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CITRIC ACID PRODUCTION FROM CAROB POD EXTRACT BY THE FUNGUS *Aspergillus niger*.

A THESIS SUBMETED IN PARTIAL
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BY

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Title of Thesis

CITRIC ACID PRODUCTION FROM CAROB POD EXTRACT BY *Aspergillus niger*

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Summary

The importance of citric acid is in using it at different food and pharmaceutical industries , many studies have focused on production of citric acid from facilitated raw materials at low cost by using the microorganisms

From the results which obtained throughout this investigation which carried out to investigate the possibility of citric acid production from carob pod extract as a substrate by ***Aspergillus niger*** . The physical and chemical factors have been examined throughout this study to determine the chemical factors for the growth of A. niger and citric acid production

The higher concentration of citric acid and biomass dry weight were obtained after six days of incubation with initial pH 4.5 and sugar content 15% .

According to the results of designated experiment to find the best concentration of the nitrogen of that stimulated citric acid production we found that 0.5 % was the best and ammonium dihydrogen phosphate was the superior nitrogen source used for production of citric acid .

Applied experiment to find out the effect of different concentrations of calcium chloride in the citric acid production it revealed that , addition of calcium chloride at 0.05 % to fermentation medium stimulate citric acid production .

Addition of different methanol concentration were also examined to determine their effect on growth and citric acid production . The results showed that 3.0 % methanol was the best concentration added to carob pod medium which stimulated citric acid production

The result revealed that , the addition of 2.0 % ethanol was also stimulated citric acid production accumulation by the fungus .

List of Figures

List of Tables

Chapter one

I.1. Introduction

Citric acid , is an intermediate compound of a Tricarboxylic acid cycle. It is found as a natural constituent of variety of citrus fruit's such as pineapple , pear , peach and fig . Citrus fruit being a particularly rich source of citric acid . (Grewal & Karlra , 1995) .

The world wide demand for citric acid is increased day by day. Since the annual production of citric acid is approximately estimated 500.000 ton's , less than 1% of total world production of citric acid , is still produced from citrus fruits in countries where citrus fruits are available economically such as Mexico and South America (Yigitoglu , 1992) .

Many different microorganisms are able to convert the carbohydrate to organic acid . Industrial-scale production of citric acid is generally carried out by fermentation process with a filamentous Fungi. *Aspergilli* are used for commercial production of citric acid ,and most effective strains are *Aspergillus niger* and *A. wentii* . Their ability to produce more citric acid per unit time ,as well as the production of undesirable side products ,such as oxalic acid, isocitric acid ,and gluconic acid ,can be more efficiently suppressed in the strain *A. niger* used to produce citric acid on commercial level (Casida , 1968) .

The yeast are also able to produce the citric acid which is fast in production compared with fungal processes but they produce undesirable amount of isocitric acid in fermentation process. Different strains of yeasts were used, such as *Hansenula pichia ,Debaromyces,Torulopsis*,

Torula ,*Kluechera* ,*Trichasporon* ,*Rhodotorula*, *sporobolomyces* ,*Endomyces*,*Nematospora*,*Saccharomyces* ,*Zygosaccharomycess* and ***candida*** which are the most used strains for citric acid production . Moreover the bacterial production of citric acid is available by some strains such as ***Brevibacterium flavum*** , ***B . lichenitormis*** , ***B. subtilis*** which used glucose as a raw material and other strains of ***Brevibacterum*** species ,***Corynebacterium Arthrobacter paraffineus*** which used the paraffin as carbohydrate source . In spite of that the accumulation of citric acid from bacterial process is not developed well compared with fungal and yeast processes (Casida , 1968) .

The bulk of produced citric acid today is used in food , beverage , pharmaceutical and cosmetic industries. Also serves in pH adjustment , antioxidant and buffering agent (Haq *et. al*., 2002). Approximately 70% of produced citric acid is used in the food and beverage industries for various purposes , as well as accident in carbohydrate and sucrose based beverage , stimulates natural fruit flavor , incorporate tartness . About 12% from pharmaceuticals companies of the world produce citric acid as effervescent in powders and tablets in combination with bicarbonates , antioxidant in vitamin preparations. For other industries approximately 18% from the world production used citric acid , such as Esters citrate finds an ever-increasing application inmoder chemical industries as plasticizers and foam inhibitors for vainyl sheeting and polyester resins (Yigitoglu, 1992) .

In recent years , many efforts have focused on the discovery of new resources and improvement of the respective production methods (Papagiani *et. al*., 1998) .

The present work is a trial to produce citric acid using carob pod extract (syrup) as raw materials . The carob pod kibble is fruit of carob tree (***Certonia siliqua***) which grow in many town's of great Jamahiriya

Economically this plant is intended to use only as animal feed and for some human consumption such as carob pod syrup . So the carob syrup was used throughout this investigation as a carbon source for the growth of the fungus ***Aspergillus niger*** and production of citric acid .

1.2. Literature review

Most people think that Biotechnology is a recent development, but in fact it is not , because microorganisms have been used to produce food such as beer, vinegar , yogurt and cheese for over 8 millennia .

Ethanol was the first chemical to be produced with the aid of biotechnology over 800 years . Until just a century ago it was not realized that microorganisms were involved in the production of alcohol and vinegar. Louis Pasteur (1871) found that yeast converts the sugar to alcohol in the absence of air and the process named fermentation.

Many different types of microorganism's posses the ability to convert carbohydrate to high yields of organic acid. This property is demonstrated by various bacteria. As for example, species of ***Clostridium*** produce acetic acid and butyric acid. ***Lactobacillus*** and ***Streptococcus*** species produce acetic acid , gluconic, 2-ketoglutric acids, and ***Pseudomonas*** species produce 2-ketogluconic and α-ketoglutric acids.

Fungal organic acids that produced commercially are , citric, fumaric, gluconic, itaconic, kojic, and gibbliric acid (Casida, 1968)

A major commodity chemical manufactured by fungal fermentation is citric acid , which was first isolated and crystallized from lemon juice by Karl Wilhelm Scheele in (1784) . This organic acid is found as a natural constituent of a variety of fruits such as citrus fruits (rich source) , pineapple, pear, peach , and fig (Al-Obaidi &Berry , 1979)

The commercial production of citric acid commenced in England in (1860) from calcium citrate imported from Italy. The discovery of biosyntheses of this acid by fermentation can be traced back to (1893) when the German Botanist, Whemer. recognized it as a metabolic product of the moulds *Citromces pfefferionis* and *Citromces glaber* . (Grewal and Kalra 1995)

Citric acid manufacture by fermentation industries has its origin in the First World War. Until the year 1893 citric acid had been extracted from citrus fruits and the major producer was Italy. As men were called to arms the citrus groves were left untended. By the time hostilities ended the industry was in rains and the price of citric acid had escalated. This paved the way for the introduction of a microbial process

According to AL-Ani , Zahorski (1913)found that , the fungus *Aspergillus niger* can be used for the production of citric acid . this fungus was previously named *Sterigmatocystis niger*. Work of Tom and Currie (1916) established that , citric acid can be produce commercially by the fungus *Aspergillus niger* using shallow fermentation technology. In (1917) Currie published a search on the use of pure materials as synthetic media to produce citric acid in commercial level using *A. niger* and by adding 0.01 g of $FeSO_4$ accumulation of citric acid in the medium was increased ; Thereafter , citric acid was produced in commercial level by Fiser Company in (1923)

Saechetti (1933) found that with high concentration of NH_4NO_3 in the culture medium fungal development was stimulated and accelerated the production of citric acid . Citric acid was also influenced by KH_2PO_4 concentration , and the presence of Mg^{+} ion in culture media appears to be necessary for the accumulation of citric acid .

Sotnikov (1934) studies concerned with the production of citric acid by *A . niger* in unchanged and changed medium . He found that the production of citric acid independent on the type of added nitrogen. Moreover , Addition of Mg ion only as a nitrate salt increases the growth of the fungus and the production of citric acid.

Quilico & Ealberto (1934) explained that the production of citric acid from row beet molasses by *A . niger* is related, particularly to the amount of phosphate in the fermentation medium prepared from different typs of molasses . He also found that , the addition of molasses at concentration of 15 % sugar to fermentation media was very necessary for the synthesis of citric acid in the presence of small amount of phosphate and nitrogen source.

Cahn (1935) found that , *Aspergillus niger* growth on solid materials under suitable environmental conditions can produce considerable amount of citric acid in a relatively short period of incubation. when solid materials impregnated with sucrose or molasses as a carbon source . Citric acid yield was 55% and 45% of the total consumed molasses and sucrose respectively.

Butkevich & Melnikova, (1936) studied the influence of nitrate and potassium ions upon the accumulation of citric acid by the fungus *Aspergillus niger* ,they postulated that synthetic media of KNO_3 a nitrogen source is better than NH_4NO_3 for citric acid production by *A. niger*

Korad & Iglaver (1936) found that , citric acid production by *A. niger* fall into two general condition : (a) internal , such as the strain organism and age of culture medium ; (b) external , such as temperature , oxygen supply , acidity and food supply .

Perguin (1938) discovered that the addition of solutions of glucose or sucrose to the preformed mycelium of *A. niger* resulted in gluconic acid production . However , some citric acid was produced when the solution contained zinc sulfate , Potassium chloride and ammonium chloride at low pH .

Jacobi & Schwarz (1939) mentioned that , the growth of *A. niger* and citric acid production was improved by addition of small amounts of various colloids to the fermentation media .

Gerhard *et. al.*, (1946) used beet molasses treated with potassium Ferro - or ferric cyanide as a substrate for mycological citric acid production . They found that, about 50 % of the consumed sugar converted to citric acid .

Perlman *et. al.*, (1946 a) studies were on the production of citric acid from cane molasses, when cane molasses was fermented by high citric

acid production strains of *A. niger* in surface culture . The yields of citric acid are very low. They explained that , the inorganic constituents of the cane molasses are responsible for the low yields of citric acid obtained .

Perlman *et. al.*, (1946 b) found that , the addition of 0.1 mg. each of (Fe^{+2} , Mn^{+2} , Al^{+3} , Cr^{+3} per liter) to the synthetic medium containing the sucrose as carbon source , stimulate the yield of citric acid two folds by the fungus *A. niger*

Shu & Johanson (1948) found that , citric acid accumulation by the fungus *A. niger* decreased if the Fe^{+3} and Zn^{+2} or P^{+3} ion's are present in the fermentation medium .

Zhyravesky (1955) reported that , production of citric acid by the fungus *A. niger* in submerged culture medium was depends on the rate of aeration.

The addition of methanol at concentration of 4% to cane molasses medium and 6% to beet molasses medium highly stimulated citric acid production by the fungus *A. niger* (Puente , 1962)

Millis *et. al.*,(1963) were studied the effect of lipids on citric acid production by mutant strains of *A. niger*. They found that fatty acids with less than 15 carbon atoms inhibited fungal growth and citric acid production .

Zhuravsky (1964) explained that , the industrial citric acid production by *Aspergillus niger* was affected by the level of carbohydrate present in fermentation media .

Liang & Tung (1965) found that , production of the citric acid by *A. niger* (C-4282) strain. increased with the addition of 2% methanol or 3% ethanol to the corn starch fermentation medium, and total acid yield was ranged between 50- 60 % of the total consumed sugar .

Erich. (1966) mentioned that, the presence of hydrogen peroxide increases the accumulation of citric acid by *A. niger* in submerged cultivation medium.

Clark *et. al.*, (1966) noted that , the stimulation effect of Mg ion's on the growth of *A. niger* and citric acid production was not affected by addition of the other metals.

Horitsu & Clark. (1966) explained that , there is no stimulation effect of ferocynaide on citric acid accumulation by blocking the reaction of the Krebs cycle.

Zdzislaw (1968) pointed out that, there's no correlation between the color of *A. niger* conidia and the production of citric acid.

Fermentation activity of *A. niger* was mutch better in synthetic medium prepared with tap water than with redistilled water (Bassalik *et. al.*, 1970) .

Sanchez *et. al.*,(1970) noted that , the production of citric acid in shaker flasks by the mutant strain of *A. niger* reached to 98.7 % in resin-treated sucrose medium while the citric acid yield is 75 % in resin-treated

treated clarified syrup medium and 68% in ferrocyanied-treatd blackstrap molasses media .

Sanchez *et. al.*, (1970) pointed out that, the activity of all diploids form of *A. niger* strains was higher than the parent strains for citric acid accumulation .

Hang *et. al.*, (1975) postulated that , brewery spent grain liquor which have economic value can be used as source for the production of single cell protein and citric acid using the fungus *A. niger*.

Wold *et. al.*, (1976) explained that , The Zn ions apparently play a role in the regulation of growth and citric acid accumulation by the fungus *A. niger*.

The presence of acetic acid at pH of 2 prevents spore germination, and stimulate citric acid accumulation in fermentation medium (Hamissa & Radwan ,1977) .

Bhate *et.al.*, (1980) mentioned that , the production of citric acid decreased to reach 6% of the biomass dry weight of the fungus *A. niger* with the addition of 300µg/ml of 5-hydroxymethylfurfural.

Cotton waste apparently can be used for saving sucrose and for increasing the yields of citric acid by *A. niger* (Kiel *et. al.*, 1981).

Kahlon (1982) found that , *A. niger* strain isolated from different soil and orange produce high amount of citric acid when cultivated on

molasses and cane sugar medium , comparing to other isolated strains of *A. niger* .

Pessoda & Angela (1982) mentioned that , the optimal initial pH values for citric acid production varied according to the strain of *A. niger*

Agrawal. *et. al.*, (1983) explained that , the addition of 0.05-1mμ sodium malonate or 0.01-0.1 mμ potassium ferricyanide , iodoacetate , sodium azide and sodium arsenate or sodium flouoride stimulated citric acid to reach (36-45 %) of the biomass dry weight .

Hossain *et. al.*, (1984) found that , the addition of 3% methanol to fermentation media of the fungus *A. niger* increased the citric acid production at pH of 4.5 .

In the presence of methanol at a concentration of 3% (vol/wt) in the fermentation medium of the fungus *A. niger* NRRL 567 was highly stimulated the production of citric acid from grab pomace. The yield was 60 % of the consumed sugar (Hang & Woodams, 1985)

Hang & Woodams (1985) explained that, the greatest amount of citric acid produced by *A. niger* NRR 567 can achieved when apple pomace was used as a substrate for fungal growth in presence of 4% methanol .

Pena & Gonzales (1994) mentioned that , the excessive growth and different morphological development of mycelium inhibit citric acid production by the fungus *A. niger* in the fermentation media containing sucrose and inorganic nutrients such as ammonium nitrate and ammonium phosphate .

In shaking culture, Autodiploid strains of *A. niger* were induced by colchicines treatment to yield higher citric acid than the parent strain when glucose was used as carbon source (Sarangbin *et.al* , 1994)

The citric acid production and it's concentration was increased when the fungus was growth in fermentation medium of carob pod extract at initial pH value of 6.0(Roukas ,1998).

Pera & Cellieri (1999) explained that , the citric acid production by *Aspergillus niger* increased from 0.37 to 3.72 when 0.5g $CaCl_2$ / l was added to fermentation media , as well , reduce the biomass concentration and loose the fungal pellet form in the fermentation medium .

Yunguo *et. al.*, (1999) mentioned that , the highest concentration of citric acid in air lift bioreactor by *A. niger* was achieved when higher air flow rate supplied to the fermentation media .

Saha *et. al.*, (1999) mentioned that, the addition of 2% ethanol to the fermentation medium , of *A. niger* the yield of citric acid was 85 % with average productivity 3.8 per liter per day after fifteen days of incubation ; moreover , the yield in repeated - batch fermentation

technique was 65% with average productivity (2.3g per liter per day) after sixteen days of incubation .

Sarangbin & Watanapokasin (1999) mentioned that , the mutant strains of *A. niger* which was cultured with soluble starch medium produced citric acid 1.5 time as much as parental strains .

Jianlong (2000) found that , the presence of n-dodecane to fermentation media had no adverse effects on the cells of *A.niger* while enhanced citric acid accumulation with addition of 5% (v/v) n-dodecane .

