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An Experimental Study on Citric Acid Production by *Aspergillus niger* Using Date Extract By-Product as a Substrate

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Mansor S. Mostafa Boufaris[1]*, Kutaiba Ibrahin Alzand[2], Sabri ÜNAL[3], Mertcan KARADENİZ[4]

[1,3,4]Department of Forest Engineering, Kastamonu University, Kastamonu, Turkey

[2]Department of Biology, University of Sulaimani, Sulaimani, Iraq.

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ABSTRACT

The date extract by-product was recycled with respect to sugar content by treating with different temperature. The extracted sugar was used as a carbon source for production of citric acid by the Fungus *Aspergillus niger*. The results of water hydrolysis of date extract by-products indicated that the high sugar was recovered after treating the by-products with 100C° for 30 min and the percentage of the yielded sugar was 8%. Different experiments were developed throughout this investigation to determine the best condition for the high initial pH value for the high accumulation of citric acid were achieved at 4. Different nitrogen sources were also applied and the results showed that the peptone was more superior to other nitrogen sources with respect to citric acid production and high yields were achieved at peptone concentration of 0.15%. The addition of calcium chloride at the concentration of 0.01 % was also stimulated citric acid production by the fungus.

INTRODUCTION

Some weak organic acids such as citric acid can be found in all citrus fruits or synthetic sources. Global production is increased day by day according to expectations of rising demands. The fermentation process produces some factors affecting in citric acid, and these process including the concentration and type of carbon source, the pH of the fermentation medium, phosphate and nitrogen limitations aeration. The morphology of the citric acid producing microorganism and the concentrations of trace elements. Some of the nutrients, such as trace metals like manganese, phosphate, and nitrogen, must be specified in a low limit to have a positive effect on the fermentation process (Shetty, 2006; Kristiansen, 2002). However, other elements, such as oxygen and sugar, are required in excess (Kamzolova, S. V, *et al*, 2003; Mostafa, 2012; Max *et al*., 2010; El-Kady, Sherif (2014). In addition, other factors that have effects on citric acid production include lipids, such as groundnut oil (Millis NF *et al*, 1963; Kumar & Ethiraj 1976; Souza *et al*. 2014), and amino acids and vitamins (Bielecki, 2000). Including citric acid production different kind and species of micro-organisms such as bacteria (Grewal, 1995; Kapoor et al., 1983), beside fungi (Mattey and Allan, El Dein Selim, 1979). In addition, yeast (Crolla and Kennedy, 2001; Okoshi, 1987). However, the main industrial producer for citric acid production is known as *Aspergillus niger*, it's cheap and better production yield (Schuster *et al*., 2002). Mostly, commercial production of citric acid is carried out by submerged fermentation using *A. niger* (Hansen, *et al*., 1950; Prasad *et al.*, 2013; Yadegary *et al*.2013). *A. niger* is generally used for citric acid production due to its well-developed enzymatic system (Navaratnam, *et al*, 2000). Moreover, it is easy to handle. Citric acid has many uses, in food and beverages (75%), pharmaceuticals (10%), (15%) dietary supplements, cosmetics, detergents, toiletries and other industries (Majumder *et al.* 2010). It is got many applications in several fields. Agricultural wastes were used as a good substrate for citric acid production due to minimizing the cost production such as apple and grape pomace, carrot waste , orange peel, kiwi fruit peel, cotton waste, okara soy-residue and cane molasses (Kumagai, 1981;Kiel *et al.*, 1981; Hang and Woodams, 1986; 1987; Khare *et al*., 1995; Haq *et al.*, 2004). Also food industrial wastes, it can be used as substrate for citric acid production (Mussatto, Solange I., *et al.*, 2012)

MATERIAL AND METHODS

Microorganism Used

Aspergillus niger EMCIIIl species was taken from the available stock culture in the Microorganism Laboratory, Ain Shams University / Egypt.

Preservation of the Strain

The wild-type *Aspergillus niger* EMCIIIl strain was conserved in potato dextrose agar (PDA) slants at 4C° and subcultured monthly.

Carbon Source

The sugar concentration of the final date extract by product was 8%, to prepare it 100g of final date extract by product was added to 1000 ml distilled water and put it in a water bath at 100 C° for 30 minutes and left to be cooled. Thereafter, the infusion was filtrated by centrifuge type NF UUVE 800 at 5000 RPM For 5 minutes and estimate of the sugar concentration was performed according to Dubois and others (1956).

