Some studies on Citric acid production by *Aspergillus niger*

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A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF PHILOSOPHY IN BIOLOGY (MICROBIOLOGY)

Department of Biological Sciences, Quaid-I-Azam University Islamabad. 2001

بسم الله الرحمن الرحيم

To my parents... for their endless efforts

CERTIFICATE

This Thesis by Qurrat- ul-Ain Khalid is accepted in its present form by the Department of Biological Sciences, Quaid-I-Azam University, Islamabad, as satisfying the Thesis requirements for the degree of Master of Philosophy in Biology (Microbiology).

Internal examiner _____ 28/2/2001

External examiner _____

Chairman Dept. Biology _____

Dated 28-2-2001

ACKNOWLEDGEMENTS

I am extremely thankful to almighty Allah for all his blessings, and help in the completion of research project.

My sincere attributes and thanks are for my supervisor Dr A. Hameed whose guidance, patience and kind attitude has helped me a lot during the whole M.Phil.

I am also very greatful to Dr Safia Ahmed as she provided me all the help which I needed.

I would like to thank Dr. Maqbool Ahmed, the Chairman of the Biological department for the providence of Better Laboratory facilities to work in.

My special acknowledgments are for Sheherzad who has encouraged me a lot and has helped me to complete my thesis on time.

Mrs Rani Faryal, Mrs Kalsoom and all the other Ph.D fellows have been very cooperative and deserve special thanks.

I am greatful to all of my lab fellows and friends specially Salma Arif for her cooperation.

I would like to thank Mr Shezad for his help and assistance during research. I would also like to thank my Brother and Sisters for all their help and love. My due regards and thanks are reserved for my Husband whose caring and supportive attitude has helped me to get out of all difficulties and to complete my thesis.

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ABSTRACT

The present study was undertaken to study Citric acid production by *Aspergillus niger*. Thirty different fungal infected Citrus fruits were taken from different areas of Pakistan. *Aspergillus niger* was isolated and then fungal growth was qualitatively tested to see weather or not the fungus produced Citric Acid. The best results came from an Orange taken from Islamabad which showed heavy precipitation when tested with Deniges reagent and rapid effervescence by the Carbonate test.

Further tests were then conducted to study the effect of Inoculum size on the size of fungal pellets produced, their dry mass weight, and amount of Citric acid produced. The effect of Incubation period on Citric acid production was also studied.

It was seen that the larger the size of the inoculum, the greater will be the dry mass weight of the pellets, and more citric acid will be produced. Whereas larger pellets are produced for smaller inoculum sizes. As a result of this study it was also seen that most citric acid is produced after longer incubation periods, therefore showing a positive correlation between incubation time and amount of citric acid produced.

INTRODUCTION

INTRODUCTION

Fungi comprise a heterogeneous collections of heterotrophic organisms which exist as saprophytes or parasites or less frequently as symbionts. They display a wide variety of morphological forms ranging from unicellular microscopic forms to macroscopic multicellular forms. The essential vegetative structure of fungi consists of hyphae, the hyphae comprise a large surface area through which substances can be interchanged with the environment, the mass of hyphae which constitutes the thallus is known as "*Mycelium*". Ecologically they are a highly successful form of life and are able to grow in an environment, which is usually found hostile to other forms of life. In fungi the cell wall primarily serves to protect and separate the cell from the environment. The cell wall is a complex, dynamic structure and is the site of diverse enzyme activity, studies have revealed that the cell wall is composed of chitin, cellulose and polysaccharides, together with proteins. Reproduction in fungi is both sexual and asexual. (Smith *et.al.,* 1975). Fungi produce spores, which are highly specialized for reproduction, survival and dispersal. Tom and Currie in 1916 showed that the black *Aspergillus* are involved in citric acid production (Miall and Rose, 1978).

A form of fermentation is "submerged fermentation" In submerged fermentation strains of families; *Aspergillus, Penicillium* and *Trichoderma* are most frequently used as host organisms (Johansen *et.al.,* 1998). Fungi of the genus *Aspergillus* are cosmopolitan and ubiquitous. The *Aspergilli* are greatly involved in the welfare of mankind. Many species cause rotting of food stuff

stored grains, vegetables fruits & Industrial products. A few species cause lungs diseases of Birds, Cattle, Horses and some times even Man, this disease is called as *Aspergillosis*. Because of great enzymatic activity *Aspergillus* is greatly employed in Industrial processes. Enzyme preparations are made commercially through the use of these fungi and a number of antibiotics have also been isolated from their cultures. In Java, *Aspergillus wentii* is employed in processing Soya beans due of its ability of softening hard tissues. In Japan, "*Sake*" an alcoholic beverage is obtained from *Aspergillus oryzae*. Citric acid and D-gluconic acid are the major metabolites of *Aspergillus niger*, it is also involved in the production of Gallic acid (Miall and Rose, 1978).

CITRIC ACID:

Citric acid is widely distributed in nature and the official form is not less than 99.7% in purity, calculated on anhydrous basis. About 60% of citric acid is used in food industries, pharmaceutical and chemical industries. Citric acid is a tribasic carboxylic acid. Molecules present in substances classified, as carboxylic acid must have a hydroxyl group attached to a carbonyl atom, the other bond from carbonyl carbon must be attached to another carbon. This group can be called carboxylic acid group or carboxyl group. It acts as an intermediate in the metabolism of acetate units, in the citric acid cycle ATP is produced and four carbon carrier molecules are regenerated.

