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CitricAcid Production from Carob Pod Extract by Aspergillus. Niger

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Abstract: *The present study was undertaken to study the production of citric acid by the fungus Aspergillus niger using carob pod extract as a carbon source for fungal growth and production of citric acid. High yields of citric acid were achieved after six days of incubation when 10% of carob pod extract as a carbon source present in fermentation media with pH value of 3.16 and the amount of yield being12.25g\l, (55.48%). The results also revealed that the accumulation of citric acid by the fungus was affected by the source and concentration of nitrogen, as well as high production of the acid was obtained in fermentation medium containing ammonium sulphate, in which the accumulated amount increased up to 18.25g\l, (70.16%). Moreover, the addition of 0.05% calcium chloride and 2% ethanol to the fermentation medium highly stimulated citric acid accumulation by the fungus to reach 21.06g\l and this amount equivalent to 113.22% of the biomass dry weight.*
Keywords: *Carob pod extract, citric acid, fungus.*

I. Introduction

Citric acid is an intermediate compound of a Tricarboxylic acid cycle**.** It is found as a natural constituent of variety of citrus fruits such as pineapple, pear, peach and fig. Citrus fruit is a particularly rich source of citric acid (1).

The worldwide demand for citric acid is increased day by day. The annual production of citric acid is approximately estimated 1,750,000 ton's and is increasing at annual growth rate of a 5% (2). Less than 1% of the 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 (3).

Many different microorganisms are able to convert the carbohydrate to organic acid. The industrial-scale production of citric acid is generally carried out by fermentation process with filamentous fungi. **Aspergilli** are used for commercial production of citric acid, and the most effective strains are **Aspergillus niger**. It is able to produce more citric acid per unit time, and suppress production of undesirable products such as oxalic acid, it is therefore used in large scale for the production of citric acid (4).

The yeasts are also able to produce citric acid in shorter period of incubation time compared with molds such as **Hansenula**, **pichia** ,**Debaromyces**,**Torulopsis**, **Rhodotorula**, and **Candida** . However, they are not commonly used for the production of citric acid owing to productions of undesirable products, isocitric acid [5]. Moreover, the bacterial production of citric acid is available by a few strains such as some species of **Brevibacterium** which utilize glucose as carbon source for growth and productions, and some species of **Corynebacterium** and **Arthrobacter** which use paraffin as carbon source. However, the accumulation of citric acid from bacterial process is not developed well compared with fungal and yeast processes (6).

The bulk of produced citric acid today is used in food, beverage, pharmaceutical and cosmetic industries. Also, it serves in pH adjustment, antioxidant and buffering agent (7, 8). Approximately 88% of produced citric acid is used in the food and industrial applications. 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 (9).

The production of citric acid by A. niger is highly affected by cultural conditions such as carbon sources and concentration [10], nitrogen sources and concentration (11), acidity of the medium (2), aeration (13), and morphology of the fungus (14).

The present work is an attempt to produce citric acid using carob pod extract (syrup) as a raw material. The carob pod kibble is a fruit of carob tree **Certonia siliqua**. Economically this plant is intended to use only as an animal feed and for some human consumption such as carob pod syrup. Thus, the carob syrup was used throughout this investigation as a carbon source for the growth of **A. niger** and production of citric acid.

II. Materials and Methods

1.1. The microorganism: **Aspergillus niger** EMCC1132 was used throughout this investigation, which had been imported from Egypt microbial culture collection, Cairo MIRCEN. It was maintained and activated at the interval of 4-5 weeks on potato-dextrose-agar (PDA) medium

1.2. 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 overnight .Thereafter, the infusion was filtrated to remove the residual kibble intended from this procedure. The filtrate solution from was boiled for 7-8 hours and left to be cooled, carob pod syrup was then obtained. The sugar concentration of the syrup was 60 %. In order to prepare 500ml of fermentation medium of carob pod syrup containing sugar at the concentration of 10 %, 83.33g carob pod extract was added to 400ml distilled water and 0.5% of $NaNO_3$ as a nitrogen source was added. The solution was mixed well and the pH was adjusted to 3.5 and finally the volume was completed to 500 ml.