Zhuang & Zhang (2001) mentioned that, the cultivated *A. niger* for the production of citric acid from livoglucosan fermentation medium needed a longer incubation time to consume the substrate .

Shojaosadati & Babaeipour (2002) explained that , under optimal cultivation conditions , the fungus *A. niger* in solid state bioreactor produced 124g of citric acid from 1 kg dry apple pomace when used as a carbon source .

Citric acid biosynthesis stimulated due to the reduction of Fe^{2+} ions by the addition of 2.0×10^{-5}M $CuSO_4$ to the fermentation medium, when cane-molasses was used as carbohydrates source (Haq *et. al.*, 2002)

The citric acid production by the mutant strains of *A. niger* was 71.4 and 92.9g/l from 200g/l corn and potato starch when they used as carbon source in the fermentation medium respectively , while the parental strain producing only 1.44 and 1.2 from the amount of corn and potato starch (Haq. *et. al.* ,2003 a)

Hag *et. al.*, (2003. b) found that, the pellet formation and increases the permeability the cell membrane of *A.niger* influences by addition of methanol to fermentation media which led to high accumulation of citric acid

1.3. Factors that effect the production of citric acid

1.3.1. Carbon sources

Many carbon sources were used as energy sources and means of the growth of the cells .Therefore , the carbon source can be obtained from different sources to be available to cells . Production of citric acid from different carbon sources has been studied intensively and these sources have marked effect on citric acid production by *A. niger*

Nature of sugars and their concentrations influence the yield of citric acid by *A. niger* (Shu & Johanson , 1948) *A. niger* can take up simple sugars such as glucose , fructose or sucrose for high yield of citric acid . Maltose and sucrose , two disaccharides , were found to be better carbon sources for production of citric acid , while *A. niger* readily assimilates of citric acid from these sugars , but the yields were lower than from glucose (Hossain *et. al.*, 1984)

Yigitoglu (1992) found that , combination of two sugars in the medium at 50% of each improved the yields of citric acid from sucrose-glucose : glucose-sorbital : glucose-xylose and xylose sorbital combinations with the mutant strains of *A. niger*

1.3.2. Nitrogen sources

The nitrogen requirements for citric acid production by the fungus was generally met by the addition of nitrogen source and biosynthesis of citric acid are highly effected by the source and concentration of the nitrogen present in the fermentation media (Grawal & Kalra. 1995)

Xu *et. al.*, (1989) investigated the effect of different nitrogen sources on citric acid production by *A. niger* ; They found that urea was the most suitable nitrogen source in which citric acid production reach to 32 kg per m^3 . Chen (1993) Found that , the best nitrogen concentration added to the fermentation medium of citric acid production by fungus ***Aspergillus foteidus*** was 2% of ammonium nitrate .while Grewal & Kalra (1995) reported that high nitrogen concentration increases fungal growth and consumption of sugars , but decreased the accumulation of citric acid . Tisnadjaja *et. al.*, (1996) Found that , the presence of 0.53 g/l ammonium chloride to fermentation medium. of ***Candida guilliermondi*** stimulated citric acid production .

Yunguo *et. al.*, (1999) investigated the ability of *A. niger* for the citric acid production when 2g/l of diammonium sulfate was added to the fermentation medium .He found that , the percent of citric acid was increased to reach 50.5 % of biomass dry weight.

1.3.3. Mineral requirements

1.3.3.1. Phosphorus source

The presence of phosphate in the medium has a profound effect on the yield of citric acid; even phosphorus compound inter in the structure of amino acid and phospholipids. The addition of phosphate to synthetic media was chosen according to the pH value and usually it supplied in the form of potassium phosphate such as (potassium dihydrogen phosphate, dipotassium hydrogen phosphate or tripotassium phosphate) (Hawker , 1968)

Grewal & Kalra (1995) reported that, phosphorus concentration (0.5-5.0 %) was required by the fungus in a chemically defined medium for maximum production of citric acid . while Sarangbin & Watanapokasin. (1999) used 1% potassium dihydrogen phosphate in yam bean starch fermentation medium to produce citric acid by protease-negative mutant strain of *A niger* .Moreover, 1% of dipotassium hydrogen phosphate was added as buffer to the fermentation medium which containing levoglucosan as carbon source (Zhuang &Zhang 2001) .

1.3.3.2. Magnesium

Magnesium is essential for growth and citric acid production due to its role as a cofactor in a number of enzyme reactions in the cell and the best source of Mg^{2+} ion's was magnesium sulfate (Yigitoglu, 1992).

Tran. *et al* ., (1998) found that. when different concentration of mg^{2+} ions added to fermentation medium of pineapple waste used as a carbon source stimulated the citric acid production and the best concentration was 20 p.p.m Mg^{2+} ions . Moreover , Sarangbin & Watanapokasin (1999) used the magnesium sulfate in the fermentation medium of citric acid production by *A. niger* .

1.3.3.3. Trace elements

Trace element nutrition is probably the main factor influencing the yield of citric acid by the fungus *A. niger* . such as Manganese , Iron . potassium and copper which are probably the main factors influencing the citric acid production (Grewal & Kalra ,1995).

Tran *et. al.*, (1998) investigated the effected of different concentration of $MnSO_4.H_2O$ on citric acid accumulation they found that , the addition of 0.05 p.p.m of Mn^{2+} ions to pineapple wastes medium promoted the biosynthesis of citric acid by the fungus *A . niger* .

Copper ions play an important role in decreasing deleterious effect of iron on citric acid production. Jerneje *et. al.*, (1982) mentioned that, copper ions is an essential requirement for citric acid production and optimum concentration of Cu^{2+} was 40p.p.m to high yield of citric acid.

Haq *et. al.*,(2002) Throughout their investigation on the different sources of copper ion's , they found $CuSO_4.H_2O$ at the concentration of $2.0 \times 10^{-5} \mu$ was the best concentration for the production of citric acid .

Tran *et. al.*, (1998) study the effect of different ion's concentration on the production of citric acid , He found that , the addition of 5p.p.m of Fe2+ ion's to pineapple waste fermentation medium . the citric acid content increased to reach 18 g / 100 g of dry weight .

Addition of 2 mg of $FeSO_4 .H_2O$ to the fermentation medium of *A . niger* highly stimulated citric acid production (Sarangbin & Watanapokasin, 1999)

1.3.3.4. Temperature

The speed of most chemical reactions increases as the temperature rises .The temperature degree depend on the type of microorganism used production method and type of medium(Tortora *et. al.*,1986) .

Mehrotra & Jyoti (1972) studied the effect of temperature on citric acid production by six strains of *A. niger* . They found that. the temperature requirement differed slightly with the strain and carbohydrate used , but the optimum range of temperature for acid production was 20 – 35 °C

Papagianini *et.al.*, (1994) found that, the temperature degree 28 °C in batch and fed-batch culture was optimal degree for citric acid accumulation by the fungus *A. niger* .

In general the range of temperature applied was 25-35 °C , whereas the regarded was 26-28 °C as the optimal temperature for citric acid production .The temperature degree more than 30 °C tend the biochemical

reactions to produce oxalic acid rather than citric acid (Ahmed & Elnaway , 1999)

Roukas (1999) found that . concentration of citric acid was increased significantly when the temperature of fermentation medium was raised from25C ° to 30 °C and the maximum yield of citric acid was at 30 °C

1.3.3.5. Hydrogen ion concentration (pH)

Hydrogen ions concentration (pH) refer to the acidity or alkalinity of the solution and maintenance of low pH is essential for maximum production of citric acid (Grewal & kalra ,1995) .

Papagianni *et .al*., (1994) Explained that . the higher pH value led to lower citric acid formation and high accumulation of oxalic acid in fermentation media ,whereas at pH 2.1 citric acid yield was 60.52 g/l. In stirred tank reactor high concentration of the citric acid was obtained at pH 2.1 and lowest citric acid production was at pH 4.5 . when used as carbon source

Roukas & Harvey (1988) found that , citric acid being the predominant product at low pH value ; whereas, at high pH the fungus *A. niger* accumulated gluconic acid .

Kan & Ghose (1973) reported that . citric acid was increased by increasing the pH value of the fermentation media of *A. niger* and the optimal initial pH was found to be 6.5 .

Roukas (1998) mentioned that , the lowest value of pH (1.5) was accompanied with greatest accumulation of citric acid in carob pod extract medium .

Watanabe *et.al.*, (1998) described that , the maximum amount of citric acid was achieved (90.02g/l) in cellulose fermentation medium when the initial pH of fermentation medium was kept at 6.0 .

1.3.3.6. Aeration and Agitation

The important of aeration and agitation in aerobic fermentation processes is to supply the necessary oxygen to microorganisms for the metabolic activities to keep the microorganisms in suspension. The stoichiometry for the microbial production of citric acid can be described by the following equations.

$$C_6H_{12}O_6 + 1.5O_2 \dashrightarrow C_6H_8O_7 + 2H_2O$$

$$C_6H_{12}O_6 + 6O_2 \dashrightarrow 6CO_2 + 6H_2O$$

The first equation represents the oxygen requirement for the oxidation of glucose to citric acid, and the second equation represents the oxygen requirement for the complete oxidation of glucose to carbon dioxide and water which is necessary for cellular maintenance and metabolism in an aerobic organism.

Clark &Lentz (1961) and Kristiansen & Sinclair (1979) found that citric acid accumulation increased significantly by increasing the concentration of dissolved oxygen . Moreover , Khan &Ghose (1973)

reported that, incubation for five days at 500 rpm was the optimal agitation speed for citric acid production.

Dawson *et. .al.*, (1986) indicated that, by increasing the concentration of dissolved oxygen from 25 to 75 %of the saturation value the citric acid yields increased from 41 to 60 %, and volumetric productivity increased from 99 mg to 324 mg citric acid.

Dawson *et .al.*, (1988) mentioned that , maximum citric acid yield and high production rates were observed at higher dissolved oxygen tension values (90% saturation) in fed batch culture .

Roukas (1991) the highest yield of citric acid 28kg/100 kg dry weight was obtained in agitated culture (300 rpm). Ran &Sims (1994) found that, during citric acid production, as the concentration of dissolved oxygen was increased from 20 to 80 % saturation, the specific oxygen uptake and Specific citric acid productivity increased to reach 160% and 71 %, respectively.

Papagianni *et.al.*(1999) found that, the high yield of citric acid (50 g/L) by the fungus *A. niger* was obtained after six days of incubation at agitation speed 200 rpm .

1.3.4 Culture media

Industrial microorganisms produce a small amount of desired product , therefore , improving the processes productivity start by using optimal fermentation condition and selecting the suitable medium . The

sciences investigated in the last few years production of citric acid by *A. niger* (Roukas,1999)

Roukas & Kotzekidou (1997) used date syrup as a sole carbon source for citric acid production . Hang & Woodams (1998) postulated that . corncobs could serve as a potential source of raw material for growth and citric acid production. While Tran *et. al* ., (1998) using pineapple waste as raw material for citric acid production . Shankaranand and Lonsane *et. al.*, (1994) applied different agro-industrial waste such as , sugar cane , bagasse , coffee husk , and cassave bagasse for their efficiency in production of citric acid by *A.niger* . On the other hand Zhuang & Zhang (2001) postulated that , levoglucosan can be using as sole carbon and energy source for citric acid production , while Haq *et .al.*, (2003 a) investigated direct production to citric acid from raw starch by *A.niger*

1.3.5 .The fungus

The *Aspergillus niger* is the fungus which commonly called black mould . The grows widely and distributed from the aretic to the tropic regions it produce large numbers of spores ,the *A. niger* capable of utilizing a enormous substances for food because it has large number of enzymes . (Elnaiiy , 1996)

To their geat enzymatic activities , Aspergill employed in several industrial processes ,such as citric acid and gluconic acids are manufactured commercially by *A. niger* , the fungus classified according to Bold et, al., (1980) as follow :

Kingdom	:	Myceteae
Division	:	Amastigomycota
Class	:	Ascomycetes
Order	:	Eurotiales
Family	:	Eurotiaceae
Genus	:	*Aspergillus*
Species	:	*niger*

1. 3.6. Carob tree

The carob tree (***Ceratonia siliqua***) is a member of ***CESALPINIACE*** family and grown in Mediterranean areas . It has a wide range of separate in many towns of Jamahiriya such as Gable Akhdar , near Derna and Ras Al-Hilal ,near Tobrouke (Ali & Jafri , 1976) . It is a large tree which grows to 15 m in 50 years of growth (Fig. 1) . The annual production is about 340.000 – 400.00 metric tons , Greece is primary producer with annual harvest of 21.000 tons . (Roukas , 1998)

Fig(1): Carob pod tree in nature (*Ceratonia siliqua*)

The carob tree doesn't have any economic value on the industrial scale in Jamahiriya except some human industries such as carob pod syrup or as animal feed . So the carob pod regard a rich carbohydrate source and can be used as substrate for citric acid production by *A. niger*

1.4. The aim of study

The aim of this study was about the citric acid production from carob pod extract by the fungus *A. niger* , by using many factors that stimulate biosynthesis of citric acid , such as effect of different incubation periods , hydrogen ion's concentration , sugar concentrations , nitrogen concentrations of $NaNO_3$, nitrogen sources , $CaCl_2$concentration , methanol and ethanol concentrations , amino acids as well as comparison between standard medium and carob pod medium with respect to citric acid production by *A. niger* .

Chapter Two

2. Material and method

2.1. The microorganism

Aspergillus niger EMCC 1132 was used throughout this investigation , which has been imported from Egypt microbial culture collection , Cairo MIRCEN . It was maintained on potato dextrose agar slants and sub cultured at intervals from 5-6 weeks (Fig. 2) .