Citric acid is mostly produced from fermentation and may be carried out with any one of over nineteen varieties of fungi belonging to *Citromyces* or

Aspergillus. The demand for citric acid in the world is more than 500,000 tons/year, today 80% of the world demand for citric acid is met through the fermentation by *Aspergillus niger* (Usami, 1978).

Citric acid has many advantages, such as its salts act as an anticoagulant of blood; salts of sodium citrate are added to draw whole blood to keep it from clotting. Citric acid is also used as an accident which is used to enhance flavor yet control acidity, viscosity and hardness in commercial products. As it is one of the major organic acids produced by fermentation, synthetic production is feasible. It is produced in both surface and submerged cultures, using strains of *Aspergillus niger*.

In case of surface culture the mycelium is grown in trays and is kept in stacks at room temperature. Oxygen is supplied by blowing air over the trays. The contents of the trays are bulked for citric acid recovery and the mycelial felt is discarded. Producing spores in submerged cultures is however more convenient, it has the great advantage of simplicity in production. In submerged cultures pellets and hyphal filaments both have distinct morphology The advantages of pellet formation is that it permits a higher oxygen solution rate than the comparatively more viscous mycelial forms. Although many fungi may suffer greater shear damage as the stirrer speed is raised, greater oxygen transfer may result in high titers. This has been demonstrated in production of citric acid by *Aspergillus niger* (Vardar and Lilly 1982). When filamentous fungi grow in the form of pellets, this growth results in an increase in radius, where as shear forces result in the release of hyphal fragments. These hyphae then act as a center for further pellet growth and

development (Tough *et.al.*, 1995). Stirring and oxygen transfer also have a great influence on product formation. Inoculum size also affects the morphology and citric acid production. Larger inocula yield white globose pellets with smooth surfaces, which produce a considerable amount of citric acid in suitable media.

The present study is focused on the production of citric acid by pure strains of *Aspergillus niger*. The relationship between number of pellets and product formation is also studied. It has been seen that the number of tips vary with the size of Inoculum. Greater the number of tips, the larger will be the product formation. Therefore a correlation can be developed between the size of Inoculum and citric acid production.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Citric acid is widely used in foods, pharmaceuticals, cosmetics and beverages. The entire worldwide demand for citric acid production is met by fermentative production mainly involving the filamentous fungi *Aspergillus niger* which is commonly called as "Black mold". This is principally an organic acid, it is produced from a feed stock of carbohydrates by fermentation with *Aspergillus niger* and yeasts of candida species. Acid is produced both by surface submerged, and by solid state fermentation. Acid is recovered by precipitation with lime. (Grewel *et.al.,* 1995). Pre selection of *Aspergillus niger* strains is done for rapid production of citric acid. A relationship between culture appearance and productivity can be established, such a solid medium is designed which gives a good level of production (Benuzzi *et.al.,* 1989). Inoculum preparation also has a great influence upon citric acid production, viability increases with incubation time. Higher production of citric acid is achieved with spores incubated for less than 7 days (Vergano *et.al.,* 1996). Fermentative conditions also have a great effect on citric acid yield. Citric acid production with *Aspergillus niger* is done on stirred tank reactors using cane juice, molasses and synthetic medium, highest yield is obtained from cane juice, rate of stirring also has a great influence upon citric acid production (Qazi *et.al.,* 1990).

Mutations in various strains of *Aspergillus niger* have been carried out to see their effect upon the citric acid yield. Four variants of *Aspergillus niger* were obtained by ultraviolet irradiations, as a result of repeated irradiations of

conidia and selection of active cultures, the variants produced more citric acid than the original strains (Imshentskii and solntseva, 1960). *Aspergillus niger*, a citric acid producing strain is treated with gamma rays, the most active and resistant mutants were produced at doses of 10-50 Krads (Golubtsova *et.al.*, 1972). Several mutants were obtained by *U.V* irradiation of spore suspension. Producers yielding large amount of citric acid were selected on agar containing methyl red as pH indicator (Pelecohova *et.al.*,1990). Auxotrophic strains were obtained by irradiation of citric acid producing strains of *Aspergillus niger* with *U.V* light. Protoplasts were isolated from young hyphae of anxotrophic strains. Prototrophic heterozygous spores were isolated from heterokaryons. Citric acid production in heterozygous strains was higher than parental strains (Martinkova *et.al.*, 1990). Mutants of *Aspergillus niger* were grown on corn starch and potato starch, the mutants grown on corn starch gave higher yields (Suzuki *et.al.*, 1996). Mutants of *Aspergillus niger* were selected which showed enhanced productivity from starch in shake culture (Ragaseel *et.al.*, 1993). Citric acid prodction from cellobiose by *Aspergillus niger* was studied by semi solid culture method. Mutant strains resistant to 2-Deoxy-D-Glucose were selected and further mutations were induced. The mutants show high citric acid yield in semi solid culture, when glucose is used as Carbon source (Suzuki *et.al.*, 1996).