1.3. Cultural conditions: The fermentation media of each experiment were distributed into 250 ml conical flasks in triplicate samples receiving each 50ml of broth medium. Thereafter, they were plugged and covered by aluminum foil before being autoclaved for 15 minutes at $121C^0$. After cooling, the culture flasks were inoculated with 2% of fungal spores' suspension (Appr. $4.5x10^6$). After that the inoculation culture flasks were incubated in shaking incubator (150RPM) for sufficient time at 30 ºC and at specifically interval, triplicates of each treatment were withdrawn for further analysis.

1.4. Analytical methods

I. Measurement of initial and final pH values: The pH value of fermentation media was achieved using hydrogen ion concentration instrument (pH METER SCHOTT TYPE CG 842)

II. Determination of biomass dry weight: The fungal biomass was separated from a fermented medium by filtration. The fungal mycelium was dried overnight at 60-65 ºC using Memmert 600 Oven. Thereafter, the biomass dry weight was measured accurately using SCALTEC SPB 63 balance.

III. Estimation of the citric acid concentration: The described method of Pearson (15)was used to determine the amount of accumulated citric acid.

IV. Estimation of the initial and residual sugar concentration: Estimation of the sugar concentration in carob pod syrup was performed according to Dubois et. al., method (16).

1.5. Experiments

Different experiments were performed through this study to determine the activity of the fungus for high production of citric acid as follow:

I. The effect of different incubation periods (2, 4, 6, 8 and 10) days on citric acid production.

II. The effect the addition of different nitrogen sources containing the same amount of nitrogen on citric acid production: [(NH_4) $_2$ SO_4, 0.38;; NH_4 H_2 PO_4, 0.67; $NaNO_3$, 0.5; peptone, 0.0.49 and urea, 0.23]%.

III. The effect of the addition of different concentrations (0.01 , 0.05 , 0.1 , 0.15 , 0.2 , 0.25 and 0.30) % of $CaCL_2$ to fermentation medium on citric acid production.

IV. The effect of the addition of different concentrations (1, 2, 3, 4 and 5) % of ethanol to fermentation medium on citric acid production.

III. Results

The effects of different incubation period on the production of citric acid have been studied to detect the most appropriate time for a maximum citric acid accumulation. The results given in table (1) showed that the amount of citric acid was increased with the incubation time reaching 12.85g/L, (55.48%) after six days of incubation and then decreased with the time reaching 8.28g/L (35.75%) after 10 days. The same condition has been observed for the biomass dry weight, whereas it is increased gradually to reach 23.16g/L after 6 days of incubation and then started to decrease again. The final pH value was also declined specially after 6 days of incubation to reach 3.16 .

Another experiment was conducted to study the effect of different nitrogen sources on citric acid production to explore the best one. Therefore, different nitrogen sources containing equivalent amount of nitrogen present in 0.5 % of $NaNO_3$ were applied and then the amount of the acid produced was calculated. The results in table (2) show that ammonium sulphate was more superior to other nitrogen sources with respect to citric acid production. The produced amount of citric acid was 18.25 g/L which represents 70.16% of the biomass dry weight. The other nitrogen sources also improved citric acid accumulation comparing to that of control. The highest yield of citric acid was associated with the lowest of final pH value and residual sugar which was obtained in fermentation medium that contains ammonium sulphate and ammonium dihydrogen phosphate. The effect of different concentration of $CaCl_2$was also studied to detect the best concentration that

stimulates citric acid production. Table (3) shows that the addition of $CaCl_2$ to the fermentation medium at concentration of 0.05 % highly stimulated citric acid production to reach 20.6 g/L (89.25%), while, the obtained amount of biomass dry weight was 23.60 g/L in fermentation medium containing 0.01 % $CaCl_2$. It is clear that the value of final pH was decreased in all treated media and the lowest pH value was 2.08 obtained in medium containing 0.01 $CaCL_2$.

