was low in comparison to phosphoric acid supplemented medium. These results agree with **Mashhoor *et al.* (1987)** and **El-Sawy *et al.* (1986)**. The highest value of citric acid concentration (32.88 g/L) was obtained with 0.30 ml/L phosphoric acid. Also at 0.30 ml/L 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%.

Low phosphorus concentrations were essential for good growth when iron and zinc were present in optimal concentrations. On the other, hand when the concentration of phosphorus, zinc or iron were low that for optimal growth, citric acid accumulation was achieved (**Kapoor *et al*., 1987**). Therefore, this concentration of phosphoric acid (0.30 ml/L) which proved to be the optimum, was used in the following experiments

Table (10): 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

The medium employed for citric acid production contained different concentrations of H_3PO_4 "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v); 4.0 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

4.2.10.Effect of $MgSO_4.7H_2O$ concentrations on citric acid production:

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 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 (11). 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)** who studied the effect of magnesium sulphate on citric acid in the fermentatiom medium.

Table (11): Effect of $MgSO_4.7H_2O$ concentrations on citric acid production by *A. niger* CA2.

$MgSO_4.7H_2O$ 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 medium employed for citric acid production contained different concentrations of $MgSO_4.7H_2O$ "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v) , 0.30 ml H_3PO_4 4.0 (v/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

The highest value of citric acid concentration (33.20 g/L) was obtained with 0.20 g/L magnesium sulfate. **Sakurai *et al.* (1999)** used 250 mg/L of $MgSO_4.7H_2O$ in the fermentation medium to preduce citric acid. 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

4.2.10.Effect of the inoculum volume (size) on citric acid production:

The effect of inoculum size on citric acid production is determined by varying the number of spores added as inoculum to the culture medium **(Maddox and Brooks, 1995)**. 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 $1x10^6$ to $20x10^6$ spores/ 100 ml. The results on the effect of inoculum size on citric acid production are presented in Table (12).

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)** who studied the effect of inoculum size on citric acid production. 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. The optimum inoculum size may vary depending on the substrate and experimental conditions used **(Maddox and Brooks, 1995)**.

Table (12): 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

The medium employed for citric acid production contained different concentrations of inoculum volume "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v) ; 0.30 ml H_3PO_4 4.0 (v/v) ; 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

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

4.2.11.Effect of the trace elements (metal ions) on citric acid production:

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)**.

Ten different trace elements were used to investigate their effect on citric acid production. Tested trace elements were added separately to basal medium No.2 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 (13). 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. 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)**.

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)**.

Table (13): 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

The medium employed for citric acid production contained different trace elements (100 µg/L) "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v) ; 0.30 ml H_3PO_4 4.0 (v/v) ; 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

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)**. 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, (1994a), Pera and Callieri, (1997), Jianlong, (1998)** and **Lesniak *et al.*, (2002)**.

4.2.12.Effect of $CaCl_2.2H_2O$ concentrations on citric acid production:

Papagianni *et al*. (1999a and b) and **Bayraktar and Mehmetogu (2000)** used $CaCl_2$ in the fermentatiom media to preduce citric acid. 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 (14). 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%.

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.

Table (14): Effect of $CaCl_2.2H_2O$ concentrations on citric acid production by *A. niger* CA2.

$CaCl_2.2H_2O$ (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 medium employed for citric acid production different concentrations of $CaCl_2.2H_2O$ "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v) ; 0.30 ml H_3PO_4 4.0 (v/v); 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

4.2.13.Effect of ethanol addition on citric acid production:

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).** 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 (15). 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. 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%.

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)**. The mycelial growth was inhibited by the increase of methanol concentration. Similar results were obtained from **Rugsaseel *et al*. (1995a)**. 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. 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 (15): 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

The medium employed for citric acid production different concentrations of ethanol "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v) ; 0.30 ml H_3PO_4 4.0 (v/v) ; 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Cultivation time (8 days). Incubation temperature (30°C at 150 rpm).

The third section

4.3.The Fermentor Experiment :

Sparging a batch culture with oxygen instead of air will lead to a reduction in the fermentation time without affecting the final yields. Thus the growth and production rates increase **(Kristiansen and sinclair, 1979)**. 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 **(Kim *et al*., 1995)**.

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

Microbial production of citric acid is a high aerobic process **(Drysdale and McKay, 1995)**. This experiment was conducted to study

effect of the air supply on citric acid producton using the fementor. The results on the effect of using fementor on citric acid production are presented in Table (16). 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)**.