Mineral requirements of *Aspergillus niger* for citric acid production have also been studied. Effect of Zinc on growth and acidogenesis of citric acid produced by *Aspergills niger* was studied, the whole process had two steps, in first phase high concentrations of zinc lead to only growth phase but when the

concentration of zinc was lowered citric acid was accumulated. Effect of both zinc and cyclic adenosine monophosphate was studied, high concentrations of zinc maintained growth phase and deficiency of zinc lead to a transition leading to accumulating phase. Cyclic adenosine monophoshate only lead to the growth in decreased concentration of zinc and did not cause transition, but enhanced growth during growth phase. At later stages cyclic adenosine monophosphate inhibited growth but citrate synthesis was augmented by the cyclic adenosine monophosphate (Suzuki et.al., 1996). Dithiocarbonates were found to enhance the production of citric acid under solid state fermentation conditions by *Aspergillus niger* (Khanna and Gupta, 1986). The effect of copper ions has been studied by various workers, copper concentration is very important at pellet stage, pellets were used as inoculum for production of acid and corresponding citric acid accumulation has been reported (Benuzzi and Segovia, 1996).

By using an appropriate ratio of carbon source to mineral components, comparable citric acid yields are obtained in media containing different concentrations of glucose (Nguyen *et.al.*, 1992). Citric acid production from xylan and xylan hydrolysate was done by *Aspergillus niger* on semisolid culture. The quantity of citric acid by strains grown on xylan was larger than those grown on arabinose and xylan hydrolysates (Kirimura *et.al.*, 1998).

Amylose like polysaccharide accumulation and hyphal cell structure was studied in relation to citric acid production. They act as a shock absorber in shake culture and increases the viscosity of broth culture, which enhanced the production of Citric acid (Kirimura *et.al.*, 1999). Various studies on citric acid

fermentations had shown that the glucose is taken up by the process of diffusion (Wayman and Mattey, 2000).

Various gases also have an influence upon citric acid yield. Effects of dissolved carbon dioxide upon citrate and gluconate production were studied. Batch culture studies show that at lag phase the cultures are sensitive to elevated CO2 levels, but in submerged cultures the results are inappropriate. (McIntyre and McNeil, 1997). Effect of oxygen tension on citric acid yield was studied. It was observed that the rate coefficient of these two is proportional, biomass concentration increases with the increased oxygen tension (Sakurai *et.al.,* 1996). It has been studied that citric acid is accumulated due to an inhibition of aconitase activity, the addition of flouroacetate which acts as an aconitase inhibitor elevated the citrate to isocitrate ratio (Kubicek and Rohr, 1985).

Initiation of citric acid accumulation in the early stages of *Aspergillus niger* growth has been studied in great detail. Glycerol which functions as an osmo-regulator in the early stages of growth of *Aspergillus niger* diffuses out of cells and enters possibly into the mitochondria. Mitochondrial nicotine adenine dinucleotide phosphate is inhibited by glycerol, and therefore citrate starts to accumulate in cells (Legisa and Kedrick, 1985).

Sudden substrate dilution induces a higher rate of citric acid production. Initially high sucrose concentration were added which caused high intracellular glycerol within the spores, so when citric acid was excreted, the substrate was diluted, resulting in tripled volume of citric acid (Legisa *et.al.,* 1995)

Carbohydrate metabolism in *Aspergillus niger* was studied in biochemical and molecular perspective. The hyphal fungus *Aspergillus niger* is one of the major sources of industrial production of citric acid, gluconic acid and of food grade enzymes. Considerable progress has been made in developing the genetics of *Aspergillus niger* which facilitates strain improvement by recombination and in developing genetic tools to genetically engineer this fungus. Biotechnology relevance of *Aspergillus niger* is to a large extent linked to carbohydrate metabolism either directly or indirectly (Visser, 1991).

MATERIALS AND METHODS

MATERIALS AND METHODS

The present research was conducted in the Microbiology Research Laboratory, Quaid-I-Azam University, Islamabad. The organism used in Citric Acid fermentation, *Aspergillus niger*, was isolated and identified in the same laboratory.

SAMPLE COLLECTION:

A total of thirty different samples were collected from different ecological sources over Pakistan. Three citrus fruits were studied for contamination by *Aspergillus niger*. Eighteen infected Oranges of different varieties were collected, ten from Islamabad and eight from Khanpur. Seven infected Apples were collected from Rawalpindi and Five infected lemons were taken from Bahawalpur. All were infected both on the skin and within the flesh of the fruit.

ISOLATION OF ORGANISM:

Six to eight loopfulls of spores were scraped from the surface of the fungal infected fruit and mixed in 50 ml of distilled sterile water. This was agitated with great force, to obtain a homogenous mixture. Five ml of this spore mixture was then poured onto prepared plates of Saborauds Dextrose agar, and placed in the incubator at 30°C for 96 hours.

Composition of Saborauds Dextrose Agar

Dextrose	40.00 gms
Peptone	10.00 gms
Yeast extract	4.00 gms
Agar agar	18.00 gms
Distilled Water	1000.00 ml
pH	5.6

IDENTIFICATION OF ORGANISM:

After 96 hours of growth the plates were studied for physical characteristics. Slides were prepared to study the ultra structure of the spores. Mycelia along with spores were collected from the surface of the plate by lightly scraping them off by an inoculating loop. They were then placed on a slide and covered with a drop of methylene blue stain. The slide was then studied under the microscope for the following morphological characteristics:

1. Mycelium stucture
2. Conidiophore shape
3. Conidia shape
4. Vesicle stucture
5. Spores

Those colonies which showed characteristics of *Aspergillus niger* were then isolated onto fresh Saborauds Dextrose plates, and placed once again in the incubator at 30C for 96 hours to obtain pure cultures.