2.2. Culture media

2.2.1. Potato-dextrose-agar (PDA) Medium

This medium used to maintain the fungus strain throughout this study , and it prepared by boiling 200 g of peeled and sliced potato in 500 ml distilled water for 1 hour , thereafter , it was filtrated and 20g of dextrose sugar and 15g of agar were added to filtrated process then the volume completed to 1 liter .The hydrogen ion concentration (pH)value of the medium was adjusted to 4.5. and sterilized by autoclave (All American model No.25x autoclave) at 1.5 lb /in^2 and 121°C for 15 minute. After that , the sterilized media distributed in Petri dishes of adequate size and stored in refrigerator at 4°C

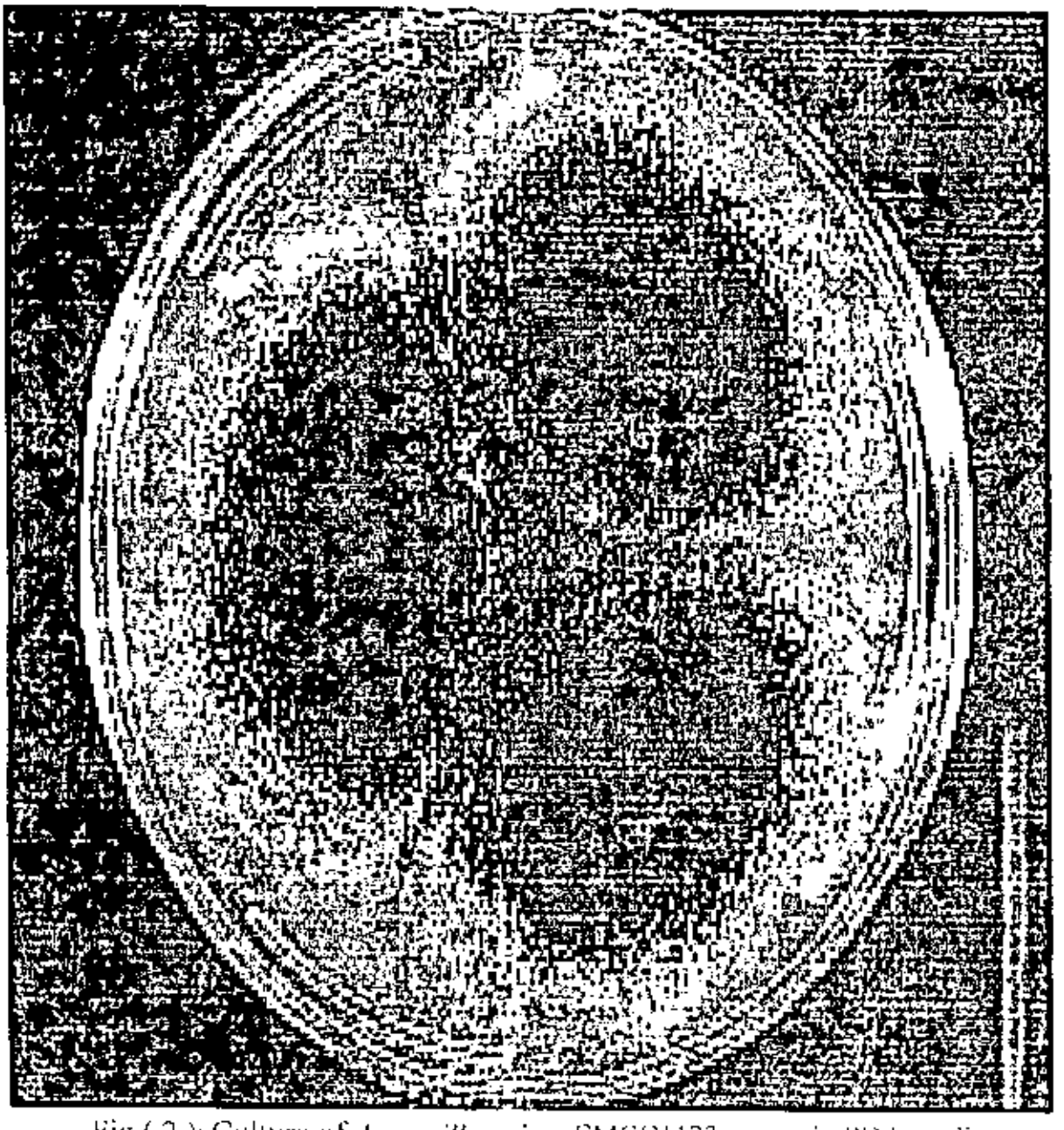


Fig (2): Culture of *Aspergillus niger* EMCC1123 grown in PDA medium

2.2.2. Standard medium

standard medium has the following composition g/l

Sucrose	1
NH_4NO_3	2
KH_2PO_4	1
$MgSO_4$	0.025
$CuSO_4$	0.06
$FeCL_3$	0.001

The pH was adjusted at 4.5 using Digital pH meter and the volume completed to one liter by sterile D-H_2O .

2.2.3. . Carob pod extracted media

The carob pod is a fruit of carob tree (***Ceratonia siliqua***) were collected from natural willed plants ,in which the carob pod syrup was used as carbon source throughout this study . After carob pod have been collected the seeds removed and the carob kibble infused in hot water over night .Thereafter , the infusion was filtrated to remove the residual kibble intended from this procedure . The residual solution from previous step boiled for 7-8 hours and leave to cool to get the carob pod syrup . The sugar concentration of the syrup was 60 % ; and used as carbon source for growth of the fungus *A. niger* and production of citric acid . In order to prepare 500ml of fermentation media of carob pod syrup containing sugar at the concentration of 10 % , 120mg carob pod extract

was added to 400ml distilled water and 0.3 % of $NaNO_3$ as a nitrogen source was added . The solution was mix well and the pH adjusted to 3.5 (using 1 N NaOH and 10 % HCL) using hydrogen ion concentration instrument . Then the volume was completed to 500 ml .

2.3. Activation of the microorganism

Aspergillus niger EMCC 1132 activated by repeating cultivation in PDA slant medium every one month .

2.4. Inoculum preparation

The cultures were incubated on PDA slants at 30 °C for 5 days . The fungal spore were transported to PDA cultural media at pH of 4.5

2.5. Cultural conditions

The fermentation media were adjusted to pH 3.5 (using 10% HCL and 1N NaOH). 0.3% $NaNO_3$ as a nitrogen source was added . after that , the media were distributed into 250 ml conical flasks in triplicate samples receiving each 50ml of broth medium . There after , plugged and covered by aluminum foil before being autoclaved for 15 min. at 121 °C . After cooling the culture flask were incubated with 2% of fungal spores suspension (Appr. $4.5x10^6$). The inoculation culture flasks were incubated for sufficient time at 30 °C and at specifically interval , three replicates of each culture flasks were withdrawn for further analysis .

2.6. Analytical methods

2.6.1. Measurement of initial and final pH value

The adjustment of the initial and final hydrogen ion concentration (pH) value of fermentation media was ob ained by using hydrogen ion concentration instrument (pH METER SCHOTT TYPE CG 842) .

2.6.2. Determination of biomass dry weight

To determine the biomass dry weight of *Aspergillus niger* after the end of incubation period , the fungal biomass was separated from fermented medium , using Bukhnar funnel . The fungal mycelium was dried overnight at 60-65 °C using Memmert 600 Oven . Thereafter, the biomass dry weight was measured accurately using SCALTEC SPB 63 MODEL balance .

2.6.3. Estimation of the citric acid concentration

To determine the citric acid concentratic n in fermented media at the end of incubation period ; 1 ml of fermented broth medium was titer against 1 ml of NaOH using phenolphthalein as an indicator in which tittered 1ml of NaOH equivalent to 62.03 of citric acid (Dubois *et. al.*, 1956) .

2.6.4. Estimation of the initial and residual sugar concentration

Estimation of the sugar concentration in carob pod syrup was performed according to the described method of (Roukas , 2000) . In which 1ml of carob pod syrup diluted to 1000 ml distilled water then 1 ml of diluted syrup ,1 ml of 5% phenol solution and 5 ml of concentrated H_2SO_4 (96 %) were added . Then the sugar concentration was calculated according to the standard curve previously prepared for different concentration of glucose 10-100 mg/ ml as shown in Fig (3). The optical density was measured by spectrophotometer using (JENWAY 6100) at 490nm .

2.6.5. Determination of chemical composition of carob pod extract

The chemical content of carob pod extract have been determined according to AOAC , 1984 procedure as follow :-

2.6.5.1. Estimation of protein content of carob pod kibble syrup

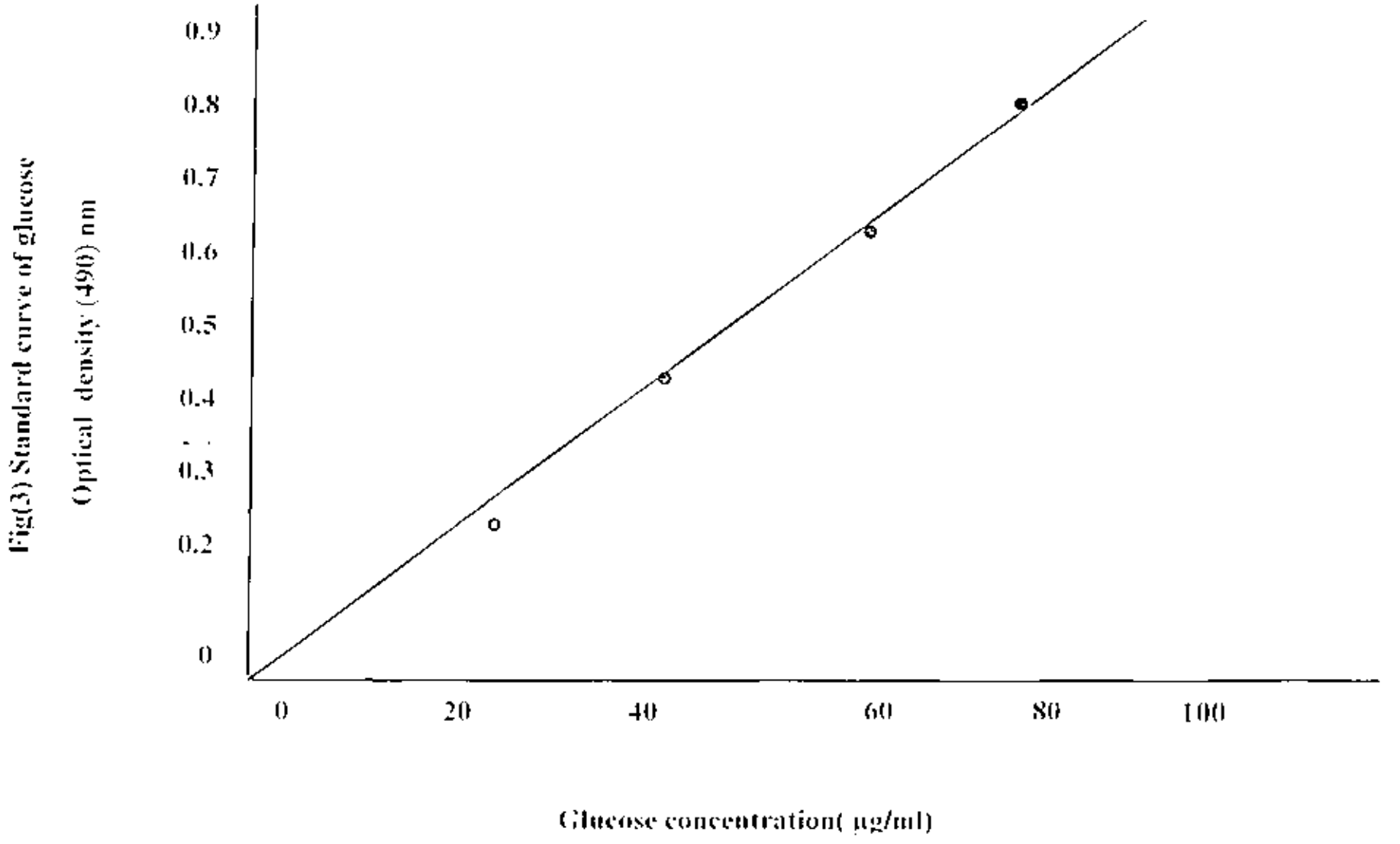
The percentage of protein content was estimated using the following equation :

$$\text{Protein percentage} = \frac{\text{Hydrogen content} \times 6.25 \times 0.14}{\text{weight of sample}}$$

Were

6.25 = protein constant

0.14 = Nitrogen constant



Fig(3) Standard curve of glucose

2.6.5.2. Estimation of soluble substances (Fat percentage) .

The amount of fat in carob pod kibble syrup was estimated by Soxholate instrument from Gerhardt Soxtherm and petroleum ether (60 - 80 °C) as organic solvent for fifteen hours .

2.6.5.3. Estimation of ash percentage .

10 g of carob pod syrup was taken and burned using Muffle Furnace electric oven at 600 °C for five hour . and ash content estimate in the weight of sample using SCALTEC SPB 63 MODEL balance .

2.7. Experiments

2.7.1. Effect of different incubation periods on citric acid production

This experiment was developed to determine the best incubation period for citric acid production by the fungus *A. niger* . The sugar concentration in the medium was prepared at 10 % and sodium nitrate at concentration of 3% as a nitrogen source were added . Then the pH was adjusted to 3.5 . The medium were distributed to 250 ml conical flasks in triplicate samples receiving each 50 ml of broth medium before being sterilized at 121 °C for 15 min . After incubation, the culture inoculation media were incubated in shaking incubator (150 rpm) at 30 °C for (2 . 4 . 6 . 8 . and 10 days).Triplicate sample's after each incubation period withdrawn randomly for determination of final pH , biomass dry weight , citric acid concentration and residual sugar.

2.7.2. Effect of different pH values on citric acid production

Many values of Hydrogen ion concentration were applied throughout this experiment . The media were adjusted to different pH value (2.5 . 3 . 3.5 . 4 . 4.5 . 5 . 5.5 . 6 . 6,5 and 7), using (1N NaOH and 10 %HCL) respectively the nitrogen source was added at concentration of 3% in the form of $NaNO_3$. After that , the media were distributed into

250 ml conical flasks in triplicate samples receiving each 50 ml of broth medium . The incubation were performed at 30 °C in shaking incubator (150 rpm)for six days , after that the final measurements were taken .

2.7.3 Effect of different sugar concentrations on citric acid production

Different sugar concentration in the form of carob pod syrup were prepared , to determine the best sugar concentration that promote citric acid accumulation . The fermentation media were prepared in triplicate samples containing (2.5 , 5 ,7.5 , 10 , 12.5 , 15 , 17.5 and 20 %) sugar. The pH of sample were adjusted to 4.5 . After six days of incubation the samples were withdrawn for farther analysis .

2.7.4. Effect of different concentration of $NaNO_3$ on citric acid production

This experiment was designed to determine the best nitrogen concentration that stimulate citric acid production .Therefore , different concentrations of nitrogen source in the form of sodium nitrate were prepared (0.1 , 0.2 , 0.3 , 0.4 , 0.5 , 0.6 , 0.7 , 0.8 , 0.9 and 1.0%) in triplicate form plus control (without nitrogen source) . The experiment continued for six days . After that the replicates were removed from incubator for the final measurements .

2.7.5. Effect of different nitrogen sources on citric acid production

This experiment was performed to investigate the best nitrogen source that stimulate citric acid production . Different nitrogen sources namely [(NH_4) $_2$ SO_4 0.5 % , NH_4CL . 0.5 % , NH_4 H_2 PO_4 , 0.5 % , $NaNO_3$, 0.5 % , Peptone , 0.5 % and urea , 0.5 %] were prepare containing equivalent levels of nitrogen with respect to that present in 0.5% of $NaNO_3$. The sources were added to fermentation media in triplicate samples . And The experiment continued for six days in shaking incubator (150 rpm) . Thereafter , final analyses were taken .

2.7.6. Effect of different concentrations of $CaCl_2$ on citric acid production

This experiment was developed to determine the effect of $CaCl_2$ on citric acid accumulation by the fungus *A. niger* . Therefore , different concentrations of $CaCl_2$ (0.01 , 0.05 , 0.1 , 0.15 , 0.2 , 0.25 and 0.30 %) were added to the fermentation media in triplicate samples , incubated at 30 °C for six days , before being removed for further analysis.

2.7.7. Effect of different concentrations of methanol on citric acid production

To determine the best concentration of methanol that stimulate citric acid production by *A. niger* , triplicate samples of fermentation

media were prepared for each concentration of methanol (0.5 , 1.0 , 2.0 , 3.0 , 4.0 and 5.0 %) the experiment was continued for six days . Then , the final analysis were taken .

2.7.8. Effect of different concentrations of ethanol on citric acid production

Different concentrations of ethanol (1 , 2 , 3 , 4 and 5 %) were added to the fermentation media (2.7.7) in triplicate samples to determine their effect on citric acid production . The experiment was performed for six days . After that , final analyses were taken .

2.7. 9. Effect of different concentrations of amino acids on dry weight and citric acid production

After the addition of the requirements of the media (2.7.8.) different concentrations (2, 3, 4 and 5 × 10^{-3} M)of amino acids in the form of (lysine , cystine ,glutamic acid and asparagine) were added separately to the culture media in triplicate samples . The experiment was continued for six days in shaking incubator (150 rpm) . Thereafter , the final analysis were taken .

2.7.10. Comparison between standard medium & carob pod medium with respect to citric acid production

This experiment was designated to compare between standard medium (2.2.2) and stimulated carob pod extract medium (2 . 7. 9) with respect to citric acid production . Triplicate samples were prepared from each medium before being sterilized at 121°C for 15 min . After cooling the medium was inoculated and incubated in shaking incubator(150 rpm)for six days at 30 °C . At the end of the experiment the culture media were removed from the incubator , for estimation of final pH . biomass dry weight , residual sugar and citric acid concentration .

Chapter Three

3. Results

3.1. The chemical composition of extract carob pod

The percentage of chemical content of carob pod extracted were recorded in Table (1) .

3.2. Effect of different incubation periods on citric acid production by *A. niger* .

The effect of different incubation period on the production of citric acid have been studied to detect the most proper time for a maximum production . The carob pod medium was used ,and citric acid had been determined at interval times of (2, 4, 6, 8 and 10 days) the results are given in table (2)and figure (4).