Table (1): Effect of different incubation periods on citric acid production by A. niger

Incubation periods	Dry weight g/ l	Citric acid		Residual Sugar g \l	Final pH
		g/ l	**%**		
2	12.20 (0.151)	8.06 (0.17)	66.86 (1.98)	0.103 (0.015)	3.83 (0.015)
4	16.41 (0.13)	11.18 (0.115)	68.13 (2.42)	0.082 (0.003)	3.55 (0.09)
6	23.16 (0.151)	12.85 (0.14)	55.48 (3.26)	0.075 (0.002)	3.16 (0.015)
8	23.21(0.103)	11.40 (0.037)	49.12 (1.3)	0.057 (0.005)	3.19 (0.104)
10	23.16 (0.115)	8.28 (0.034)	35.75 (1.309)	0.054 (0.006)	3.46 (0.105)

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

Table (2): Effect of different nitrogen sources on citric acid production by A. niger

Nitrogen Sources	**Dry Weight g/ l**	**Citric acid**		**Residual Sugar g\l**	**Final pH**
		g/ l	**%**		
Control	20.0(0.529)	11.20 (0.07)	56.00 (0.52)	0.03 (0.009)	2.20 (0.02)
$(NH_4)_2SO_4$	26.01(0.057)	18.25 (0.10)	70.16 (2.15)	0.017 (0.008)	2.01 (0.063)
$NH_4 H_2PO_4$	22.0(0.021)	14.85 (0.032)	67.50 (7.06)	0.012 (0.007)	1.99 (0.015)
$NaNO_3$	25.03(0.025)	13.50 (0.09)	53.93 (1.75)	0.02 (0.005)	2.15 (0.04)
Peptone	19.0(0.113)	11.58 (0.08)	60.94 (1.01)	0.080 (0.005)	3.81(1.28)
Urea	17.21(0.1)	7.160 (0.08)	41.70 (2.07)	0.076 (0.002)	3.04 (0.09)

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

Table (3): Effect of different concentrations of $CaCl_2$ on citric acid production by A. niger

$CaCl_2$%	**Dry Weight g/ l**	**Citric acid**		**Residual Sugar g\l**	**Final pH**
		g/ l	**%**		
Control	22.0 (1.0)	16.73 (0.057)	76.04 (0.31)	0.018 (0.003)	2.70 (0.049)
0.01	23.6 (0.304)	17.16 (0.142)	72.71 (1.012)	0.024 (0.013)	2.08 (0.094)
0.05	23.08 (0.043)	20.60 (0.087)	89.25 (0.398)	0.015 (0.011)	2.13 (0.042)
0.10	22.06 (0.64)	16.16 (0.17)	73.25 (1.72)	0.025 (0.011)	2.15 (0.042)
0.15	21.70 (0.41)	13.90 (0.15)	64.05 (0.55)	0.015 (0.003)	2.27 (0.007)
0.20	21.22 (0. 15)	7.50 (0.11)	35.34 (0.7)	0.014 (0.005)	2.28 (0.13)
0.25	21.16 (0.12)	5.47 (0.09)	25.85 (0.64)	0. 012 (0.001)	2.90 (0.049)
0.30	20.62 (0.24)	5.27 (0.19)	25.55 (0.70)	0.01 (0.002)	2.92 (0.09)

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

The results of the effect of different ethanol concentrations added to carob pod medium on the production of citric acid indicates that the highest amount of citric acid accumulation by the fungus was 21.06 g/L (113.22%), obtained in culture medium containing 2% ethanol table (4). Moreover, it is clear that there is an inverse relationship between biomass dry weight and concentration of added ethanol and the lowest amount of dry weight (11.33g/L) obtained in medium containing 5% ethanol. Explicitly, the lowest final pH values were increased with the amount of added ethanol to fermentation medium.