INOCULUM PREPERATIONS:

The surface of the colony was scraped with a sterile inoculating loop and the spores were added to 10 ml autoclaved distilled water in a screw capped test tube and shaken well to obtain a spore suspension. This was then vortexed to get a homogeneous suspension. One ml of this suspension was taken and the number of spores present in it, was determined by a Heamocytometer. This is an apparatus consisting of a special glass slide with a grid of lines engraved on the bottom of a shallow rectangular trough so that if a coverslip is placed over the through the grid demarcates known volumes, 1 ml of the sample was placed in the space and the number in the grid squares were counted under the microscope.

After the spore count was completed, 1 ml of suspension was taken and further diluted by serial dilutions to obtain Inoculum ranges of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 spores/ml for further studies four of these concentrations (10^2, 10^4, 10^6, $10^{7)}$ were taken. The effect of these concentrations upon citric acid production is to be studied.

SHAKE FLASK FERMENTATION:

The suitable broth medium for citric acid production was prepared with the following composition:

Sucrose	8.00 gms
$NH_4 No_3$	2.5 gms
$KH_2 PO_4$	2.5 gms
$Mgso_4 7HO_2$	0.25 gms
Distilled Water	1000 ml
pH	3.5

Citric acid accumulation experiments were carried out in Erlenmeyer flasks of 500 ml. 250 ml of autoclaved medium was poured into the flask and was inoculated with *Aspergillus niger* isolated previously. The flasks were then placed in shake flask fermenter and were agitated at the speed of 150 rpm for 48 hours, the temperature was maintained at 28 °C. Growth of the fungi was in the form of pellets. The suspension was then filtered onto Whatman filter papers.

STUDIES ON DIFFERENT FACTORS AFFECTING CITRIC ACID YIELD.

1. Incubation period:

In order to study the effect of incubation period upon the yield of Citric acid, an Inoculum from the above mentioned medium was incubated for 120 hours and then agitated at a speed of 150 rpm at 28°C. samples were collected after every 24 hours.

2. Dry mass weight of pellets:

After fermentation, the pellets were removed and kept in an oven over night at 60 °C in order to remove any excess moisture, these were weighed after drying to study the effect of their dry weight upon production rates.

QUALITATIVE ANALYSIS:

The following tests were conducted to analyze the quality of the filtrate.

1. Carbonates test

The filtrate obtained after fermentation was treated with 5 % $CaCO_3$ salt solution to test the presence of citric acid in the filtrate. (5 % of $CaCO_3$ solution was obtained by dissolving 5 gms of $CaCO_3$ salts in 100 ml distilled water.

2. Deniges Reagent test

The filtrate was also treated with Denigues reagent and then with $KmnO_4$ solution so as to test the production of citric acid.

QUANTITATIVE ANALYSIS:

Quantitatively the amount of citric acid produced was determined by the following tests:

1. Calcium salt test

50 ml of the filtrate obtained after fermentation was treated with 5 % $CaCl_2$ salt solution, the solution was continuously stirred so that all of the calcium reacts with the citrate ions and then the mixture was subjected to centrifugation at 4°C for 30 minutes at 10,000 rpm. 5 % of $CaCl_2$ is prepared by dissolving 5 gms of $CaCl_2$ in 100 ml of distilled water.

2. Titration

The filtrate taken after shake flask fermentation was titrated against 0.1N NaOH, using 0.1 % phenolphthalein as an indicator.

RESULTS

RESULTS

The organism isolated was identified as *Aspergillus niger*. A total of 30 different samples were collected from various locations. The strain used in the present study was isolated from an Orange taken from Khanpur. This strain was primarily identified by observing the colonies which were grown on Saboraud Dextrose plates.

The mycelial mass was whitish green, mature colonies were characterized by blackish spores. Other morphological characteristics observed were as follows:

Conidiophores:	Non septate
Conidial heads:	Fuscous and black
Conidia:	globose
Vesicle:	globose

QUALITATIVE TEST RESULTS

1. Carbonates test

The filtrate obtained after 48 hrs of incubation in a shake flask fermenter was treated with 5 % $CaCO_3$ salt solution, effervescence took place which indicated the presence of citric acid as the citrates effervesces with carbonates. The greatest effervescence was produced from a strain isolated from Orange # 9, taken from Islamabad (Table 7).

2. Deniges reagent test

When the citrate reacted with Deniges reagent, a yellow colour appeared, when $KmNo_4$ was added, the purple colour of $KmNo_4$ diminished and white precipitates appeared indicating the presence of citric acid. Orange # 9, taken from Islamabad showed the greatest amount of precipitates formed. (Table 7)

SPORE COUNT

The number of spores present were counted by using a heamocytometer. The number counted for Orange #9, which showed the best qualitative results, was 1210.