As shown ,the amount of citric acid increased with the incubation time reaching 4.7g/L ,(24.4 %) after six days of incubation and then decreased again with the time reaching 4.28g/L , (17.79 %) after 10 days.

The same trained have been observed for the biomass dry weight ,whereas , it is increased with the time up to 23.6g/L after 6 days ,and then started to decrease again .

Table (1) The percentage of chemical content of carob pod extract .

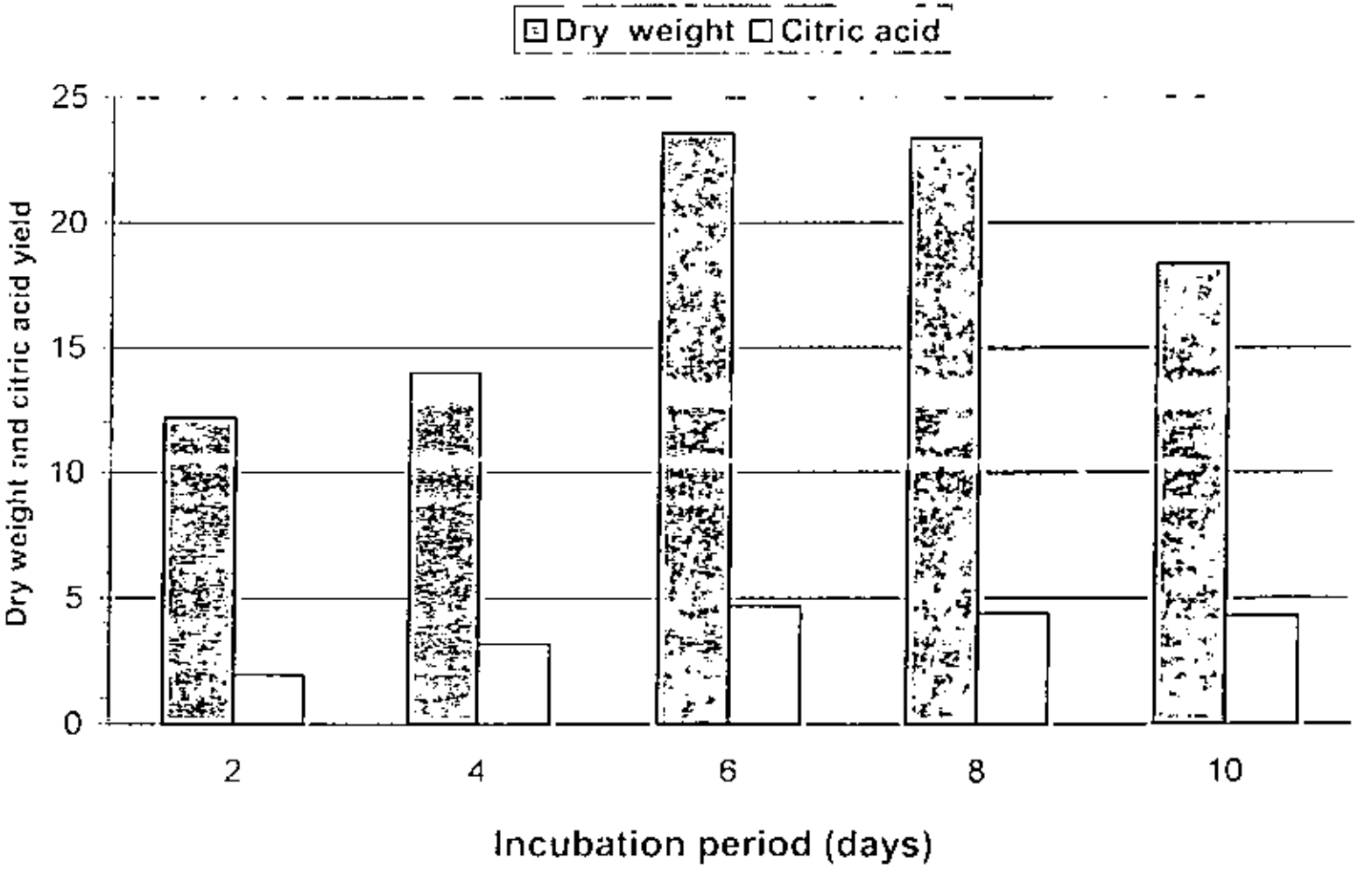
Chemical content	%
Sugar	60
Protein	3.0
Fat	1.0
Ash	1.2

Table (2) Effect of different incubation periods on dry weight and citric acid production by *A. niger* .

Incubation period	Dry weight g/l	Citric acid		Residual sugar	Final pH
		g/l	%		
2	12.2 (0.151)	1.96 (0.17)	16.25 (1.98)	0.103 (0.015)	3.83 (0.015)
4	14 (0.13)	3.18 (0.115)	20.6 (2.42)	0.082 (0.005)	3.55 (0.09)
6	23.6 (0.151)	4.70 - (0.14)	24.4 (3.26)	0.075 (0.002)	3.16 (0.015)
8	23.4 (0.103)	4.40 (0.037)	18.07 (1.3)	0.057 (0.0047)	3.19 (0.104)
10	18.4 (0.115)	4.28 (0.034)	17.79 (1.309)	0.054 (0.0058)	3.46 (0.105)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± S D)

FIG(4)Effect of incubation periods on dry weight and citric acid production



It is clear that the most of the carbon source present in the fermentation media was utilized by the fungus during the incubation periods . The final pH value was also declined specially after 6 days of incubation to reach 3.16 .

The statistical analysis program One-way ANOVA test at significance level ($\alpha = 0.05$) showed that , there are a significant differences ($P = 0.000$) within treatments of biomass dry weight and citric acid production . List significance differences L S D test revealed a significant differences in the biomass dry weight of *A. niger* at different incubation period except (2 & 4) and (6 & 8) treatments . While a significant differences are observed for citric acid production at all incubation period .

3.3. Effect of different pH values on citric acid production by *A. niger*

The acidity of the medium is a one of the most important parameters which affect on the amount of the acid produced. The amount of citric acid has been determined at different pH- values varied from 2.5 up to 7 after six days of incubation time at 30 °C. The results are given in table (3) and by Fig (5) .

In general , the amount of citric acid produced and the biomass dry weight increased as the pH value increases and the yields was 5.06 g/L , (21%)at pH value of 4.5 and the minimum yields 2.71g/L . (11.6 %) at pH 7.0 , whereas , the highest amount of biomass dry weight of *A. niger* was obtained at pH 5.5 (25.13 g/L)

On the other hand , the amount of residual sugar decreased with increases of pH values to reach 0.028 g/L at pH 7.0 .

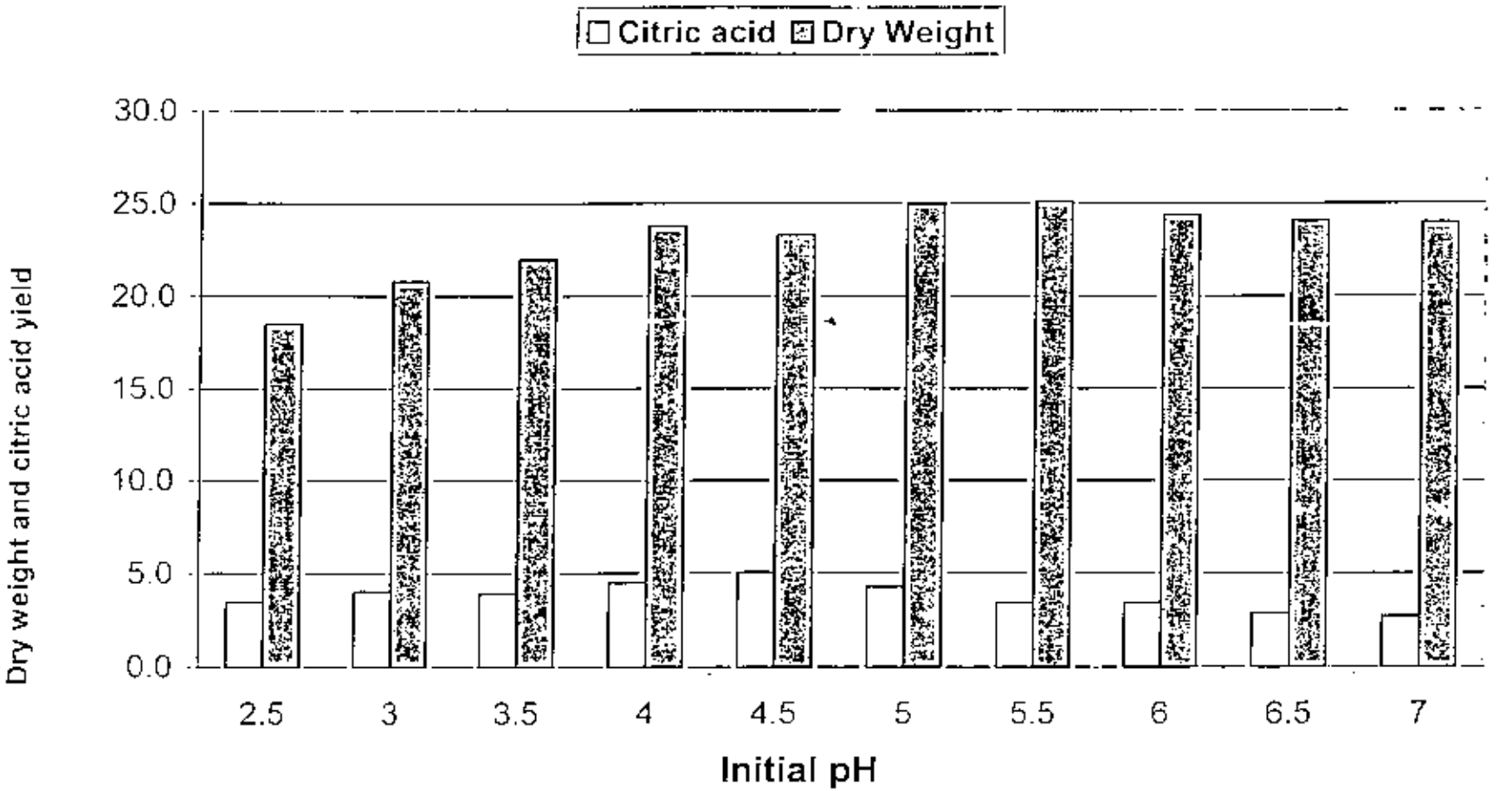
The statistical analysis program one–way ANOVA test at significance level (α = 0.05) was used and the result showed that there are significance difference (P = 0.000) within treatments in biomass dry weight and amount of citric acid produced . LSD Test revealed no significant differences in dry weight between treatments (2.5 & 3.0) , (3.0 & 3.5) , (3.5 & 4.0 to 7.0) , (48 & 4.5 to 7.0) , (4.5 & 5.0 to 7.0) , (5.0 & 5.5 to 7.0) , (5.5 & 6.0 to 7.0) , (6.0 & 6.5 to 7.0) and (6.5 & 7.0) . In case of citric acid production, no significant differences obtained

Table (3) Effect of different pH values on dry weight and citric acid production by *A. niger*

Initial pH	Dry weight g/ l	Citric acid		Residual sugar	Final pH
		g/ l	%		
2.5	18.5 (0.65)	3.48 (0.035)	18.60 (1.2)	0.065 (0.003)	3.0 (0.058)
3.0	20.8 (0.304)	4.05 (0.180)	19.40 (1.13)	0.064 (0.0037)	3.6 (0.0431)
3.5	22.0 (0.277)	3.91 (0.17)	16.40 (0.51)	0.057 (0.01601)	3.47 (0.022)
4.0	23.8 (0.30)	4.50 (0.057)	18.70 (0.30)	0.048 (0.0015)	3.70 (0.087)
4.5	23.33 (0.34)	5. 06 (0.15_)	21.0 (0.608)	0.045 (0.001)	4.06 (0.035)
5.0	25.0 (0.2)	4.28 (0.088)	16.80 (0.628)	0.041 (0.0017)	4.38 (0.020)
5.5	25.1 3 (0.41)	3.43 (0.099)	14.10 (0.67)	0.03 (0.00301)	4.38 (0.020)
6.0	24.40 (0.30)	3.44 (0.099)	14.10 (0.67)	0.03 (0.00301)	4.30 (0.25)
6.5	24.07 (0.229)	2.85 (0.15)	12.10 (0.34)	0.03 (0.0025)	4.50 (0.045)
7.0	24 (0.34)	2.71 (0.138)	11.6 (0.191)	0.028 (0.001)	4.58 (0.026)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± S D)

FIG(5)Effect of initial pH on dry weight and citric acid production



between citric acid and pH treatments (2.5 & 5.5) , (2.5 & 6.0) ,(3.0 & 3.5) and (5.5 & 6.0).

3.4. Effect of sugar concentration on citric acid production by *A. niger*

This experiment has been carried out to find the effect of different sugar concentrations in the form of carob pod extract on the production of citric acid by *A. niger* . The results are shown in table (4) and figure (6) .

The results evident that , the amount of citric acid produced increased with sugar concentration up to 11.5g /L , (45.89 %) at 15% sugar content and then decrease again with further increases the sugar percent in fermentation medium . This indicated that , the most suitable sugar concentration is 15% to get the maximum amount of citric acid produced . While the lowest amount of the acid 8.16 g/L , (28.9 %) was obtained at sugar concentration of 20% .

As shown in presented data that ,the lowest value of final pH was detected at highest yields of citric acid which reached 2.06 and only (0.015 g/L) concentration of residual sugar was obtained in the same fermentation medium .

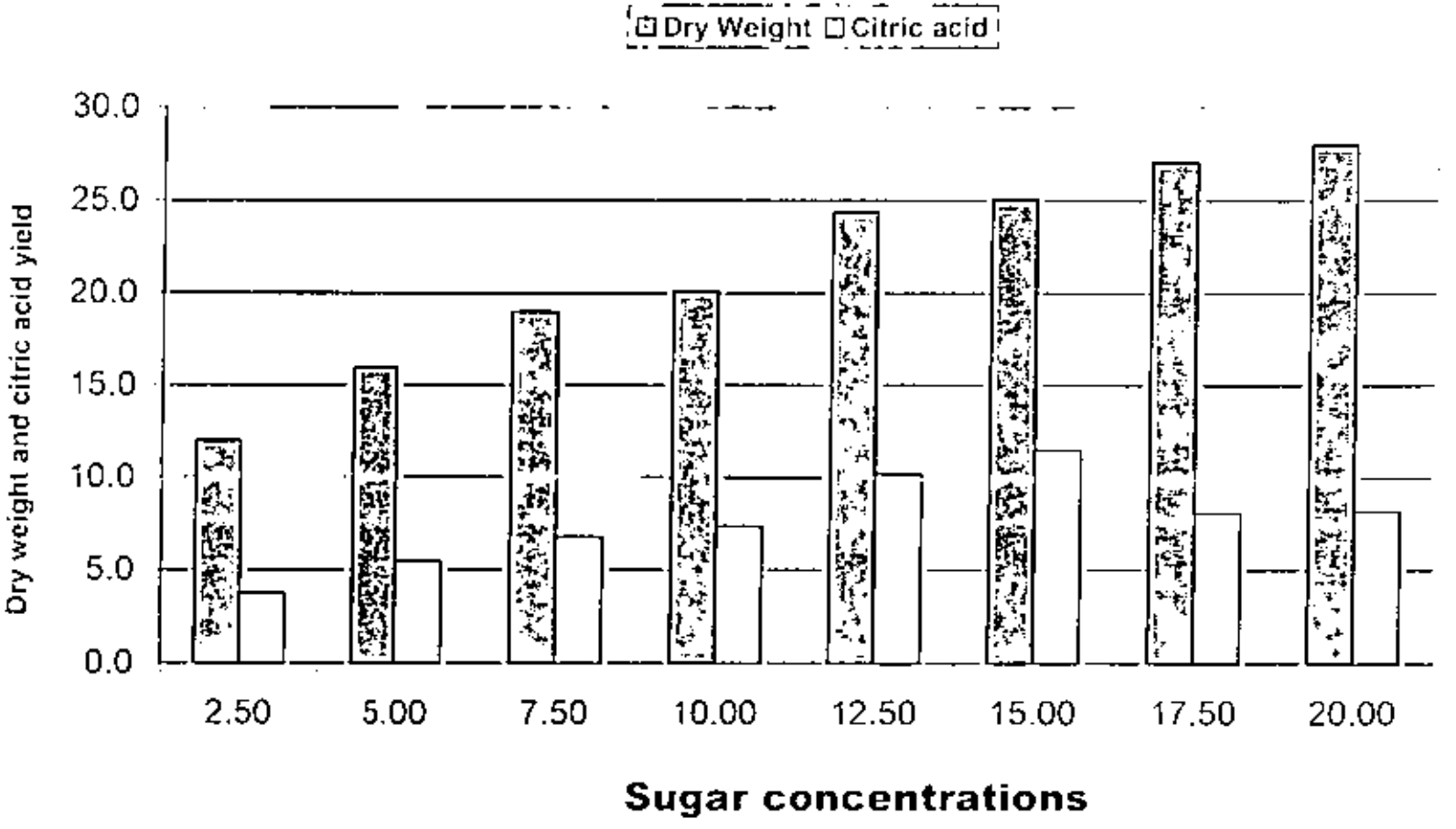
It is clear that there is a direct relationship between biomass dry weight of *A. niger* and sugar concentration in the culture media in which the dry weight increased gradually to reach 28.0 g/L , in the culture median containing 20 % .

