

## Citric Acid Production from Pretreating Crude Date Syrup by *Aspergillus niger* NRRL595

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**Abstract:** The production of citric acid by *Aspergillus niger* NRRL595 and *Aspergillus niger* NRRL599 in crude date syrup was evaluated. *Aspergillus niger* NRRL595 produced higher concentration of citric acid (5.28g/l) than *Aspergillus niger* NRRL599 (1.0g/l), while *Aspergillus niger* NRRL599 synthesized biomass dry weight (31.98g/l) higher than synthesized by *A. niger* NRRL 595 (16.36g/l) with sugar consumption (61.83%), whereas, sugar consumption by *A. niger* NRRL595 was 72.91%, which were achieved at an initial sugar concentration of 150 g/l and pH value of 6.5. The concentration of citric acid produced by *A. niger* NRRL 595 was increased as fermentation progressed up to 360h (6.84g/l). Biomass dry weight also increased as fermentation progressed up to 360h (16.73g/l). Citric acid concentration increased as pH values increased from 3.5 to 6.5, reaching its maximum at pH 6.5(6.55g/l) with a sugar utilization value of 79.49%. The highest biomass dry weight was obtained at pH value of 3.5 (20.53g/l). Citric acid concentration increased as sugar concentration increased from 90 to 240g/l. The highest concentration of citric acid (11.53g/l) was obtained at a sugar concentration of 240g/l. High biomass dry weight (33.9g/l) and sugar utilization (79.74%) were achieved at the same sugar concentration. The production of citric acid from pretreated date syrup(240g sugar /l, pH6.5 and 360h) using different chemical and biological methods was highly increased in comparison with crude date syrup except the treatment by sulfuric acid. Maximum citric acid concentration (31.10g/l) was obtained in culture grown in 3%(v/v) methanol treated medium. On the other hand the highest biomass dry weight (41.90g/l)was achieved in culture grown in 2%(w/v) tricalcium phosphate treated medium. Generally, the order of the treatments of date syrup to produce high citric acid production was : methanol 3%(v/v) > methanol 5% > potassium ferrocyanide (25µg/ml) > methanol 1% (v/v) > methanol 4% (v/v) > boiled *Rhizopus* sp. NRC 5 biomass.

**Key words:** *Aspergillus niger*, citric acid, dates, fermentation, chemical and biological treatments

### INTRODUCTION

Dates are produced in tropical and subtropical countries. The semidried date called tamer<sup>[14]</sup> contains the following (expressed as g 100<sup>-1</sup> of date): moisture, 16; total sugars (glucose and fructose), 65; fiber, 6; protein, 2.5; ash, 1.5; other compounds, 9.0. The dates and date syrup are used for human consumption, in bakery and ice-cream products, and for the production of caramel color, ethanol, vinegar, and single cell protein<sup>[14]</sup>. Because of the high concentration of sugars in the date, it is important to develop new and more attractive uses of these sugars.

Citric acid is one of the organic acids of which world market in growing every year and is extensively used in food and pharmaceutical industries. It is produced mainly by submerged, surface and soiled state fermentation using *Aspergillus niger* from different sources of carbohydrates, such as molasses; date syrup and date pulp<sup>[6,10,17,13,23,8,19,20,2,21]</sup>. It is well known that citric acid fermentation is greatly affected by presence of some trace metals in toxic concentrations and it can be a significant

problem during the fermentation of crude substrates into useful chemical products<sup>[4]</sup>. Heavy metals such as iron, zinc and copper which are contained in great amounts in date syrup can cause a critical problem during citric acid fermentation<sup>[1]</sup>. Concentrations of trace metals in culture media may be controlled in either of two ways. The first method is to purify the medium so as to remove certain metal ions. The second method is to add metal chelating agents to the medium to decrease the concentration of free metal ions to desired values<sup>[4,3,19]</sup>.

The present investigation describes the effect of pretreating crude date syrup with easy applicable techniques on its heavy metals content and production of citric acid when using this substrate as a carbon source in fermentation experiments using *Aspergillus niger* NRRL 595.