$= 1210 \times 10^3/ml$

$=1.21 \times 10^7/ml$

QUANTITATIVE TEST RESULTS:

1. Calcium salt test

5 % $CaCl^2$ reacted with the filtrate to give white precipitates as a result of the reaction between calcium and citrate ions and these precipitates were centrifuged. The supernatant was discarded and the pellets obtained were weighted. The weight of acid produced in 50 ml of filtrate is 3.02 gms. (Table 6)

2. Titration

When the filtrate was titrated against 0.1N NaOH solution, using 0.1 % phenolphthalein, the end point was pink. It was seen that an inoculum size of 10^7 gave the highest productivity in which 30 ml of NaOH neutralized 50 ml of acid.

EFFECT OF INOCULUM SIZE ON THE SIZE OF PELLETS

It was seen that the smaller the inoculum size, larger is the size of pellet formed after incubation. The largest pellets were obtained from the smallest inoculum size (Table 1, Fig 1)

EFFECT OF INOCULUM SIZE ON DRY MASS WEIGHT OF PELLETS

The larger the inoculum size, the greater is the dry mass weight of the pellets. (Table 2, Fig 2)

EFFECT OF INOCULUM SIZE ON CITRIC ACID PRODUCTION

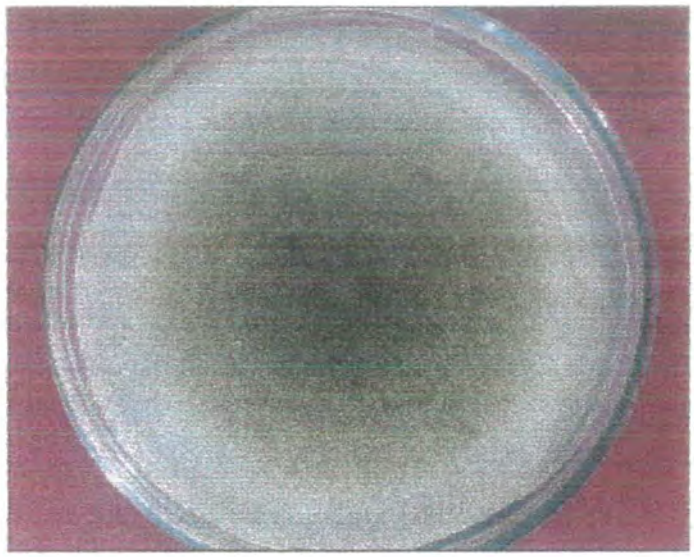
It has been seen that the larger the size of the inoculum, the more the amount of citric acid produced. (Table 3, Fig 3)

EFFECT OF INCUBATION PERIOD ON CITRIC ACID PRODUCTION

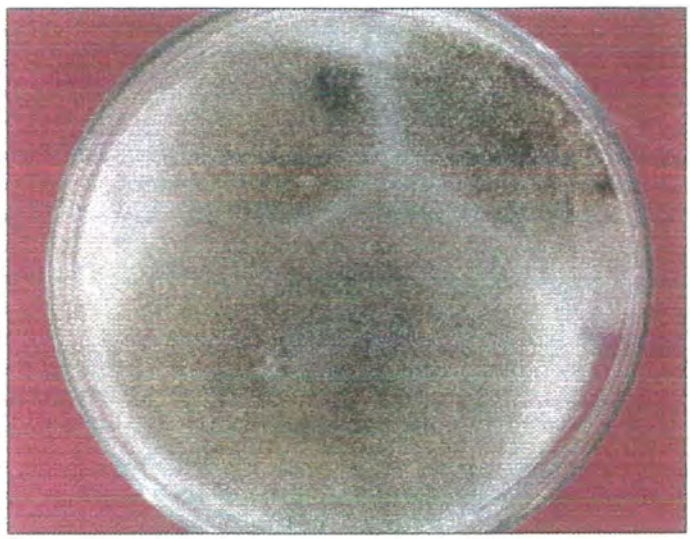
The greatest quantity of citric acid was produced for the longest time period incubated. Therefore there is a positive correlation between incubation time and citric acid produced. (Table 4, Fig 4)

EFFECT OF INOCULUM ON NUMBER OF PELLETS

It was seen that for the largest inoculum size, the most number of pellets were produced. An inoculum of 10^7 gave the most pellets (Table 5)



Aspergillus niger growth after 96 hours



Aspergillus niger growth after 120 hours

GROWTH IN INCUBATOR AT 37° C

Table 1. Effect of Inoculum size on size of pellets

Inoculum size. (Spores/ ml)	Size of Pellets (ml)
100 (10^2)	7
10000 (10^4)	5
1000000 (10^6)	3
10000000 (10^7)	1.5