PDA Medium

Potato Dextrose Agar (PDA) was used by taking 200 g of sliced potatoes fresh and placed in a 500 ml of distilled water and been boiled until maturity (30 minutes) and added 20g of dextrose to complete to 1000 ml with distilled water, then separated equally in test tubes at a (5-7 ml) of each tube. They were plugged and covered with aluminum foil before being autoclaved for 15 minutes at 121 C°.

Fermentation Conditions

The fermentation media were distributed into 250ml conical flasks in triplicate samples receiving each 50 ml of broth medium. Then, plugged and covered with aluminum foil before being autoclaved for 15min at 121 C°. After cooling the culture flasks were inoculated with 2% of fungal spores suspension. The inoculation culture flasks were incubated for sufficient time in the

rotary incubator (150 RPM) at 30 C° and at a specifically interval; three replicates of each treatment were withdrawn for further analysis.

Analytical methods

Measurement of initial and final pH values

The initial and final pH of the culture media was adjusted using hydrogen ion concentration instrument pH METER SCHOTT TYPE (CG842).

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 C° using Mummers 600 Oven. Thereafter, the biomass dry weight was measured accurately using SCALTEC SPB 63 balance.

Estimation of the citric acid concentration

The described method of Pearson was used to determine the amount of accumulated citric acid.

Estimation of the initial and residual sugar concentration

Estimation of the sugar concentration in the date extract by-product was performed according to Dubois *et. al*., (1956).

Experiments

Three experiments were performed as follow:

1. The effect of different pH values (3, 3.5, 4, 4.5,5 and 5.5) on citric acid production.

2. The influence of different nitrogen sources [NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, $NaNO_3$, peptone, urea, and $NaNO_3$) on citric acid production

3. The effect of the addition of different concentrations (0.01, 0.05, 0.1, 0.15, 0.2 and 0.25) % of $CaCl_2$ to fermentation medium on citric acid production.

RESULTS

Effect of pH on citric acid production

The results of the effect different pH concentrations added to medium on the production of citric acid indicate that the highest amount of citric acid accumulation by the fungus was (1.57 g/L, 36 %), obtained in culture medium containing pH value of 4 (Table 1). It was more superior to other concentrations with respect to citric acid production. Similar results have been previously described by Haider (2014) when they grew *Aspergillus niger* on date syrup as a carbon source. Rao, P. R., & Reddy, M. K. (2013) reported similar observations when using Oat Bran as substrate.

Table 1. Effect of different pH on citric acid production by *A. niger*.

pH	Residual Sugar	Dry weight	Citric acid		Final pH
	g/ l	g/ l	g/ l	%	
0.3	0.226	4.03	0.55	14.29	3.50
	(0.0057)	(0.0057)	(0.057)	(0.275)	(0.05)
3.5	0.246	5.30	1.32	29.80	3.41
	(0.0057)	(0.05)	(0.025)	(0.453)	(0.028)
4.0	0.253	4.35	1.57	36.09	3.23
	(0.0057)	(0.05)	(0.017)	(0.793)	(0.0288)
4.5	0.460	4.70	1.15	24.46	3.73
	(0.01)	(0)	(0.05)	(1.06)	(0.028)
5.0	0.400	4.51	1.21	26.93	3.62
	(0.064)	(0.076)	(0.05)	(0.256)	(0.036)
5.5	0.39	4.63	1.11	24.09	3.47
	(0.0057)	(0.0057	(0.028)	(0.317)	(0.025)

Each number represent the mean of three replicates and the number between brackets represent (S. D.).

These results are confirmed when they are statistically analyzed at significance (α=0.05). LSD test also revealed that there a significant difference in case of production in all treatments.

Effect of different nitrogen sources

Nitrogen content in the media is of extreme importance for synthesis of citric acid. For this reason, it was investigated which nitrogen source would be of most value in this experiment. All nitrogen sources also improved citric acid accumulation comparing to that of control.