### MATERIALS AND METHODS

**Organism and culture conditions:** *Aspergillus niger* NRRL 595 and *Aspergillus niger* NRRL 599 were obtained

from the culture collection of Northern Regional Research Laboratory, Department of Agriculture, Peoria, Illinois, U.S.A., *Rhizopus* sp. NRC 5 a local isolated strain from aged acidic whey was identified by plant pathology Dept. National Research Center, Dokki, Cairo, Egypt. The fungi were maintained on potato dextrose agar (PDA) slants at 4°C and subcultured at intervals from 15-30 days. *Aspergillus niger* strains were incubated on PDA Petri dishes at 30°C for 5 days. Circular mycelial plug (4mm in diameter) was transferred to each of a sterile date syrup flask.

*Rhizopus* sp. NRC5 was grown in a sterile medium containing (g/l): Yeast extract, 3; Peptone, 10 and glucose syrup, 30 with shaking (150rpm) for 5 days at 28°C.

**Preparation of date syrup:** Semi-dried dates were obtained from the local market. Dates were chopped into small particles and heated in distilled water (solid/ liquid ratio, 1:3) at 80°C for 2h. and the mixture was maintained at room temperature for 24h., after that the mixture was homogenized in warring Blender for 3 min. The extract was filtered in a filter press. The extract was diluted with distilled water to obtain 25% sugars.

**Fermentation conditions:** The fermentation was performed in 250 ml conical flasks containing 50 ml untreated or treated date syrup. The medium was sterilized at 121°C for 15 min. and inoculated with one disk of the inoculum. The flasks were incubated static at 30±1°C under stationary conditions.

**Effect of initial pH:** A series of conical flasks each containing 50 ml of the production medium (15% initial sugars) adjusted at different initial pHs (3.5, 4.5, 5.5, 6.5 and 7.5) were inoculated with the fungus and incubated at 30±1°C for 15 days. The pH was adjusted with 3N NaOH or 1N HCl to avoid the change of the initial sugar concentration.

**Effect of initial sugar concentration:** The date syrup (pH 6.5) 25% total sugars, was diluted with distilled water to obtain 9, 12, 15, 21 and 24% initial sugars concentration.

**Pretreatment of crude date syrup:** Date syrup was diluted with distilled water in order to obtain 24% (w/v) total sugar concentration. The date syrup solution was used for all pretreatment experiments as described below.

**Tricalcium phosphate treatment (TPT):** Date syrup solution was adjusted to pH 7.0 with 1N NaOH and treated with 1 or 2% (w/v) TP. The mixture was heated at 100°C for 5 min. After cooling the mixture was centrifuged

at 4,000g for 20 min. and the pH of the supernatant was adjusted to 6.5 with 1N HCl. The insoluble matter was discarded.

**Potassium ferrocyanide:** Date syrup solution was adjusted to pH 6.5 with 1N NaOH and sterilized at 121°C for 15 min. The liquid was treated while hot with 25 or 50 µg/ ml potassium ferrocyanide to encourage the precipitation of heavy metals<sup>[16,4]</sup>.

**Methanol concentration:** A series of conical flasks each containing 50 ml sterilized date syrup (24% initial sugars, pH 6.5) and different methanol concentrations (1,2,3,4 and 5% v/v) were inoculated each with one disk of the inoculum and incubated at 30°C for 15 days. Methanol was added on the third day of fermentation.

**Sulfuric acid:** Date syrup solution was adjusted to pH 3.0 with 1N H<sub>2</sub>SO<sub>4</sub>. The liquid was allowed to stand for 12 h. and then centrifuged at 4,000g for 20 min. The supernatant was adjusted to pH 6.5.

**Boiled *Rhizopus* sp. biomass:** The precultured fungal biomass (2.5g blotted between two filter papers) were added to 50 ml portions of crude date syrup in 250 ml flasks and incubated shaken (150rpm) for 1h. By the end of experiment, the biomass were separated by filtration.

**Analytical techniques:** At appropriate time intervals, the contents of the fermentation flasks were analyzed. Mycelial dry weight was determined by filtering, washing with distilled water and drying at 105°C to constant weight. Citric acid was determined by the method of Marier and Boulet<sup>[12]</sup>. Residual sugars were determined as glucose by the method of Dubois *et. al*<sup>[5]</sup>.