Fig. 1 EFFECT OF INOCULUM SIZE ON SIZE OF PELLETS

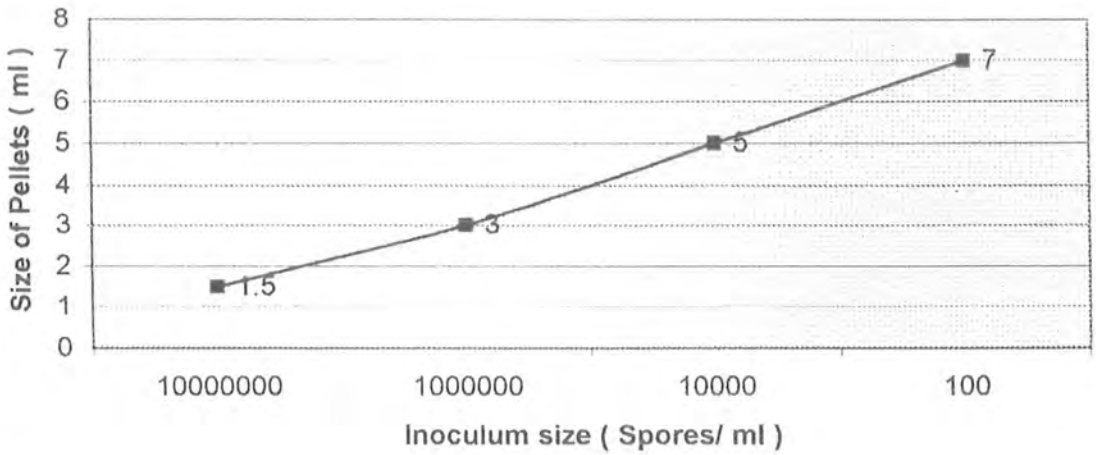


Table 2. Effect of Inoculum size on dry mass weight of pellets after 96 hrs	
Inoculum size. (Spores/ ml)	Dry mass weight (gms)
100 (10^2)	1.17
10000 (10^4)	1.6
1000000 (10^6)	2.4
10000000 (10^7)	2.67

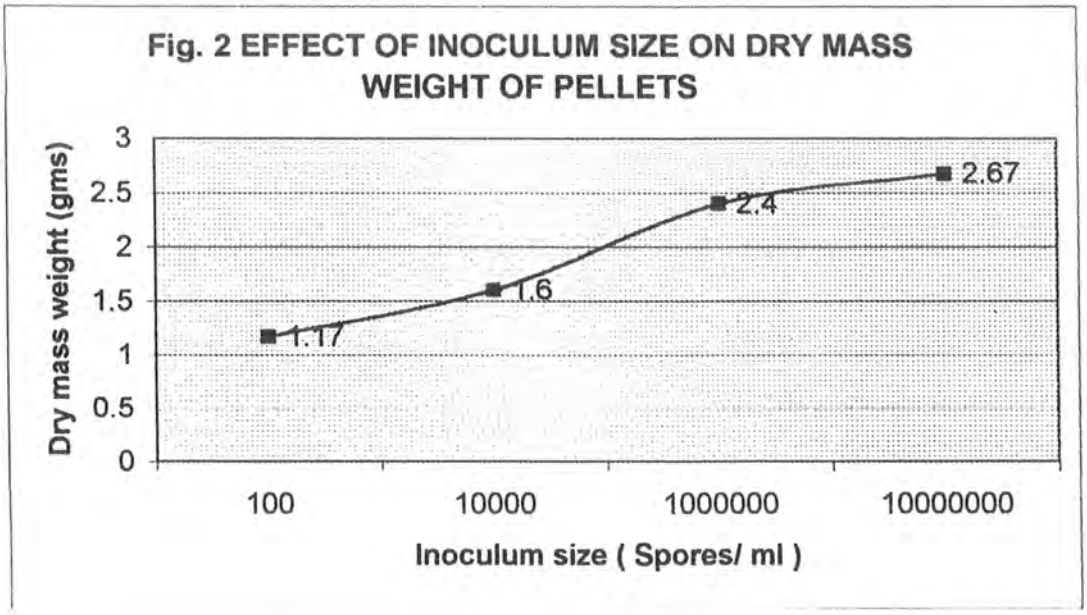


Table 3. Effect of Inoculum size on citric acid production	
Inoculum size. (Spores/ ml)	Citric acid produced (gms)
100 (10^2)	2.6
10000 (10^4)	2.8
1000000 (10^6)	2.9
10000000 (10^7)	3.02