Table (4) Effect of sugar concentrations on citric acid production by *A. niger*

Sugar Concentration%	Dry Weight g/l	Citric acid		Residual Sugar	Final pH
		g/l	%		
2.5	12.0 (0.113)	3.77 (0.68)	30.90 (0.151)	0.025 (0.015)	2.64 (0.19)
5.0	16.0 (0.17)	5.46 (0.135)	34.06 (0.889)	0.054 (0.0081)	2.26 (0.031)
7.5	19.0 (0.0961)	6.77 (0.046)	35.52 (0.073)	0.051 (0.014)	2.13 (0.034)
10	20.13 (0.141)	7.35 (0.459)	37.70 (0.173)	0.038 (0.025)	2.1 (0.04)
12.5	24.40 (0.40)	10.20 (0.117)	41.80 (1.60)	0.028 (0.025)	2.09 (0.08)
15.0	25.06 (0. 30)	11.50 (0.090)	45.89 (0.90)	0.015 (0.0014)	2.06 (0.028)
17.5	27.07 (0.67)	8.08 (0.034)	29.70 (0.36)	0.037 (0.018)	2.09 (0.071)
20.0	28.0 (0.30)	8.16 (0.27)	28.90 (0.99)	0.039 (0.0056)	2.10 (0.02)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± S D)

FIG(6)Effect of different sugar concentration on dry weight and citric acid production



One-way analysis of variance ANOVA test at significance level ($\alpha = 0.05$) showed that , there are significances difference ($P = 0.000$) within treatment in dry weight and citric acid . LSD Test revealed that , there is significant differences in biomass dry weight between treatments except (2.5 & 5.0) , (5.0 & 7.5) , (5.0 & 10.0) , (7.5 & 10.0) . (10.0 & 12.5) , (12.5 & 15.0) . (12.5 & 17.5) . (12.5 & 20.0) , (15.0 & 17.50) , (15.0 , 20) and (17.5 & 20) . All treatments showed significant difference in case of citric acid production except for (17.5 & 20.0) .

3 .5. Effect of different concentrations of $NaNO_3$ on dry weight and citric acid production by *A. niger*

To determine the best nitrogen concentration required to get the maximum production of citric acid . different concentrations of $NaNO_3$ are used . The amount of citric acid and the biomass dry weight of *A. niger* after six days of incubation at 30 °C , pH 4.5 and 15 % sugar are calculated and given in table (5) and Fig. (7) .

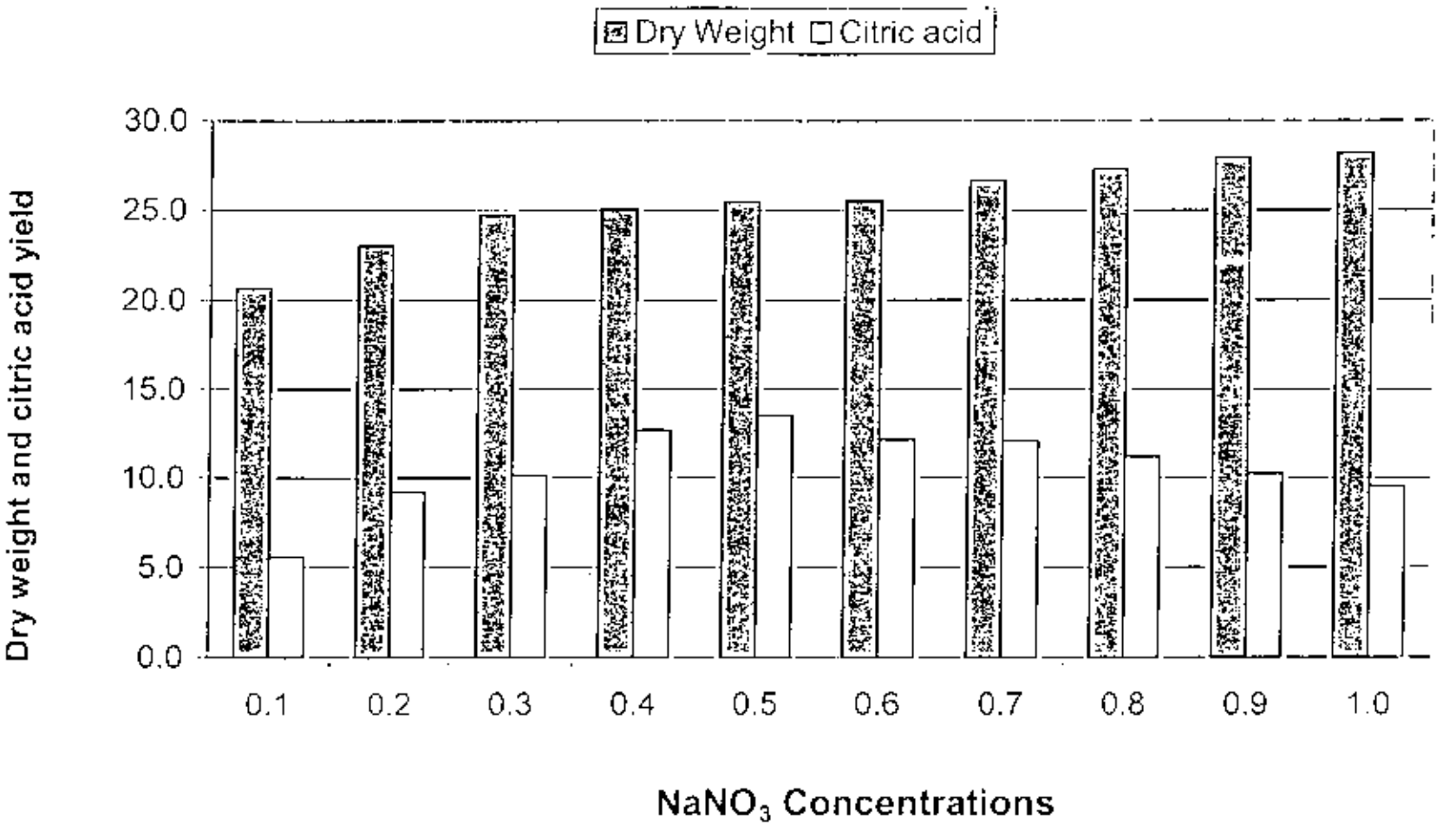
As shown . the highest a mount of citric acid production was detected in the culture medium containing 0.5 % sodium nitrate which reach to 13.5 g /L . (53.0 %) and biomass dry weight (25.47 g /L) .By Increasing of salt concentration , above 0.5 % have suppression effect on citric acid yield and promoted fungal growth to reach 28.2 g/L at concentration of 1 % $NaNO_3$.

Table (5) Effect of different concentrations of $NaNO_3$ on dry weight and citric acid production by *A. niger*

$NaNo_3$ Concentration %	Dry Weight g/ l	Citric acid		Residual Sugar	Final pH
		g/ l	%		
0.1	20.67 (0.50)	5.59 (0.15)	27.30 (1.0157)	0.062 (0.0063)	3.15 (0.106)
0.2	23.07 (0.05)	9.22 (0.132)	42.30 (3.60)	0.046 (0.035)	2.7 (0.014)
0.3	24.73 (1.135)	10.14 (0.316)	42.40 (5.35)	0.039 (0.0041)	2.15 (0.084)
0.4	25.06 (0.61)	12.69 (0.120)	50.60 (2.51)	0.026 (0.087)	2.11 (0.024)
0.5	25.47 (0.61)	13.50 (0.090)	53.0 (0.43)	0.017 (0.0032)	2.00 (0.339)
0.6	25.53 (0. 32)	12.14 (0.229)	48.10 (0.346)	0.021 (0.0025)	2.05 (0.289)
0.7	26.67 (0.36)	12.09 (0.151)	45.5 (0.506)	0.015 (0.00371)	2.35 (0.240)
0.8	27.30 (0.150)	11.20 (0.410)	41.60 (1.403)	0.012 (0.0034)	3.47 (0.007)
0.9	27.93 (0. 53)	10.26 (0. 251)	36.20 (1. 076)	0.011 (0.002)	2.69 (0.131)
1.0	28.20 (0. 53)	9.56 (0. 207)	34.50 (0.98)	0.009 (0.0021)	2.8 (0.014)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± D S)

FIG(7)Effect of nano3 concentration on dry weight and citric acid production



Lowest recorded of final pH value (2.0) was obtained with highest amount of accumulated citric acid . The amount of sugar was decreased by increasing the amount of nitrogen sources in the fermentation medium .

The statistical analysis program one-way ANOVA test at significance level ($\alpha = 0.05$) showed that , there are significance deference ($P = 0.000$) within treatments in biomass dry weight and citric acid result . LSD test revealed that , there are significant differences in biomass dry weight between treatment except (0.1 & 0.2) . (0.2 & 0.3). In case of citric acid production all treatments showed a significant difference.

3.6. Effect of different nitrogen sources on citric acid production by *A. niger*

This experiment was conducted to study the effect of different nitrogen sources on citric acid production to explore the best one .There for , different nitrogen sources containing equivalent amount of nitrogen present in 0.5 % of $NaNO_3$ were applied and then amount of acid produced was calculated .

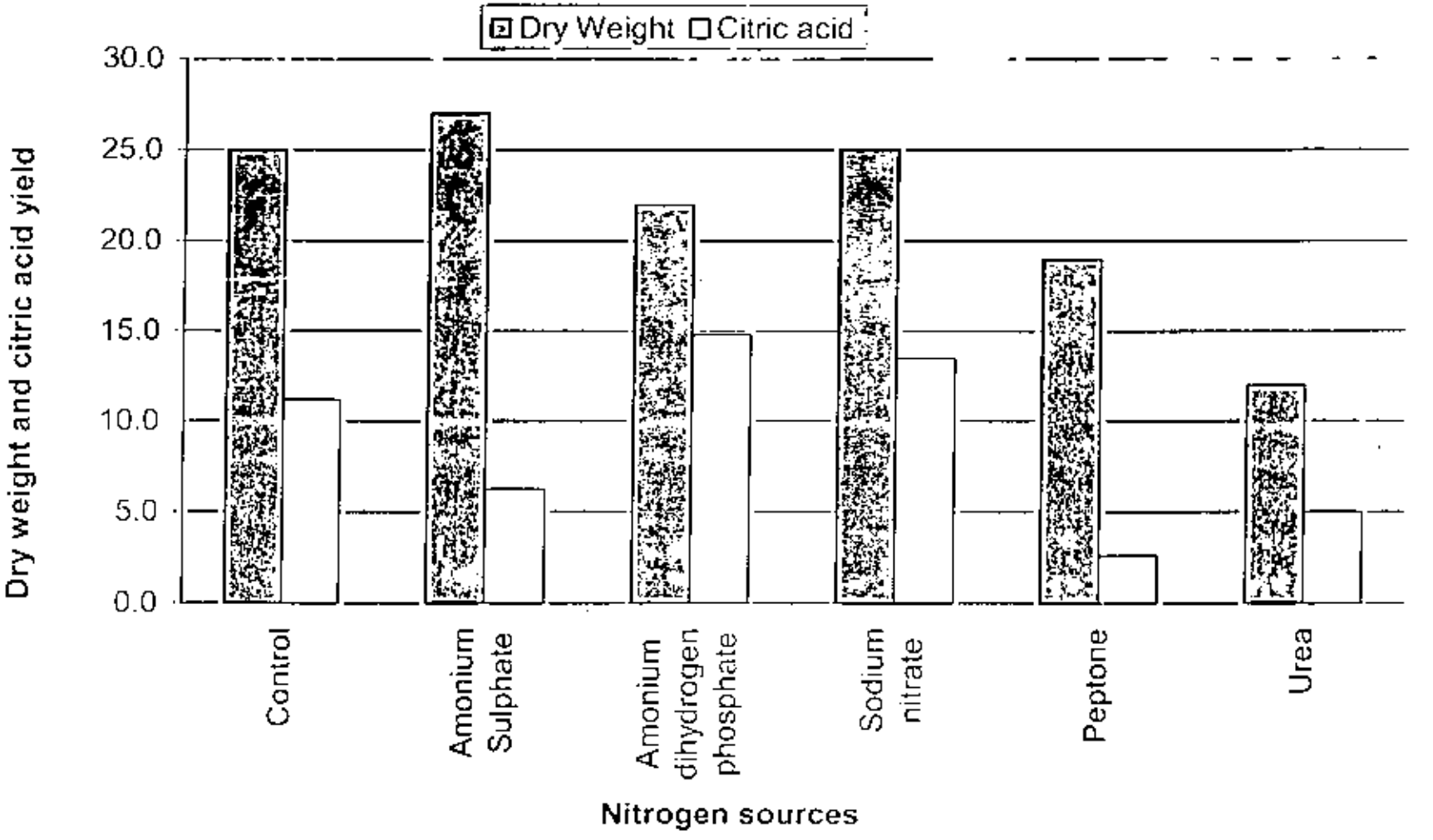
The result in table (6) and fig (8) showed that , ammonium dihydrogen phosphate was more superior than other different nitrogen sources with respect to citric acid production and the produced amount was 14.85 g / L (67.8 %) and biomass dry weight 22.0 g / L . Followed

Table (6) Effect of different nitrogen sources on dry weight and citric acid production by *A. niger*

Nitrogen Sources	Dry Weight g/ l	Citric acid		Residual Sugar	Final pH
		g/ l	%		
Control	25.0 (0.529)	11.20 (0.07)	44.70 (0.52)	0.03 (0.009)	2.20 (0.02)
$(NH_4)_2SO_4$	27.0 (0.057)	6.25 (0.10)	23.16 (2.15)	0.027 (0.008)	2.61 (0.063)
$NH_4H_2PO_4$	22.0 (0.0217)	14.85 (0.032)	67.80 (7.06)	0.012 (0.007)	1.96 (0.015
$NaNO_3$	25.03 (0.025)	13.50 (0.09)	53.90 (1.75)	0.02 (0.005)	2.15 (0.04)
Peptone	19.0 (0.113)	2.58 (0.08)	13.50 (1.01)	0.080 (0.0049)	3.81 (1.28)
Urea	12.0 (0.1)	5.01 (0.08)	41.70 (2.07)	0.076 (0.0021)	3.04 (0.09)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

FIG(8)Effect of nitrogen sources on dry weight and citric acid production



by sodium nitrate where 13.5 g / L (53.9 %) and biomass dry weight 25.03 g/L were obtained of citric acid

The other nitrogen sources namely peptone , ammonium sulphate and urea proved to be unsuitable sources for citric acid accumulation comparing to that of control .