Table (16): 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

1.5 liter of the clear supernatant diluted cane molasses was supplemented with (g/L) [2.00 $NH_4H_2PO_4$ (w/v), 0.30 ml H_3PO_4 4.0 (v/v) ; 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Incubation temperature (30°C at 200 rpm and the air flow rate 1 vvm.).

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

The fourth section

4.4.Citric acid production using immobilized spores:

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)**.

4.4.1.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 (17). 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 (17): 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

The medium employed for citric acid production "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v), 0.30 ml H_3PO_4 4.0 (v/v) ; 0.20 $MgSO_4.7H_2O$ (w/v)] Initial pH (5.5). Incubation temperature (30°C at 150 rpm).

4.4.2.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 (18). 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 (18): 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

The medium employed for citric acid production "supplemented with" (g/L) [2.00 $NH_4H_2PO_4$ (w/v); 0.30 ml H_3PO_4 4.0 (v/v); 0.20 $MgSO_4.7H_2O$ (w/v)] Incubation temperature (30°C at 150 rpm). Cultivation time (8 days).

5. SUMMARY

Studies on the microbial production of citric acid

Citric acid is one of the most important organic acids in the foods and pharmaceutical industries, citric acid had extracted from natural resources specially citrus fruits, then citric acid was begin to produced with using *Aspergillus niger*. This study aim to use *Aspergillus niger* to produce citric acid by screening some strains which can produce citric acid and screening some media which used for citric acid production, then to reach the maximum citric acid production were studied some factors affected on citric acid production.

This study divided into four sections :-

The first section :-

Screening the most active strains which can produce citric acid, and to achieve this propose nine stains of *Aspergillus niger* were used, six of it were local CA1, CA2, CA3, CA4, CA5, CA6 and three of it were exported from the Microbial Properties Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, USA. It were namely NRRL 2270, NRRL 3 NRRL 67. Six cultural media were used as basal media for citric acid production cultivation was made in 250 ml. Erlenmeyer flasks, each containing 100 ml of sterile medium. Inoculum containing $5x10^6$ spores was transferred to the culture medium. The flasks were incubated at 30°C on a rotary shaker at 160 r.p.m. for incubation period 6 days. It could observe that the best strain of citric acid producer was *A. niger* CA2 on medium No.2, which had cane molasses, and the production was 22.9 g/L citric acid.

The second section :-

Study the facores affecting on citric acid productionas follows:- The best time course was after eight days of fermentation and the production was 23.2 g/L citric acid. The optimumpH was 5.5 and the production was

29 g/L citric acid. Total sugars was 15% and the production was 29.3 g/L citric acid. The production of citric acid wes 29.84 g/L when 1.5 g/L $K_4Fe(CN)_6$ was used. 10 g/L ammonium oxalate was used and the citric acid concentration was 29.88 g/L. The effect of nitrogen sources were investigated and the best source was ammonium phosphate and the production was 31.4 g/L citric acid. The ptimum concenteration of ammonium phosphate was 2 g/L and the production was 32.03 g/L citric acid. The effect phosphorus sources were investigated and the best source was phosphoric acid and the production was 31.78 g/L citric acid. The highest amount of citric acid was 32.88 g/L when the concentration of phosphoric acid was 0.3 ml/L. 0.2 g/L magnesium sulfate was used and the production was 32.2 g/L citric acid. The optimum inoculum volume was $5x10^6$ Spores/ 100 ml and the citric acid production was 32.88 g/L. Several trace metal ions such as Fe^{++} ,Cu^{++}, Zn^{++}, Mn^{++} ,Ca^{++} ,Ba^{++}, K^{+} ,Mo^{++}, Co^{++} and Ni^{++} was studied and the addetion of ethanol were investigated and there were no effect on the production citric acid.

The third section :-

In this section we study the use of best factores affecting on citric acid production, and used it to produce the citric acid using the fermenter, and 51.1 g/L citric acid after eight days of fermentation was recorded.

The fourth section :-

In this section we study the effect of immobilized the spores with calsium algenate, and use this immobiliezd spores as an inoculum to produce citric acid. The best day was the sixth the best pH was 5.5 and the production of citric acid was 35.71 g/L. The possibility of citric acid production was 51.1 g/L in fermentor by the selected stain No, 2, meduim which containing (g/L): sugar cane molasses 272, ammonium phosphate 2, phosophoric acid 0.3 ml, magnesium sulfate 0.2, pH 5.5, at 30°C, after eight days of fermentation.

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