Fig. 3 EFFECT OF INOCULUM SIZE ON AMOUNT OF CITRIC ACID PRODUCED

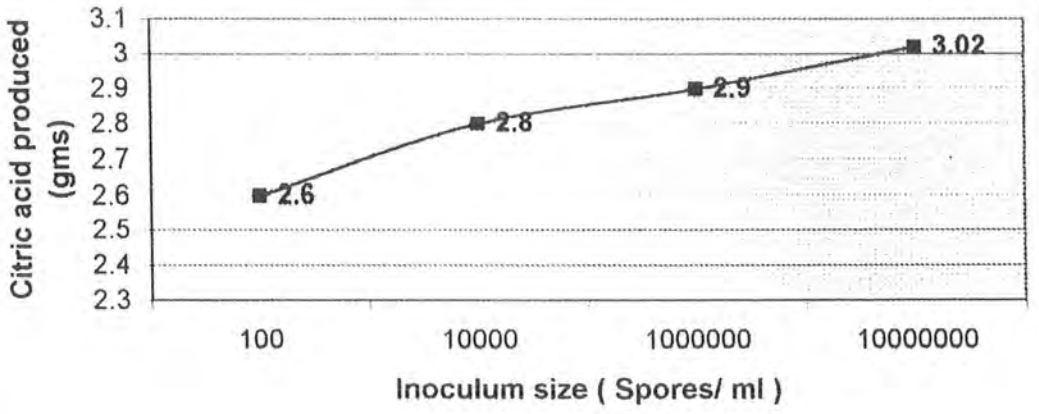


Table 4. Effect of incubation period on citric acid production

Incubation period (hrs)	Citric acid produced (gms)
24	2.0
48	2.4
96	3.0

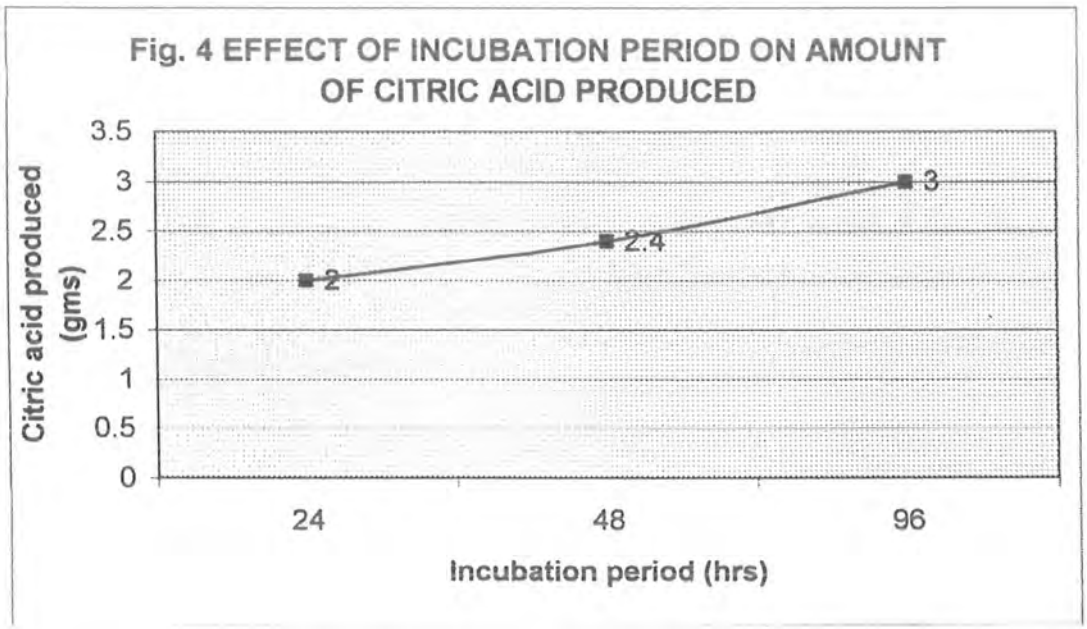


Fig. 4 EFFECT OF INCUBATION PERIOD ON AMOUNT OF CITRIC ACID PRODUCED
Citric acid produced (gms)
3.5
3
2.5
2
1.5
1
0.5
0
2
2.4
3
24
48
96
Incubation period (hrs)

Table 5. Effect of Inoculum on number of pellets after 96 hrs	
Inoculum size (spores/ ml)	Approx. number of pellets
100 (10^2)	150
10000 (10^4)	280
1000000 (10^6)	too numerous
10000000 (10^7)	too numerous

Table 6. Results of calcium salt test		
Inoculum size (spores/ml)	Vol. of filtrate (ml)	Wt. of citric acid (gms)
100 (10^{2})	50	2.5
10000 (10^{4})	50	2.75
1000000 (10^{6})	50	2.89
10000000 (10^{7})	50	3.02

Table 7. ISOLATION OF ASPERGILLUS NIGER FROM FRUIT

CITRUS FRUIT		QUALITATIVE TEST	
Serial no.	Fruit	Carbonates test	Denigues test
(Isb 01)	Orange	+ +	+
(Isb 02)	Orange	–	–
(Isb 03)	Orange	–	–
(Isb 04)	Orange	+	–
(Isb 05)	Orange	+ +	+ +
Isb 06)	Orange	–	–
(Isb 07)	Orange	+	–
(Isb 08)	Orange	+	+
(Isb 09)	Orange	+ + +	+ + +
(Isb 10)	Orange	+ +	+
(Kpr 01)	Orange	+	+
(Kpr 02)	Orange	+ +	+ +
(Kpr 03)	Orange	–	–
(Kpr 04)	Orange	–	–
(Kpr 05)	Orange	–	–
(Kpr 06)	Orange	+ +	+
(Kpr 07)	Orange	+	–
(Kpr 08)	Orange	+	–
(Bwpr 01)	Lemon	–	–
(Bwpr 02)	Lemon	–	–
(Bwpr 03)	Lemon	–	–
(Bwpr 04)	Lemon	–	–
(Bwpr 05)	Lemon	+ +	+
(Rwp 01)	Apple	–	–
(Rwp 02)	Apple	–	–
(Rwp 03)	Apple	–	–
(Rwp 04)	Apple	+	–
(Rwp 05)	Apple	–	–
(Rwp 06)	Apple	–	–
(Rwp 07)	Apple	–	–

DISCUSSION

DISCUSSION

The present investigation has been carried out to study the production of citric acid by *Aspergillus niger*. There are many strains of *Aspergillus niger* which produce citric acid. The acid in the present study is produced by a strain which was isolated and cultured in the Microbiology Research Laboratory of Quaid-I-Azam University, Islamabad. This strain was grown on Saboraud's Dextrose agar. Dextrose agar is supposed to yield high citric acid titres as compared to other types of agar and better viability of spores (Vergano *et.al.*, 1996).