The highest yield of citric acid was associated with the lowest value of final pH and residual sugar which obtained in fermentation medium contain ammonium dihydrogen phosphate as a nitrogen source .

The statistical analysis program one-way ANOVA test at significance level (α = 0.05) showed that , high significant differences (P = 0.000) within treatments in case of biomass dry weight and citric acid production . Dunnett t - test revealed that , no significant difference between biomass dry weight and control . While , illustrated high significant differences between(control & sodium nitrate) and (control & ammonium dihydrogen phosphate) .

3.7. Effect of different concentrations of $CaCl_2$ on citric acid production by *A. niger*

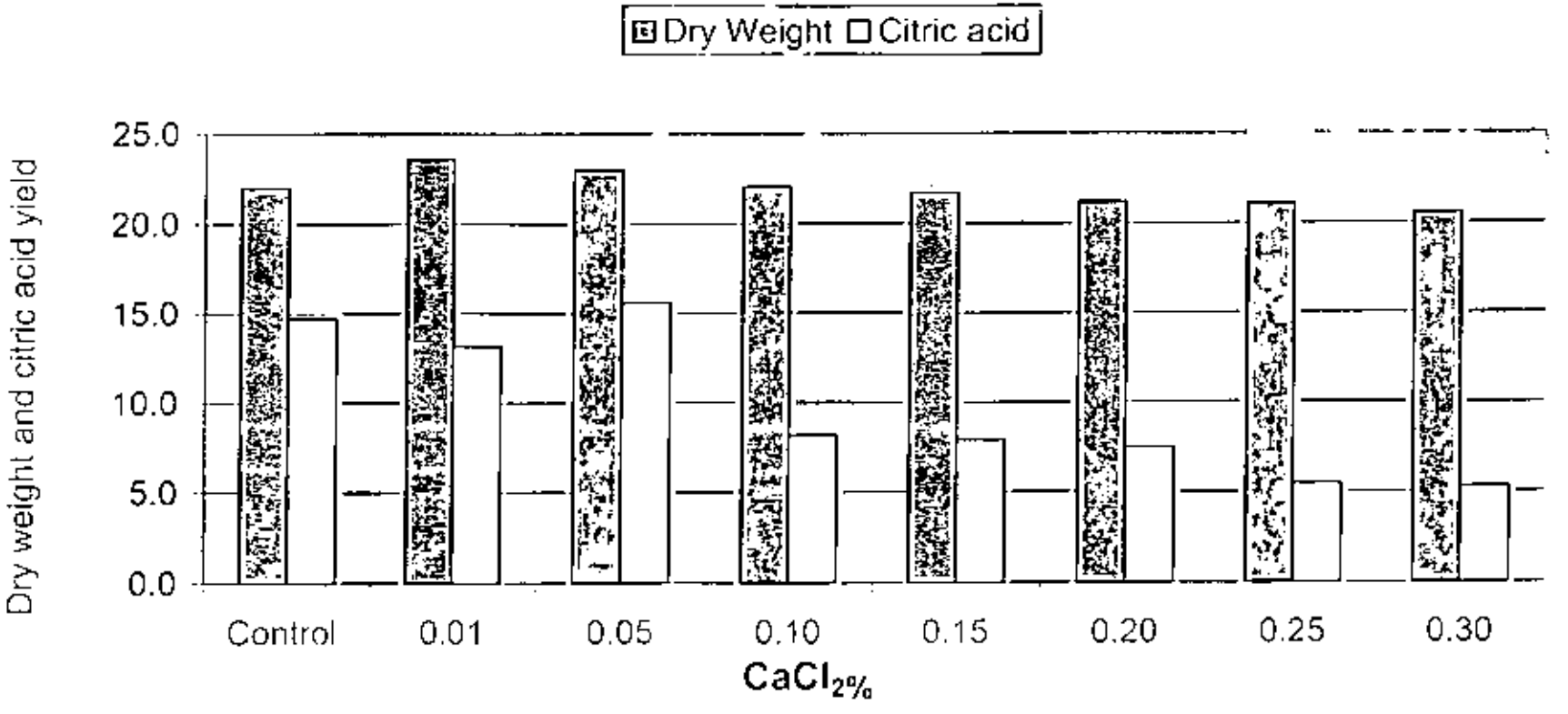
This experiment was carried out to detect the best concentration of $CaCl_2$ that stimulate citric acid production . Table (7) , and Fig . (9) showed that , the addition of $CaCl_2$ to the fermentation at concentration of 0.05 % highly stimulated citric acid production to reach 15.6 g/L (73.0%) . While , the amount obtained biomass dry weight was 23.6 g/L in fermentation medium containing 0.01 % $CaCl_2$.

Table (7) Effect of different concentrations of $CaCl_2$ on citric acid production by *A. niger*

$CaCl_2$%	Dry Weight g/l	Citric acid		Residual Sugar	Final pH
		g/l	%		
Control	22.0 (1.0)	14.73 (0.057)	6.69 (0.31)	0.018 (0.003)	2.00 (0.049)
0.01	23.6 (0.304)	13.16 (0.142)	56.14 (1.012)	0.024 (0.013)	1.78 (0.094)
0.05	23.0 (0.043)	15.60 (0.087)	73.0 (0.398)	0.015 (0.011)	1.88 (0.0421)
0.10	22.06 (0.64)	8.16 (0.17)	37.00 (1.72)	0.025 (0.011)	1.88 (0.042)
0.15	21.70 (0.41)	7.90 (0.15)	36.30 (0.55)	0.015 (0.003)	1.87 (0.007)
0.20	21.20 (0. 15)	7.50 (0.11)	35.60 (0.70)	0.014 (0.005)	1.88 (0.13)
0.25	21.10 (0.12)	5.47 (0.09)	25.80 (0.64)	0. 012 (0.0014)	1.9 (0.049)
0.30	20.6 (0.24)	5.27 (0.19)	25.50 (0.70)	0.01 (0.002)	1.92 (0.09)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

FIG(9)Effect Of $CaCl_2$ On dry weight and citric acid production



It is clear that , the value of final pH was decreased in all treated media and the lowest pH value was 1.78 obtained in medium containing 0.01 $CaCL_2$.

The statistical analysis program one-way ANOVA test at significance level (α = 0.05) showed that , high significant differences (P = 0.000) within treatments of biomass dry weight and citric acid production. Dunnett t–test indicate that , there's significance difference between . (0.01 , 0.05) comparison with control of dry weight . While

,the result of citric acid showed that , high significance difference between 0.05 % concentration and control .

3.8. Effect of methanol concentration on citric acid production by *A. niger*

This experiment was carried out to determine the most suitable concentration of Methanol to be added to the fermentation medium for citric acid production by *A . niger* .

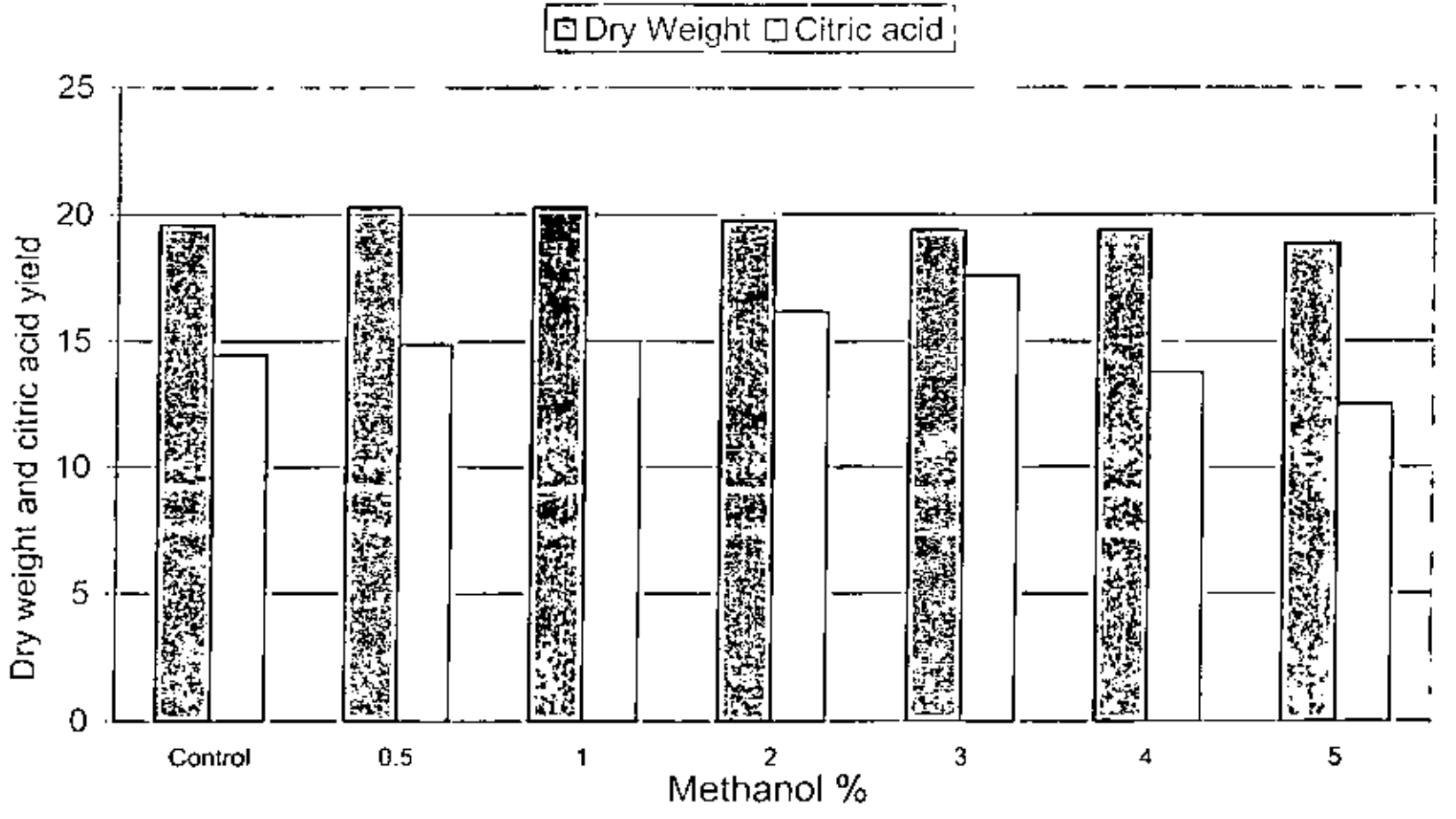
The result in table (8) and Fig . (10) showed that , the presence of 3.0 % methanol to fermentation medium increased the citric acid production to reach 17.61 g / L (92.02 %) and the biomass dry weight was 19.4 g / L . By increasing the methanol concentration above 3.0 % reduced citric acid production when compared to the amount of citric acid produced in control medium .

Table (8) Effect of methanol concentrations on dry weight and citric acid production by *A. niger*

methanol %	Dry Weight g/l	Citric acid		Residual Sugar	Final pH
		g/l	%		
Control	19.60 (0.83)	14.46 (0.153)	72 (3.2)	0.011 (0.007)	1.94 (0.02)
0.5	20.30 (0.41)	14.86 (0.169)	62.03 (2.905)	0.013 (0.0014)	1.99 (0. 036)
1.0	20.30 (0.46)	15.02 (0.196)	65.2 (1.305)	0.026 (0.0012)	2.04 (0.0529)
2.0	19.80 (0.17)	16.17 (0.116)	71 (1.906)	0.012 (0.0034)	2.00 (0.173)
3.0	19.40 (0.30)	17.61 (0.139)	92.02 (2.7)	0.019 (0.009)	2.02 (0.069)
4.0	19.44 (0.34)	13.76 (0.214)	64.2 (1.74)	0.023 (0.002)	2.05 (0.13)
5.0	18.90 (0.14)	12.51 (0.153)	57.06 (0.988)	0.025 (0.0018)	2.1 (0.2)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

FIG(10)Effect of methanol concentrations on dry weight and citric acid production *A. niger*



The biomass dry weight was slightly increased in the medium containing 0.5 % and 1 % of methanol respectively to reach (20.30 g/L) in both media .

It is not worthy to stat that , the final pH values was decreased in all treated fermentation medium . The best value of residual sugar was achieved at 2% methanol (0.012%)

The statistical analysis program one-way ANOVA test at significance level ($\alpha = 0.05$) showed that , high significant difference ($p = 0.000$) within treatments , Dunnett t-test revealed , there are no significance difference between treatments of dry weight compared with the control . But there is a significant differences in the concentration of citric acid production between treatments (control &2.00 %) and (control & 3.0 %)

3.9. Effect of ethanol concentration on citric acid production by *A. niger*

This experiment has been carried out to find the effect of the addition of different ethanol concentration to carob pod medium on the production of citric acid by *A . niger* . The experiment was continued for six days at 30 C° and the results presented in table (9) and Fig (11) .

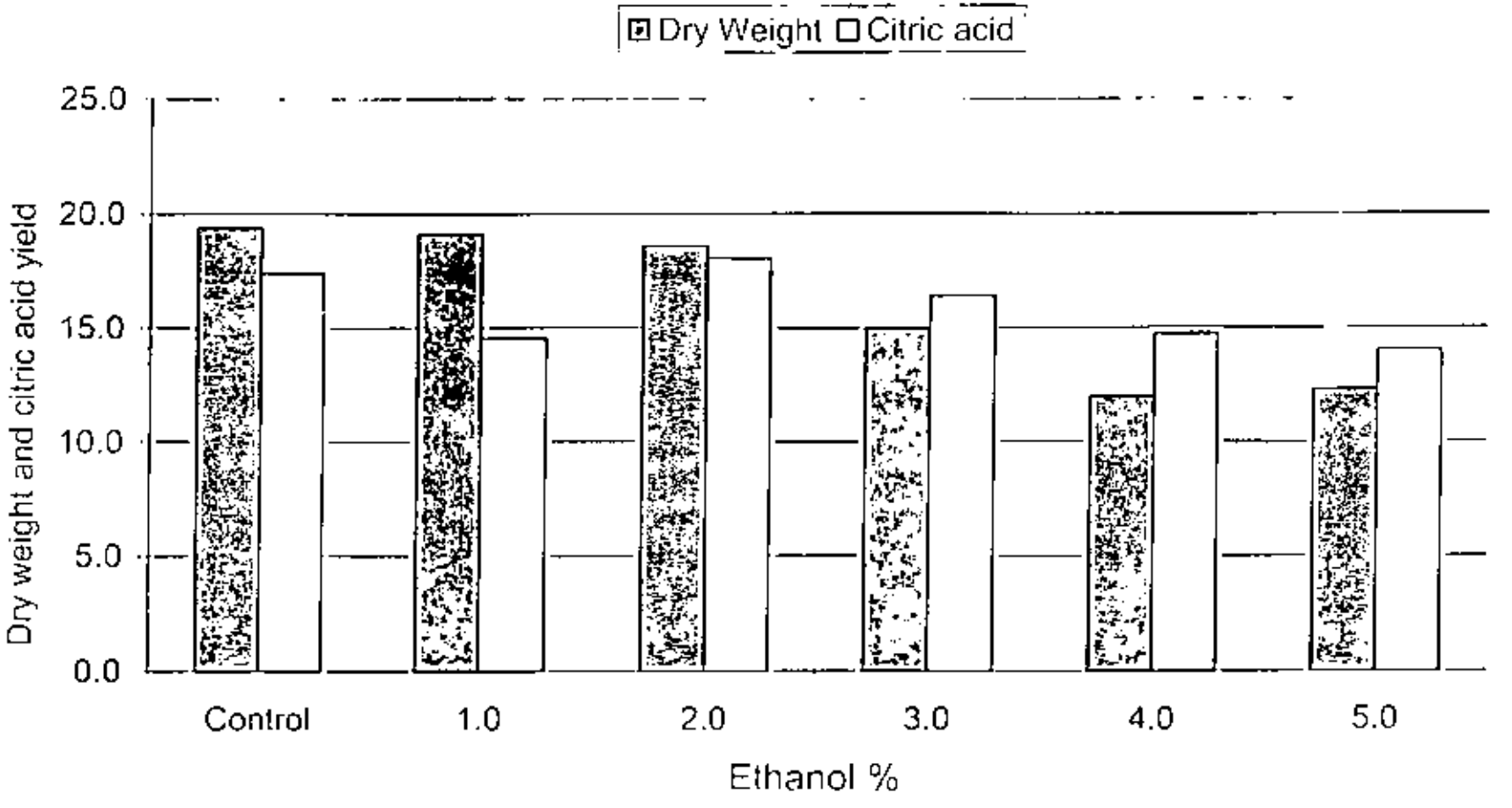
The results indicated that , the highest amount of citric acid accumulation by the fungus 18.06 g / L , (91.6 %) was obtained in culture medium containing 2 % . Moreover , it is clear that there is a divers relationship between biomass dry weight and concentration of added

Table (9) Effect of ethanol concentrations on dry weight and citric acid production by *A. niger*

Ethanol %	Dry Weight g/l	citric acid		Residual sugar g/l	Final pH
		g/l	%		
Control	19.40 (0.80)	17.40 (0.091)	90 (3.72)	0.01 (0.003)	2.07 (0.059)
1	19.10 (0.032)	14.52 (0.085)	76.03 (3.68)	0.014 (0.0052)	2.09 (0.062)
2	18.60 (0.02)	18.06 (0.153)	91.6 (1.14)	0.019 (0.004)	2.12 (0.079)
3	15.0 (0.062)	16.40 (0.45)	109.70 (1.60)	0.01 (0.0091)	2.29 (0.149)
4	12.0 (0.031)	14.75 (0.092)	122.90 (4.20)	0.022 (0.005)	2.49 (0.083)
5	12.30 (0.045)	14.01 (0.092)	120.4 (2.94)	0.038 (0.01)	2.57 (0.06)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

FIG(11)Effect of ethanol concentrations on dry weight and citric acid production



ethanol , and the lowest amount of dry weight was 12.0g/l, at 5 % ethanol .