## RESULTS AND DISCUSSIONS

**Citric acid production via surface fermentation:** The production of citric acid from crude date syrup (initial sugar conc. 15%, pH 6.5) by *Aspergillus niger* NRRL595 and *Aspergillus niger* NRRL599 is shown in Table (1). *A. niger* NRRL595 and *A. niger* NRRL599 were examined for the production of citric acid. *Aspergillus niger* NRRL595 was the best organism for the production of citric acid after 288h (12 days) of fermentation period. The strain consumed 72.91% of initial sugar and synthesized 5.28g/l citric acid. This strain also synthesized 16.36g/l dry biomass. On the other hand, *Aspergillus niger* NRRL599 synthesized citric acid less than *Aspergillus niger* NRRL595 (1.0g/l), and synthesized dry biomass higher than that produced by *A. niger* NRRL595 (31.98g/l) with

**Table 1:** Citric acid production from crude date syrup by *Aspergillus niger* NRRL595 and *Aspergillus niger* NRRL599 in surface fermentation at 30 ± 1°C.

Parameters	Organisms	
	<i>Aspergillus niger</i> NRRL595	<i>Aspergillus niger</i> NRRL599
Initial sugar conc. (g/l)	150	150
Initial pH	6.5	6.5
Process time (h)	288	288
Final pH	3.05	4.21
Consumed sugar (%)	72.91	61.83
Citric acid conc. (g/l)	5.28	1.00
Product yield (%) <sup>*</sup>	3.52	0.667
Productivity (g/l/h)	0.018	0.0035
Biomass dry weight (g/l)	16.36	31.98

\* Product yield (%), expresses % citric acid formed of initial sugar in medium.

Each value is an averaged of results from two individual determinations and four experiments.

suger consumption 61.83%. Roukas and Kyriakides<sup>[20]</sup> found that *A. niger* ATCC10577 and *A. niger* B60 differed considerably in their capacity to produce citric acid (8.0 and 5.0 g/l, respectively) when grown on Fig water extract. Also Saad<sup>[21]</sup> found that *A. carbonarius* NRRL67 and *A. niger* NRRL599 differed in their capacity to produce citric acid (2.21 and 1.47 g/l, respectively) when grown on crude date syrup (initial sugar 12%, pH 4.5) for 10 days. The above results show the *A. niger* NRRL595 produced more citric acid than *A. niger* NRRL599. Thus, the strain *A. niger* NRRL595 was selected for all subsequent experiments.

**Time course of citric acid production by *A. niger* NRRL595 from crude date syrup:** Table (2) shows the time course of citric acid production at 30 ± 1 °C by *A. niger* NRRL595 from crude date syrup. Citric acid production increased as fermentation period progressed to 360h. (6.84g/l). Total sugar was reduced from an initial concentration of over 150 g/l to about 30g (consumed sugar 80.09%) after 360h. of fermentation with yield of

**Table 2:** Effect of fermentation period on citric acid production by *Aspergillus niger* NRRL595 grown on crude date syrup at 30 ± 1°C.

Parameters	Fermentation (h)				
	72	144	218	288	360
Initial sugar conc. (g/l)	150	150	150	150	150
Initial pH	6.5	6.5	6.5	6.5	6.5
Final pH	3.28	3.27	3.12	3.05	2.91
Consumed sugar (%)	8.727	38.49	65.73	72.91	80.09
Citric acid conc. (g/l)	2.382	3.256	4.465	5.28	6.84
Product yield (%) <sup>*</sup>	1.588	2.171	2.980	3.52	4.56
Productivity (g/l/h)	0.033	0.0226	0.0204	0.018	0.019
Biomass dry weight (g/l)	7.42	11.100	14.71	16.36	16.73

\* Product yield (%), expresses % citric acid formed of initial sugar in medium.

Each value is an average of results from two individual determinations and four experiments.

citric acid 4.56%. The productivity (g citric /l/h) decreased as fermentation period progressed to 360h. The biomass dry weight increased by the increasing of fermentation period until reached the maximal level after 360h (16.73g/l).

As expected, the fermentation of consumed sugars increased during fermentation, coinciding with an increase in citric acid and biomass. The concentration of consumed sugars fell rapidly from 72 to 360h of fermentation. This was due to rapid increase of citric acid concentration and biomass observed at the same time.