As illustrated in Table 2, the results revealed that the sources of nitrogen affected the synthesis of citric acid. The results of the presence of different nitrogen sources in fermentation media revealed that the source of nitrogen in the form of peptone was more superior to other nitrogen sources. Maximum production of citric acid was (5.1 g/L, 117 %). These results disagree with the results reported by Yigitoglu, *et al* (1992) and El-hashmy (2004). They found that the addition ammonium dihydrogen phosphate to the fermentation influential catalyst to citric acid production. Whereas Hader (2014) shows that ammonium sulfate was superior to another nitrogen source for the highest production of citric acid. It is clear that the value of final pH and residual sugar were decreased in all treated media comparing with control.

Statistical analysis showed that increased effect citric acid production by peptone was highly significant at level (α = 0.05)

Table 2. Effect of different nitrogen sources on citric acid production by *A. niger*

Nitrogen Sources	Residual Sugar g/ l	Dry weight g/ l	Citric acid g/ l	%	Final pH
Control	0.89 (0.01)	3.43 (0.0577)	0.49 (0.0057)	14.34 (0.040)	3.45 (0.040)
NH_4CL	0.471 (0.0076)	3.86 (0.0288)	4.21 (0.0152)	10.59 (0.069)	1.96 (0.057)
$(NH4)_2SO_4$	0.440 (0.01)	3.91 (0.0288)	2.40 (0)	61.27 (0.450)	1.96 (0.057)
$NH_4 H_2PO_4$	4.33 (0.00577)	4.11 (0.028)	3.583 (0.02862)	85.03 (0.66)	2.05 (0.05)
$(NH_4)_2HPO_4$	0.296 (0.005774)	4.16 (0.057)	3.63 (0.028)	87.20 (0.519)	3.65 (0.05)
Peptone	0.346 (0.0057)	4.255 (0.103)	5.10 (0)	117.42 (0.311)	1.88 (0.026)
Urea	0.376 (0.0057)	4.03 (0.0577)	3.96 (0.0281)	98.35 (0.687)	1.9 (0)
$NaNO_3$	0.513 (0.0057)	3.73 (0.0577)	1.49 (0.01)	39.91 (0.406)	2.53 (0.0577)

Each number represent the mean of three replicates and the number between brackets represent (S. D.)

Effect of calcium chloride on citric acid production

Table 3 shows that the addition of $CaCl_2$ to the fermentation medium at concentration of 0.01 % highly stimulated citric acid production to reach 5 g/L (131%) when the $CaCl_2$ was further increased during fermentation, the production of citric acid decreased, gradually. It is concluded that clear the value of biomass dry weight was 3.86 g/L in fermentation medium containing 0.01

% $CaCl_2$, It is gradually reduced to 3.61 g/L with 0.25% $CaCl_2$. Observably the value of final pH was decreased in all treated media and the lowest pH value was 1.79 obtained in medium containing 0.01 $CaCl_2$. The highest final pH values were increased with the amount of added calcium chloride to fermentation medium.

These results are confirmed when they are statistically analyzed at significance (α=0.05). LSD test also revealed that there a significant difference in case of production in all treatments.

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

$CaCl_2$ %	Residual Sugar	Dry weight	Citric acid		Final pH
	g/ l	g/ l	g/ l	%	
Control	0.40	0.73	4.15	111.16	1.916
	(0.0057)	(0.0577)	(0.05)	(0.875)	(0.041)
0.01	0.34	3.86	5.03	130.73	1.79
	(0.0057)	(0.0288)	(0.057)	(0.875)	(0.0057)
0.05	0.40	3.73	3.37	90.48	1.92
	(0.0057)	(0.0577)	(0.028)	(0.445)	(0.015)
0.10	0.40	3.73	2.83	75.88	1.93
	(0.0057)	(0.0577)	(0.0577)	(0.369)	(0.015)
0.15	0.40	3.70	2.53	68.01	1.97
	(0)	(0)	(0.028)	(0.779)	(0.030)
0.20	0.41	3.59	2.36	65.91	1.97
	(0.0057)	(0.036)	(0.057)	(0.995)	(0.025)
0.25	0.41	3.61	1.80	49.81	2.00
	(0)	(0.023)	(0)	(0.317)	(0)

Each number represent the mean of three replicates and the number between brackets represent (S. D.)