It is clear that , the lowest final pH value were increased with the amount of added ethanol in medium .The lowest value of residual sugar was obtained at 3 % of ethanol

One-way ANOVA test at significance level ($\alpha = 0.05$)showed that , there are significant differences (p = 0.000) within treatments in biomass dry weight and citric acid production . Dunnett t-test revealed that there is no significant difference between treatments biomass dry weight compare with the control . For citric acid production revealed significant difference between treatment (control & 2.0 %) .

3.10. Effect of amino acid concentrations on citric acid production by *A. niger*

This experiment was developed to determined the effect of different concentrations of some amino acid on citric acid production in carob pod medium by *A niger*.

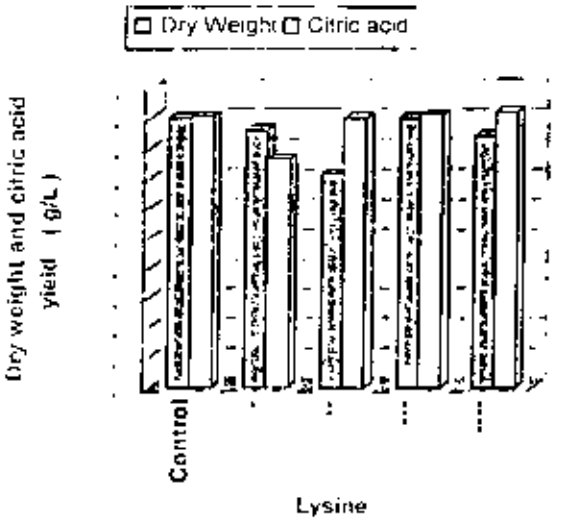
The result in table (10) and Fig (12) showed that , glutamic acid was found to be the best selected one among the amino acids . studied in the present investigation with respect to the citric acid .

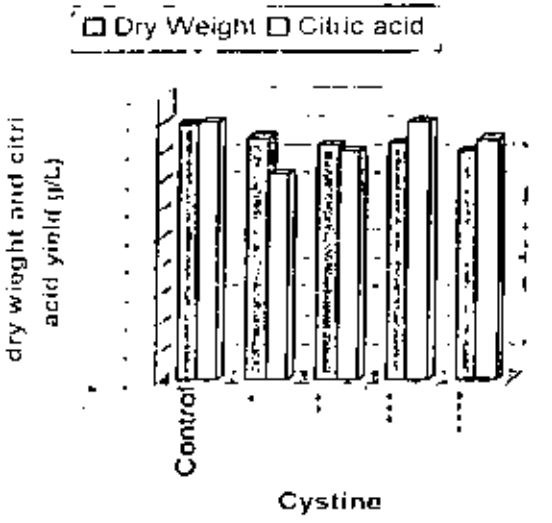
Table (10) Effect of different concentrations of amino acids on citric acid production by *A. niger*

Amino acid concentration / M / Measurement	Control	cystine				lysine				glutamic acid				asparagine			
		$^{-2}10\times2$	$^{-3}10\times3$	$^{-4}10\times4$	$^{-5}10\times5$	$^{-2}10\times2$	$^{-3}10\times3$	$^{-4}10\times4$	$^{-5}10\times5$	$^{-2}10\times2$	$^{-3}10\times3$	$^{-4}10\times4$	$^{-5}10\times5$	$^{-2}10\times2$	$^{-3}10\times3$	$^{-4}10\times4$	$^{-5}10\times5$
Final PH	2.49 (0.08)	2.04 (0.001)	2.01 (0.03)	2.05 (0.0018)	2.11 (0.02)	2.08 (0.06)	2.11 (0.04)	2.17 (0.065)	2.,6 (0.103)	2.12 (0.079)	2.11 (0.02)	2.14 (0.052)	198 (0.055)	2.10 (0.2)	2.20 (0.03)	2.12 (0.106)	2.03 (0.01)
Residual Sugar g/l	0.033 (0.004)	0.024 (0.003)	0.023 (0.001)	0.011 (0.005)	0.013 (0.0015)	0.005 (0.01)	0.016 (0.0037)	0.02 (0.0026)	0.016 (0.003)	0.012 93)	0.028 (0.0053)	0.018 (0.0052)	0.01 (0.0091)	0.009 (0.002)	0.0018 (0.004)	0.21 (0.001)	0.019 (0.004)
Dry Wight g/l	18.0 (0.58)	17.0 (0.026)	16.48 (0.212)	16.6 (0.035)	16.0 (0.055)	17.20 (0.05)	14.30 (0.015)	18.0 (0.026)	16.83 (0.023)	17.0 (0.026)	18.20 (0.001)	14.0 (0.308)	14.0 (0.043)	14.70 (0154)	14.0 (0.061)	13.7 (0.017)	14.3 (0.017)
Citric acid g/l	18.08 (0.093)	14.36 (0.091)	16.0 (0.01)	18.08 (0.1)	18.86 (0.10)	15.24 (0.30)	18.05 (0.60)	18.21 (0.03)	18.40 (0.48)	14.36 (0.091)	17.72 (0.178)	20.83 (0.016)	19.99 (0.091)	14.36 (0.091)	18.34 (0.09)	19.17 (0.09)	19.18 (0.09)
%	100.4 (1.2)	84.21 (2.52)	122.2 (3.64)	108.6 (1.97)	118.2 (8 84)	88.4 (5.45)	127.3 (4.53)	137.2 (3.55)	108 60 (1.97)	86.6 (2.09)	96.3 (0.551)	161.0 (1.87)	142.7 (2.87)	86.36 (1.500)	131.5 (10.1)	108.5 (4.13)	137.7 (3.55)

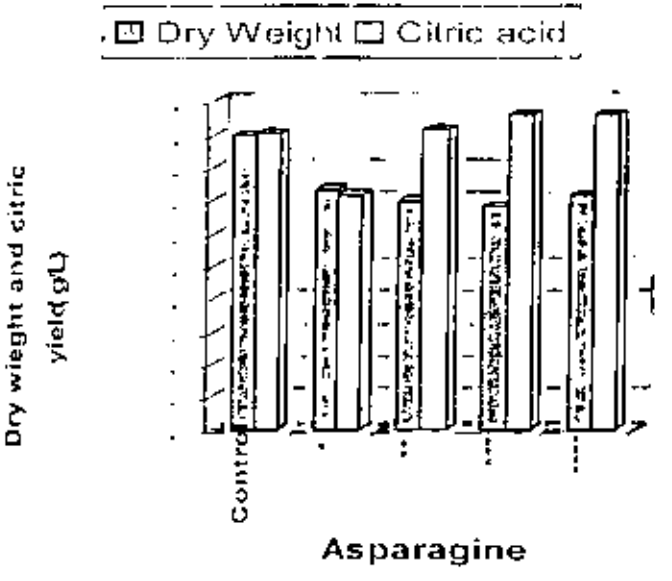
Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

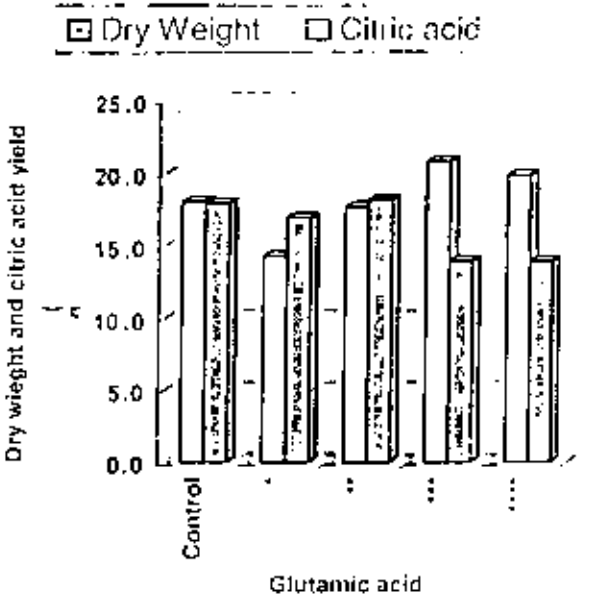
FIG(12) Effect of amino acids on citric acid production by *A niger*





* = 2×10^{-2} MOLARITY
** = 3×10^{-2} MOLARITY
*** = 4×10^{-2} MOLARITY
**** = 5×10^{-2} MOLARITY





The highest concentration of citric acid was 20.83 g/L ,(108.50 %) was obtained in fermentation medium contain 4×10^{-1} M glutamic acid ,followed by asparagine at concentration of 5×10^{-3} M in which the amount of accumulated citric acid equal 19.99 g/L , (142.7 %) . On the other hand lysine and cystine slightly stimulated citric acid production specially at concentration of 5×10^{-3} M when compared with control .

The highest amount of biomass dry weight 18.20 g/L was obtained in fermentation medium containing 3×10^{-3} M glutanic acid . The final pH value was decreased in all fermentation media , the lowest residual sugar was 0.009 g/L in fermentation medium containing 2×10^{-3} asparagine .

The statistical analysis program One-way ANOVA test at significance level($\alpha = 0.05$), showed that, high significance difference ($p=0.000$) within treatments. Dunnet t-test revealed, there is a significance between glutamic acid and control at concentration 4.0×10^{-3}M compared with control, while asparagine , glutamic acid and cystine at concentration 5×10^{-3}M showed significance difference in citric acid production while , there's no Significant difference between all treatments in biomass dry weight.

3.11. Comparison between standard medium and carob pod extract medium with respect to citric acid production.

The result in Table(11) and Fig (13) ,showed that , the carob pod medium was more superior than standard medium with respect to citric acid production by the fungus *A. niger* : in which the amount of produced citric acid in carob pod medium is about 15 folds more than of standard medium . The recorded amount of citric acid was 20.97 g/L (159.79 %) in the case of carob pod medium and 1.36 g/L (103.1%) in case of standard medium respectively .

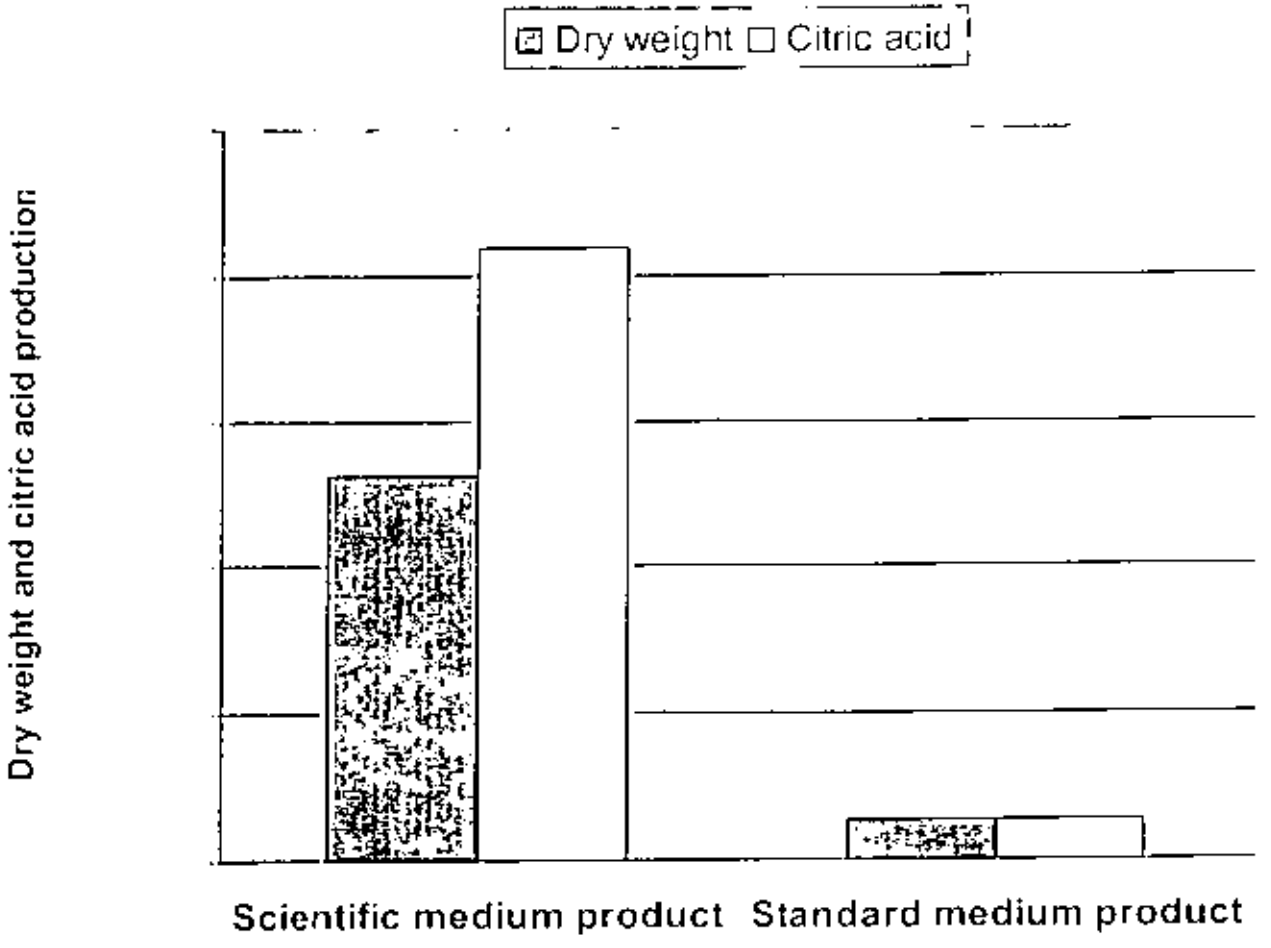
One-way ANOVA test, showed that , high significance difference within treatments. The statistical programs T-test revealed there is a significant difference between standard medium carob pod extract medium .