**Effect of initial pH:** As shown in Table (3), citric acid concentration increased with the increase of initial pH from 3.5 to 6.5 and reached the maximal level at pH 6.5 (6.55g/l) with consumed sugar, 79.49% and citric acid yield, 4.366%, also the productivity increased as pH values increased until reached the maximal level at pH 6.5 (0.0182). The highest biomass dry weight was obtained at pH 3.5 (20.53g/l).

An important factor that affects the performance of date syrup fermentation is the initial pH of the substrate<sup>[19]</sup>. These results agree with Roukas and Kotzekidou<sup>[19]</sup>, who found that, the highest values of the fermentation parameters (citric acid concentration, citric acid yield and consumed sugars) were achieved at an initial pH of 6.5.

**Effect of initial sugar concentration:** The experiments were performed with initial sugar concentrations of date syrup (pH6.5) to obtain the best sugar concentration which gives the highest citric acid production. Table (4) shows citric acid concentration and the productivity increased with the increase of sugar concentration from 90 to 240g/l. The highest concentration of citric acid and productivity (11.53g/l and 0.032, respectively) were obtained at a sugar concentration of 240 g/l after 360h. Consequently, consumed sugar increased from 70.64 to

**Table 3:** Effect of initial pH on citric acid production by *Aspergillus niger* NRRL595 grown in crude date syrup at 30 ± 1°C.

Parameters	Initial pH				
	3.5	4.5	5.5	6.5	7.5
Initial sugar conc. (g/l)	150	150	150	150	150
Process time (h)	360	360	360	360	360
Consumed sugar (%)	39.31	67.23	73.89	79.49	66.92
Citric acid conc. (g/l)	3.53	5.64	6.164	6.55	5.60
Product yield (%) <sup>*</sup>	2.353	3.76	4.110	4.366	3.733
Productivity (g/l/h)	0.0098	0.0156	0.0171	0.0182	0.0156
Biomass dry weight (g/l)	20.53	18.36	17.81	16.84	13.88

\* Product yield (%), expresses % citric acid formed of initial sugar in medium.

Each value is an average of results from two individual determinations and four experiments.

**Table 4:** Effect of initial sugar concentration on citric acid production by *Aspergillus niger* NRRL595 grown on crude date syrup at 30 ± 1°C.

Parameters	Initial sugar concentration					
	90	120	150	180	210	240
Initial pH	6.5	6.5	6.5	6.5	6.5	6.5
Process time (h)	360	360	360	360	360	360
Final pH	3.14	3.0	3.03	2.95	2.91	2.91
Consumed sugar (%)	70.64	72.35	81.20	86.23	88.33	79.74
Citric acid conc. (g/l)	3.17	3.82	6.80	7.55	9.60	11.53
Product yield (%) <sup>*</sup>	3.522	3.183	4.533	4.194	4.571	4.80
Productivity (g/l/h)	0.0088	0.0106	0.0189	0.0210	0.0267	0.032
Biomass dry weight (g/l)	11.27	12.91	16.41	18.21	20.95	33.90

\* Product yield (%), expresses % citric acid formed of initial sugar in medium.  
Each value is an average of results from two individual determinations and four experiments.

**Table 5:** Citric acid production from pretreatment crude date syrup by *Aspergillus niger* NRRL595.

Parameters	Control	Pretreatment of crude date syrup											
		Potassium ferrocyanide (ug/ml)		Tricalcium phosphate (%)		Sulfuric acid (1N)	Boiled <i>Rhizopus</i> sp. NRC5 Biomass	Methanol added (%)					
		25	50	1%	2%			1	2	3	4	5	
Initial pH	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Initial sugar conc. (g/l)	240	240	240	240	240	240	240	240	240	240	240	240	240
Process time (h)	360	360	360	360	360	360	360	360	360	360	360	360	360
Final pH	2.91	1.93	1.87	2.06	2.05	1.35	1.94	1.4	1.39	1.51	1.79	2	
Consumed sugar (%)	79.74	75.83	86.59	69.32	67.27	52.73	80.83	76.82	68.94	90.21	78.52	89.93	
Citric acid conc. (g/l)	11.53	19.52	8.66	14.34	6.84	4.06	16.47	17.3	10.66	31.1	17.04	27.02	
Product yield (%) <sup>*</sup>	4.8	8.133	3.61	5.975	2.85	1.692	6.863	7.21	4.442	12.54	7.1	11.26	
Productivity (g/l/h)	0.032	0.0542	0.024	0.04	0.019	0.01128	0.0458	0.0481	0.023	0.0836	0.0473	0.0751	
Biomass dry weight, (g/l)	33.9	31.18	28.72	39.7	41.9	27	38.7	28	27.8	30.2	35.7	39.9	

\* Product yield (%), expresses % citric acid formed of initial sugar in medium.  
Each value is an average of results from two individual determinations and four experiments.