DISCUSSION

CA accumulation is strongly influenced by the type and concentration of carbon source (Grewal, 1995; Xu, 1989). Max (2010) that the factors mainly affecting the citric fermentation are the type and concentration of carbon source, nitrogen and phosphate limitation, pH, aeration, oligo elements concentration, and morphology of the producing microorganism. The pH of a culture may change in response to microbial metabolic activity.

The first experiment was designed to determine the best value of PH that stimulates of citric acid by the fungus *A. niger*. A pH value of 4 was found optimum for producing maximum amounts of citric acid. This is consistent with the results obtained by (Rao, 2013; Ganne, 2008). The pH is more stable between 4.0 and 5.0 (Soccol, 2006).

A low initial pH, generally below 2.0, is extremely important for maximum production of CA and has the advantages of limiting contamination and inhibiting oxalic acid formation (Vandenberghe *et al.*, 1990; Kubicek, 1986). A pH of approximately 3.0 was reported to be optimum for both fungal growth and the production of CA using synthetic medium or pure carbon sources. Higher pH values of approximately 5.0 or 6.0 have been found to be optimum for CA production in molasses medium (Vandenberghe *et al*. 1990; Soccol, 2006).

Al-Shehri and Mostafa (2006) and Afifi (2011) reported that the initial pH of 5.5 was optimum for citric acid production.

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Xu, (1989) and Max (2010) they reported that the factors mainly affecting the citric fermentation are the type and concentration of carbon source, nitrogen and phosphate limitation, pH, aeration, oligo elements concentration, and morphology of the producing microorganism.

The concentration of nitrogen has been found to have a strong effect on the production of citric acid, as nitrogen is not only part of a cell's proteins, but also necessary for cellular metabolism (Ali *et al.* 2002a; Kareem, 2010).

The results of the presence of different nitrogen sources in fermentation media revealed that the source of nitrogen in the form peptone was more superior to other nitrogen sources. Similar results have been explained by (Kurbanoglu & Kurbanoglu, 2004), who postulated that peptone found to be the best nitrogen sources that provide an acidic condition to the fermentation and stimulated citric acid accumulation by *A. niger*, due to the activation of the enzymes involved in the metabolism.A high nitrogen concentration increases the consumption of sugar and fungal growth while decreasing the amount of citric acid produced (Hang et al. 1977).

On Ali *et al.* (2002b), Rao, (2013) and Kareem (2010), they reported that ammonium nitrate was superior to another nitrogen source for the highest production of citric acid.

Mattey (1992) reported that the high purity media, used in laboratory scale research, were supplemented usually with ammonium salts which reduced slightly the pH value of the media to an acidic condition which favored fermentation. A high nitrogen concentration increases the consumption of sugar and fungal growth while decreasing the amount of citric acid produced (Hang *et al*. 1977).

The results showed that this factor plays an important role in the production of citric acid, the addition of $CaCl_2$ to the fermentation medium at concentration of 0.01 % highly stimulated citric acid production. Moreover, Pera and Callieri (1997) who reported that the addition of 0.5 g/L $CaCl_2$ to the fermentation medium increased the production of citric acid. An addition of $Ca+^2$ induced a pelleted form of growth, highly branched hyphae and numerous bulbous cells. Bulbous cells growing in the presence of Ca^{+2} exhibited cell walls composed of laminated layers and featured vesicles associated with the wall and/or the cell membrane, containing numerous inclusions. The cytotoxic effect of high concentrations of citric acid in the medium as well as an increase of the activity of N-acetyl-beta-D-glucosaminidase, a lytic enzyme, might be involved in these morphological changes. While, Haider, 2014 revealed that the highest production of citric acid by the fungus A. niger was achieved when the concentration of calcium chloride in the fermentation media was 0.05%, while, Mazhar, (2003) reported that calcium chloride (1.5%)

were optimized for enhanced citric biosynthesis. This resales disagree with (Papagianni *et al.* 1999a and b; Bayraktar and Mehmetog 2000; El-Kady, 2014; Banik 1976) were used $CaCl_2$ in the fermentation media to produce citric acid. Calcium chloride did not prove to be increasing for citric acid.

CONCLUSION

In this study, the main emphasis is given on the techniques by which citric acid can be produced at low cost. The study revealed that these parameters effect citric acid production extremely. The use of these wastes might represent an efficient method of reducing the environmental problem due to their disposal and also help in the reduction of the substrate cost.

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