Table (11) Comparison between standard medium and carob pod extract medium with respect to citric acid production

Treatment	Dry Weight g/ l	citric acid		Residual sugar g/l	Final pH
		g/ l	%		
Scientific medium product	13.12 (0.10)	20.97 (0.114)	159.79 (1.83)	0.02 (0.004)	2.02 (0.03)
Standard medium product	1..35 (0.004)	1.36 (0.016)	103.1 (1.76)	0.015 (0.0014)	2.09 (0.033)

Each number represent the mean of three replicates and the numbers between brackets represent standard deviation (± SD)

FIG(16)Comparison between standard medium and carob pod medium



Chapter Four

4. Discussion

It has been recorded that citric acid production by microorganisms , specially *A. niger* affected by chemical and physical properties of the fermentation medium (Papagianni , *et .al* ., 1998 ; Jianlong , 2000 ; Haq, *et .al* ., 2003 a) . therefore , different experiments were designated throughout this study to determine these effects .

The first experiment was applied to determine the effect of incubation periods on the production of citric acid by the fungus *A. niger* and the results showed that this factor play an important rule in citric acid production . It is found that six days of incubation period was very suitable for this process .

The obtained data was agreed with the results by (Yigitoglu . 1992 ; Jianlong . 2000 and Hang & Woodams . 2000, 2001) . They recorded that six days of incubation was the best period for citric acid production by the *A. niger* using different carbon sources .

On the other hand, Chen ,(1993 ;1996) Saha *et. al*., (1999) , Roukas , (1999) and Shojaosadati & Babaeipour (2002) found that , the suitable period of incubation for production of citric acid by *A. niger* were 8, 7, 14,12 and 5days respectively

The decline in citric acid concentration after six days of incubation might be due to a gradual decay in the enzyme system responsible for the production of citric acid upon the decrease of the fermentable sugar (Firedrich *et . al* ., 1993 : Roukas .1998) .

The decreased in citric acid production could be attributed to it's break down due to the toxic effect of accumulated waste products and also exhaustion of the available energy source (Roukas . 1999) .

The data obtained from different investigations indicated that , there is no agreement about optimal initial pH to citric acid production . The rate of almost all enzymes catalyzed reaction exhibit a significant dependence on hydrogen ion concentration so the pH affects the activity of key enzymes and metabolic pathway in vivo cells (Tortora *et. al.*, 1986 : bozakouk . 2002)

The data present in table (3) and fig (5) showed that , initial pH of the medium greatly effected citric acid production by *A. niger* . The optimum initial pH value for the highest accumulation of citric acid by the fungus was obtained at pH value of 4.5 .

The recorded pH value in this experiment came in a close agreement with the result reported by Papagianni *et. al.*, (1998) and Haq *et.al.*, (2003 b)They found that , the optimum initial pH value in the fermentation medium was detected to be 4.5 .

On the contrary (Yigitoglu 1992 ; Jerneje *et. al.*, 1982 ; Saha *et. al.*, 1999 ; Haq *et. al.*, 2003 a) found the optimum pH values for production

of acid were 2.8, 2.5 , 3 and 6 respectively . These difference might be due to the nature of the substrate and type of fungal strain .

Initial pH value above 4.5 inhibited citric acid production . This finding might be due to the accumulation of Na ion's in the fermentation media which inhibit secretion of citric acid from the fungal cells (Elgbory ,1997) or might be due to accumulation of the waste product which consider as toxic for the fungus and furthermore decrease the productivity of the *A. niger* for citric acid production or even stop the production process (bozakouk , 2002)

The recorded data also indicated that citric acid production by the fungus was highly stimulated by increasing initial sugar concentration in fermentation media . The maximum yield of citric acid achieved in 15% sugar

Such results are similar to that obtained by Sarangbin & Watanapokasin (1999) and Jernje & Legisa (2002) . They observed that the best sugar concentration in fermentation medium for stimulation of citric acid production by *A. niger* was 14 % . Moreover , Rugsaseel , *et .al.*, (1995) and Kirimura *et .al.*, (1999) stated that . 12 % sugar in fermentation media was the best

On the other hand Chen , (1993) and Jianlong , (2000) studied the effect of sugar concentration on citric acid production by *A. niger* , and they found that , the presence of sugar at concentration 20 % greatly stimulated citric acid production

The observed results in this experiment could be attributed to the presence of different monosaccharides and disaccharides contents of the carob pod . This monosaccarides and disaccharides includes (glucose . Fructose . maltose and sucrose) This types of sugars consider as the best source of carbon which used for production of citric acid by *A. niger* (Roukas , 1998) .

The presence of initial sugar more than 15 % in fermentation media slightly inhibited citric acid accumulation but stimulated fungal growth , due to the conversion of the sugar to biomass rather than citric acid . similar results have been explained by Roukas (1998) .

The effect of nitrogen concentration in the form of sodium nitrate on fungal growth that stimulated biomass of citric acid by the fungus *A. niger* was also studied . the results revealed that the presence of sodium nitrate at concentration of 0.5 % was very suitable for fungal growth for the production of maximum amounts of citric acid

This result was agreed with those reported by Chen . (1993) . Saha *et. al.*, (1999) and Jianlong . (2000) . They observed the best concentration of nitrogen source ($NaNO_3$) that stimulated citric acid production by the fungus *A. niger* was 0.5, 0.5and 0.6 % respectively .

On the contrary Chen . (1996) and Haq . *et. al.*, (2002) recorded that . 0.2 % of ammonium sulphate was best concentration of nitrogen source added to fermentation medium which stimulated citric acid production by *A. niger*

Therefore , one can concluded that nitrogen concentration play a important role in citric acid production and having two effects . one effect was negative . since in excess of nitrogen promoted a bigger growth and consequently diverted the source of carbon toward energy and biomass production . The other effect was positive . because a moderate in put of nitrogen contributed to the maintenance of citric acid production (Chen . 1993: Saha *et.al*., 1999 ; Jianlongt . 2000 ; Haider &Al-bargathy.2003)

The results of the presence of different nitrogen sources in fermentation media , revealed that , the source of nitrogen in form of ammonium salts was more superior than other nitrogen sources . Similar results have been explained by Yigitoglu (1992) who postulated that ammonium compound found to be the best nitrogen source that provide acidic condition to the fermentation and stimulated citric acid accumulation by *A. niger* due to activation of the enzymes involved in the metabolism .

Moreover , the results showed that ammonium dihgdrogen phosphate was superior that other nitrogen source for the highest production of citric acid . This result agreed with the results obtained by several investigators (Roher.*et.al.*,1983; Kubiek & Roher , 1985 ; Chen . 1999). While Xu, *et. al*.,(1989)reported that nitrogen source in the form of urea greatly stimulated citric acid production comparing to other nitrogen source . This finding might be due to composition of fermentation media or due to the nature of fungal strain

It has been contributed that the presence of calcium ion in the fermentation media of the fungus *A. niger* played an important role in tricarboxylic acid cycle (citric acid cycle) . because the activity of pyruvate dehydrogenas responsible for the production of Co-enzyme A (Voet & Voet , 1995) .Therefore different calcium ion concentration were applied throughout this experiment and the results revealed that 0.05 % of $CaCl_2$ highly stimulated citric acid production Pera & Callieri , (1997 , 1999) explained that the addition of $CaCl_2$ at 0.05 % to the fermentation medium inhibit cell builching and increases the absorption of phosphate and carbon by the fungus *A. niger* and highly stimulated citric acid accumulation .

The recorded result in the present study was similar to the results explained by El-hashimy , (2004) who noted that the addition of 0.05 % $CaCl_2$ to the acid hydrolysis of sawdust powder fermentation medium was greatly stimulated citric acid accumulation by *A. niger* Pera & Callieri , (1999) stated that , the positive effect of calcium might be related to the increase of the mycelial branching level which probably favour the formation of pellet and improving the process .

The effect of the addition different methanol concentration to the carob pod medium was also studied . The results revealed that methanol inihibit fungal growth and delay spore formation but stimulate citric acid production . This finding might be related to effect of methanol on the permeability of cell membrane without affecting metabolic pathway of citric acid accumulation and allowed citrate to excreted from the cell (Roukas & Kotzekidou , 1997) . Also stimulate the activity of the enzyme citrate synthase which catalyze the condensation between acetyl Co-A and oxaloacetate to produce citric acid (Haq *et , al.*, 2003 b) .

The result indicated that , high accumulation of the citric acid was achieved at the presence of 3 % methanol . Similar results have been obtained by many investigators (Hang & Woodams , 1998 ; Navartham , *et. al.*, 1998 ; Roukas , *et. al.*, 2002)

Methanol concentration above 3 % inhibited citric acid accumulation , due to the disruption of fungal metabolism and mycelial morphology (Haq *et. al.*, 2003 b) .

On the other hand , Roukas , (2000) found that , the accumulation of citric acid in fermentation media of fungus *A. niger* increasing by increasing methanol concentration up to 6 % .

While the addition of different ethanol concentration to the carob pod medium showed inhibition effect on fungal growth and promotion in the biosynthesies of citric acid specially in fermentation medium containing 2 % ethanol .

This results came hand to hand with result of many investigators (Hamissa , 1978 ; Haq & Deng 1995 ; Hang & Woodams , 1986) . Who recovred that the highest production of citric acid by the fungus *A. niger* was achieved when the concentration of ethanol in the fermentation media ranged between 1-3 % .

This result might be related to the difference in metabolic process of the fungi which leading to production of acid . This metabolic process include increases in citric acid synthase activity which responsible for

synthesis of citric acid and decrease aconitase activity as a result of addition of ethanol (Manonmani & Sreekantiah , 1989).

Robert *et. al.*, (2003) observed that the activities of other TCA cycle enzymes increased slightly and citric acid firstly after addition of ethanol with slow degradation of citric acid consequent to reduce in aconitase activity . Also (Bhat *et. al.*, 1980) have stated that ethanol might be converted to acetyl-co A which is required for citric acid formation . Ethanol also act as carbon source which increases the inflow of carbon through the citric acid cycle (Roukas , 1998) .

The present investigation also revealed that the addition of amino acids at certain concentration also stimulate citric acid accumulation by the fungus *A. niger* and the highest production was achieved when glutamic acid was added to the fermentation media at concentration of 4×10^{-3} M. This results agreed with results of Lai & Srivastave , (1982) who stated that , the presence of glutamic acid (4×10^{-3}) highly stimulated citric acid production and the optimum yield was 76.7 % of the total fermented sugar .

The stimulation effect of glutamic acid for highest production of citric acid by *A. niger* appears to be due to the fact that glutamic acid gives rise to α-ketoglutrate by the reversible reductive amination , since α-ketoglutrate are will known intermediate of Krebs cycle which then converted to oxaloacetate (Conn & Stumpf , 1978) .

The citric acid cycle is not only a pathway for oxidation of two-carbon units ,it is also a major pathway for intereconversion of metabolites arising from transamination and deamination of amino acids . It also provides the substrates for amino acid synthesis by transamination and transaminase reactions for pyruvate from alanine , oxaloacetate from aspartae and α-ketoglutrate from glutamate .(Robert *et. al.*, 2003)

The result of the comparison between carob pod extract and standard medium showed that carob medium was supported fungal growth (13.1 g/L) and highly stimulated citric acid production (20.97 g/L , 159.79 %) when compared to the results obtained in the case of standard medium in which the fungal growth was inhibited (1.35 g/L) which was directly affected on the amount of citric acid accumulation in the fermentation medium (1.36 g/L , 103.1 %) .

The amount of citric acid produced by the fungus in carob pod medium was about 15 folds more than produced in the standard medium . This mean that carob pod medium was highly superior than standard medium with respect to fungal growth and citric acid production due to the optimization of the carob pod medium by addition most of the factors that stimulate biosynthesis of citric acid and also might be due to the presence of certain factors in carob pod extract which stimulate the process.

Conclusion

1- The mean aim of this study , which is the benefit from carob pod extracts as a raw material for the production of citric acid by *A. niger* has come to true and been achieved .

2- The Carob pod extract medium illustrated high productivity in production of citric acid by *A. niger*

3- The study proved that the carob pod extract contain (60 %) of sugar .

4- Addition of some factors (Nitrogen , alcohol and amino acid) to fermentation media have profound effect on productivity of *A. niger*

Recommendations

1- Many investigations should be done to determine the production of citric acid by *A. niger* from cheep different carbon sources .

2- The productivity of fungus *A. niger* to citric acid should by improving by inducing genetic mutations .

3- the incorporeal and finical support should be provided for this kind of studies

4- Many studies should be made . depending on the result's of this work as economical program to importance of the citric acid in many industries .

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الجماهيرية العربية الليبية الشعبية الاشتراكية العظمى

جامعة التحدي

كلية العلوم

قسم النبات

إنتاج حامض الليمون من مستخلص ثمار نبات الخروب بواسطة الفطر *Aspegillus niger*

قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة

الاجازة العليا (الماجستير)

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الخلاصة

نظرا لاستخدام حمض الليمون المنتج من الكائنات المجهريه في العديد من الصناعات الغذائية والدوائية والصيدلانية والكيميائية لذلك تركزت الدراسة في هذا البحث على أمكانية أنتاج حامض الليمون من عصير ثمار نبات الخروب باستخدام الفطر *Aspergillus niger* حيث تضمنت الدراسة اختبار تأثير بعض العوامل الفيزيائية والكيميائية على إنتاج الحمض وذلك لإيجاد الظروف المثلى لإنتاج الفطر حمض الليمون بواسطة الفطر *A. niger* حيث أتضح أن أفضل فتره زمنيه لإنتاج أعلى كمية من حمض الليمون هي ستة أيام عند تركيز أيون الهيدروجين 4.5 pH وباستخدام 15 % سكر على هيئة عصير ثمار نبات الخروب في وسط التخمر .

حيث بينت هذه الدراسة أن أفضل تركيز من النيتروجين لنمو وإنتاج الفطر لحامض الليمون وجد أن التركيز 0.5 % من النيتروجين يعطي أعلى قيمة لنمو وإنتاج الفطر.

وجد في هذه الدراسة أن أفضل مصدر نيتروجيني يحفز إنتاج حامض الليمون بواسطة الفطر *A. niger* هو فوسفات الأمونيوم ثنائية الهيدروجين أفضل مصدر نيتروجيني بينما كان لليوريا والببتون تأثير سلبي على إنتاج حامض الليمون .

و لبيان تأثير الكالسيوم على إنتاج حامض الليمون حيث وجد أن إضافة 0.05% من كلوريد الكلسيوم إلى وسط التخمر حفزا إنتاج الحمض بواسطة الفطر *A. niger* .

كذلك تمت دراسة تأثير تراكيز مختلفة من الميثانول على إنتاج حمض الليمون حيث وجد أن إضافة 3% من الميثانول في وسط التخمر لعصير الخروب أدى إلى زيادة في إنتاج حامض الليمون كما لوحظ أن زيادة تركيز الميثانول اعلى من 3% يؤدي إلي تثبيط إنتاج الحمض .

وكما أوضحت التجربة التي أجريت لبيان تأثير الأيثانول على إنتاج حامض الليمون أن إضافة الأيثانول عند تركيز 2% له تأثير محفز على إنتاج الحمض كما لوحظ أن زيادة تركيز الأيثانول عن 2% انخفض إنتاج الحمض .

ولبيان تأثير إضافة بعض الأحماض الامينية علي إنتاج حمض الليمون أوضحت الدراسة التى أجريت أن جميع التراكيز المنخفضة للأحماض الامينية التي استخدمت في التجربة ليس لها تأثير مثبط لإنتاج الحمض وعلى العكس فان التراكيز العالية من الأحماض الامينية كان لها تأثير محفز على إنتاج حامض الليمون حيث وجد أن حامض الجلوتاميك عند تركيز 4×10^{-1} ذات تأثير محفز لإنتاج الحامض

وأظهرت هذه الدراسة على أن وسط مستخلص ثمار نبات الخروب أفضل بكثير من الوسط القياسي من حيث نمو وإنتاج حامض الليمون ، حيث أن كمية الحمض المنتج من وسط مستخلص الخروب أكثر بحوالي خمسة عشر مرة من الوسط القياسي .