79.74% and the pH value was strongly decreased from 6.5 to 2.91 by increasing the production of citric acid. Also progressive increase of biomass dry weight (from 11.27 to 33.9g/l) was obtained with the increase of sugar concentration.

The results agree with those of Saad<sup>[21]</sup> who reported that citric acid and biomass dry weight increased with the increase of initial sugar concentration from 90 to 210g/l when *Aspergillus carbonarius* grown on date syrup.

**Effect of treatment of date syrup on citric acid production:**

Date syrup was treated by using different chemical and biological methods to precipitate or chelate the heavy metals which affected citric acid production. Data

presented in Table(5) observed that most treatments gave a remarkable increase in citric acid concentration, biomass dry weight productivity and consumed sugars in comparison with untreated date syrup. This means that date syrup contained undesirable substances, which affected citric acid production. The highest value of citric acid concentration, citric acid yield and productivity (31.10g/l, 12.54% and 0.0836, respectively) were obtained in culture grown in 3%(v/v) methanol treated medium, while citric acid production from untreated date syrup by *A. niger* NRRL 595 was 11.53g/l. On the other hand, the highest biomass dry weight (41.90g/l) was obtained in culture grown in 2% (w/v) tricalcium phosphate treated medium. The lower values of citric acid and biomass dry

weight production (4.06 and 27g/l, respectively) were obtained in 1N sulfuric acid treated medium. Generally, the order of the treatments of date syrup to produce high citric acid production was : methanol 3% (v/v) > methanol 5% (v/v) > potassium ferrocyanide (25µg/ml) > methanol 1% (v/v) > methanol 4% (v/v) > boiled *Rhizopus* sp. NRC5 biomass. Hang *et. al*<sup>[7]</sup> and Roukas and Kotzekidou<sup>[18]</sup> reported that the addition of methanol at concentrations of 1-4% (v/v) resulted in a marked increase in the amount of citric acid formed by *A. niger* on spent grain liquor and brewery wastes, respectively. The mechanism by which methanol stimulates citric acid production from sugars is not clear. The high stimulation effect of methanol can be attributed to the inhibition of spore formation and the increase of the microorganisms tolerance to high levels of minerals contained in different sources of carbohydrates<sup>[6]</sup>. Madox *et al*<sup>[11]</sup> reported that the effect of methanol is at the cell permeability level: it allows citrate to be excreted from the cell. The cell then responds by increasing its citrate production via repression of 2- oxoglutarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite.

The use of boiled *Rhizopus* sp. NRC5 biomass as a biosorbent agent for the biological treatment of date syrup in comparison with other chemical treatments to produce high citric acid is a new method which performed recently by us, since *Rhizopus* sp. NRC5 have long been recommended for use as a source of food<sup>[9]</sup>. Data in Table (5) shows that treatment of date syrup by boiled *Rhizopus* sp. NRC5 biomass gave citric acid production higher (16.47g/l) than that produced from treated date syrup by potassium ferrocyanide (50 µg/ml), tricalcium phosphate (1and 2% (w/v)) or sulfuric acid (1N), Table (5).

Saad and Moataza<sup>[22]</sup> found that *Rhizopus* sp. NRC5 biomass markedly removed Ni, Cu, Co and Zn from their solutions better than *A. parasiticus* NRRLA-17995. The taxonomic grouping of the fungus is an important factor which influences bio-adsorption. *Rhizopus* is a member of the order Mucorales. The major macromolecular constituents of Mucorales cell wall are chitosan and chitin. Chitosan, which is distinctive for Mucorales, has been reported to be implicated in the uptake of heavy metal ions<sup>[15]</sup>.

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