Table (4): Effect of different ethanol concentrations on 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)	89.69 (3.72)	0.01 (0.003)	2.07 (0.059)
1	19.10 (0.032)	18.52 (0.085)	96.96 (3.68)	0.014 (0.005)	2.09 (0.062)
2	18.60 (0.02)	21.06 (0.153)	113.22 (1.14)	0.019 (0.004)	2.12 (0.079)
3	15.03 (0.062)	17.40 (0.45)	115.77 (1.60)	0.01 (0.009)	2.29 (0.149)
4	12.07 (0.031)	14.75 (0.092)	122.20 (4.20)	0.022 (0.005)	2.49 (0.083)
5	11.33 (0.045)	13.01 (0.092)	114.82 (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)

IV. Discussion

It has been reported that citric acid production by microorganisms, especially **A. niger** was affected by chemical and physical properties of the fermentation medium (17, 18). 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**. The results showed that this factor plays an important role in the production of citric acid. It was found that six days of incubation period were very suitable for this process. This is consistent with the results obtained by (19, 20, 21) using different carbon sources. On the other hand, (22, 23, 24) found that the suitable periods of incubation for production of citric acid by **A. niger** were 8, 14, 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 (25). The reduction of citric acid production could also be attributed to the toxic effect of accumulated waste products that breaks down citric acid, and also exhaustion of the available energy source (26).

The results of the presence of different nitrogen sources in fermentation media revealed that the source of nitrogen in the form of ammonium salts was more superior than other nitrogen sources. Similar results have been explained by 9[3), who postulated that ammonium compounds found to be the best nitrogen sources that provides acidic condition to the fermentation and stimulated citric acid accumulation by **A. niger,** due to the activation of the enzymes involved in the metabolism. Moreover, the results demonstrate that ammonium sulphate was superior than other nitrogen source for the highest production of citric acid. This result agreed with the results obtained by (27), while, Xu, et. al., (28) reported that the nitrogen source in the form of urea greatly stimulated citric acid production comparing to other nitrogen sources. This finding might be owing to composition of fermentation media or the nature of fungal strain.

It has been concluded 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), due to the activation of pyruvate dehydrogenase which is responsible for the production of Co-enzyme A (29). Therefore, different calcium ion concentrations were applied throughout this experiment, and the results revealed that 0.05 % of $CaCl_2$ highly stimulated citric acid production. Pera & Callieri (30) explained that the addition of $CaCl_2$ at 0.05 % to the fermentation medium inhibits cell building and increases the absorption of phosphate and carbon by the fungus **A. niger** and highly stimulated citric acid accumulation. The result of present study was also similar to the results explained by (31) who noticed that the addition of 0.05 % $CaCl_2$ to the acid hydrolysis of sawdust powder fermentation medium greatly stimulated citric acid accumulation by **A. niger**. Moreover, Pera & Callieri (32) stated that the positive effect of calcium might be related to the increase of the mycelial branching level which probably favors the formation of pellet and improving the process. Moreover, the addition of different ethanol concentrations to the carob pod medium showed inhibitory effect on fungal growth and promotion in the biosynthesis of citric acid specially in fermentation medium containing 2 % ethanol. This result came hand to hand with the result of other investigators (33, 34) who reported 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%. The increased production of citric acid is potentially attributed to the increased activity of the enzyme citric acid synthetase and decreased aconitase activity after addition of ethanol (35). Robert et. al., (36) also observed that the activities of TCA cycle enzymes were increased slightly after the addition of ethanol with slow degradation of citric acid consequent to reduction of aconitase activity.

V. Conclusions

Carob pod extract can be used as a raw material for the production of citric acid by **A. niger**. Moreover, additions of some factors such as calcium chloride and ethanol at specific concentration to the fermentation media have stimulatory effect on citric acid accumulation by the fungus.

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