Different factors were considered to be responsible for the better accumulation of citric acid by *Aspergillus niger* and a lot of work has been done in this regard. One of the main and important factors is the preparation of the Inoculum. The size of Inoculum has a great influence upon citric acid production. 10^2, 10^4, 10^6 and 10^7 spores/ml of the Inoculum concentrations were used as a controlled parameter to observe quantity of citric acid produced by these concentrations. Highly concentrated Inoculum i.e. 10^7 spores/ml results in the formation of smaller pellets as compared to lesser concentrated ones but as far as productivity is concerned, less concentrated inoculum i.e. 10^2 spores/ml give low yield where as 10^6 spores/ml and 10^7 spores/ml which are concentrated inoculum give high citric acid yield. High yield of citric acid in case of concentrated inoculum is due to a very large number of pellets, as a result of this, the number of tips are also greater.

Whereas in the case of less concentrated inoculum the number of pellets is less, resulting in lesser number of tips thereby giving low productivity of acid.

It was also studied that mold morphology in submerged culture also has an effect on the production of acid. The general consensus seems to be that the formation of small dense pellets is a prerequisite for the formation of the citric acid, this may not be completely correct but this could be true due to the fact that the formation of pellets is a result of environmental conditions which favour the formation of citric acid. Incubation period is one of the other major factors, which improves the productivity of citric acid.

It was also observed that the viability of spores increased with the increase in the period of incubation. The favourable time period of incubation for highest yield was also observed. The spores were incubated for 24 hrs, 48 hrs, 96 hrs and 120 hrs. The best results were obtained by the sample collected after 96 hrs interval. Spores show high citric acid yield and viability when incubated for less than 7 days (Vergano *et.al.*, 1996).

Another important factor was agitation. As the culture was subjected to shake flask fermentation, the speed of agitation had a marked effect upon pellet formation as well as product formation. Agitation of a culture broth can have different effects on filamentous fungi which includes variation in the growth rate, variation in efficiency of growth rate and also in the rate of product formation (Smith and Lilly, 1989).

Agitation has the following influences.

1. Improved mixing.
2. Heat transfer.
3. Mass transfer.

Good bulk mixing of the fermentation broth is very crucial, as it is needed to minimize nutrient concentration gradients and to ensure adequate flow rates of heat transfer. Oxygen is one of the important requirements of microorganisms. In aerobic fermentations agitator is aerated (Smith and Lilly, 1989). Oxygen requirement is an essential factor. As the Oxygen tension was increased i.e. 74% to 100% by Volume; rate of citric acid production decreased whereas with the decrease i.e. 21% to 74% by Volume in oxygen tension, the production of acid increased (Sakurai *et.al.,* 1996).

In our present study a pure strain of *Aspergillus niger* was isolated through which citric acid was produced in suitable media under appropriate conditions. Certain other alterations have also been tested in the media. The effect of various mineral nutrients upon citric acid production was also studied. In another study sucrose was used in higher concentration but as citric acid began being produced by Aspergillus *niger*, sucrose was diluted which enhanced the production of citric acid (Legisa *et.al,* 1995). Besides sucrose glucose, corn starch and potato starch were also used as a substrate and carbon source during citric acid production. Different results have been obtained with different substrates. In the present study sucrose was the

substrate which acted as the carbon source, and gave a considerable yield of citric acid.

The biochemical mechanism responsible for citric acid production has been the subject of investigation for over 40 years. The presence of the Embden Meyerhof pathway as glycolytic system is well established in *Aspergillus niger* by many studies. Aconitase, the enzyme of the citric acid cycle, catalyzes the equilibrium between citrate, cis-aconitate and iso-citrate. This is the immediate enzyme catalyzing citrate breakdown, within the cycle, several workers have claimed that the disappearance of this enzyme or its inhibition causes citric acid to accumulate. According to some other scientists, the inhibition of ketoglutarate dehydrogenase is the cause of citric acid accumulation while some investigators have observed that once the citrate is accumulated, it inhibits its own catabolism and can further be accumulated (Kubicek, 1985). According to another investigation, Glycerol which functions as an osmoregulator in the early stages of growth diffuses out of the cell and enters the mitochondria. Mitochondrial nicotine adenine dinculeotide phosphate specific isocitrate dehydrogenase is inhibited by glycerol and citrate starts to accumulate.

Quantitative analysis of citric acid was done by titrating the filterate with 0.1M NaoH and phenolphthalene was added, another method which has been used is the calcium salt test. In both these tests the inoculum size of 10^7 has given the best results. Which shows that the size of inoculum greatly influences the productivity.

In this study a pure strain was isolated and was subjected to produce citric acid, but other methods have also been applied to improve the production of the above mentioned acid. One such method is the production of citric acid by variants obtained by ultraviolet irradiation. The variants produced citric acid in larger quantities as compared to the original ones (Kuranova *et.al.*, 1960). Another study was carried out in which citric acid yield of 8 different strains of *Aspergillus niger* in pure and mixed culture was investigated but the yield was higher in pure culture than in mixed culture (Khan and Shaukat, 1990).

So we can conclude that a number of factors are involved in citric acid production such as size of inoculum, incubation period, agitation and kind of media. Citric acid production is a vast project and a lot of work is still going on to enhance its production due to the ever increasing demand of citric acid in the industrial world.

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