Effect of some physiological factors on citric acid production by three isolates of *Aspergillus niger* acid - hydrolyzed sawdust as a carbon source

Efecto de algunos factores fisiológicos en la producción de ácido cítrico por tres aislamientos de *Aspergillus niger* empleando aserrín hidrolizado como fuente de carbono.

Autor:

Muna, B. Abdulazeez [1]
Mustafa M. Haider [2]

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ABSTRACT

Introduction: Citric acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid) was first isolated from lemon juice in 1784. It is a primary metabolic product which is formed in the tricarboxylic acid (Krebs) cycle. It is estimated that the market value of citric acid will exceed two billion dollars in 2019. About 70% of total citric acid produced globally is utilized in food industry, while about 12% is utilized in pharmaceuticals and cosmetic industries and the remainder in other industrial purposes. The industrial production of citric acid is undertaken by fermentation process in the presence of filamentous fungi for large scale of production. *Aspergillus niger* is the most efficient fungus due to its ability to produce more citric acid per unit time and ferment different inexpensive raw materials. **Materials and Methods:** Three isolates of the fungus *Aspergillus niger* (An1, An2, An3) were used throughout this study using different carbon source concentration in the form of sawdust acid hydrolysis supplemented with different concentration of $(NH_4)_2H_2SO_4$ as a nitrogen source. The effect of hydrogen ion concentration and addition of methanol to the fermentation medium was also investigated. **Results and Discussion:** The results indicated that the optimization of carbon and nitrogen concentration had stimulating effect on citric acid production by the three used isolates. Moreover, addition of methanol at concentration of 1% at pH of 3.5 highly increased citric acid production. **Conclusion**: we concluded that the agriculture waste was a favorable substrate for the production of citric acid especially it is cost effective and easily obtainable.

[1] Department of pharmacy, Duhok Technical Institute, University of Duhok Polytechnic, Duhok, Iraq, muna.abdelaziz@dpu.edu.krd.
[2] Department of Biology, College of Science, University of Duhok, Duhok , Iraq, Mustafa.haider@uod.ac.

INTRODUCCTION

Citric acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid) which name originates from the Latin word *citrus*, it was first isolated from lemon juice in 1784. Citric acid is a tricarboxylic acid with a molecular weight of 210.14 g/mol, which contains three carboxylic functional groups with three different values of p*K*a (3.1, 4.7, and 6.4). It is a primary metabolic product formed in the tricarboxylic acid (or Krebs) cycle. The annual production of citric acid reaches up to 1.6 million tons worldwide [(1)] with an annual growth demand/consumption rate of 3.5-4% [(2)]. It is estimated that the market value of citric acid will exceed two billion dollars in 2019 [(3)]. About 70% of total citric acid produced globally are utilized in food industry while about 12% is utilized in pharmaceuticals and cosmetic industries and the remainder in other industrial purposes [(4)]. The industrial production of citric acid is undertaken by fermentation process in the presence of filamentous fungi for large scale of production. *Aspergillus niger* is the most efficient fungus due to its ability to produce more citric acid per unit time and ferment different inexpensive raw materials [(5)]. In addition, its ease of handling and high yields of the acid. Refined sugars such as glucose and sucrose are the most commonly used substrates for the production of citric acid by fermentation processes, however, owing to their high costs they have been replaced by costlier effective substrates such as molasses, carob pod extract, rape seed oil, corn cobs, apple and grape pomace which are utilized as a carbon source for fungal growth and the production of citric acid [(6,7,8)] Moreover, raw agro waste, such as sawdust, possesses high carbon content, approximately 60-70% that render it as an important and cheap carbon source for citric production [(9)].

The productivity of fermentation products is greatly influenced by the type of substrate as well as the fermentation conditions like temperature, fermentation time and the type of culture/strain [(4)]. The carbon source for citric fermentation has been the subject of many studies, especially regarding the use of polysaccharides. In general, only the sugars that are quickly assimilated by the microorganism allow high final yield of citric acid [(10)].

The nature and concentration of nitrogen source directly influence the citric acid production. Nitrogen sources are usually derived from ammonium salts such as ammonium nitrate and sulphate [(11, 12)].

Belen *et al.*, [(10)] explained that low pH during the production phase reduces the risk of contamination by other microorganisms and suppresses the production of unwanted organic acids such as gluconic and oxalic acids. On the other hand, high pH values induce the production of a large amount of oxalic acid. Several researches have attempted to explain the biochemical role of alcohol on citric acid production by *A. niger*[(13,14)]. Rugsaseel *et al.*, [(15)] have found that the addition of methanol to the production medium remarkably depressed cellular protein synthesis without inhibiting nitrogen uptake, thus causing an increase of amino acids, peptides and low-molecular-mass protein pooled in the mycelium especially at the early stage of cultivation. Also, it has changed the activity of some enzymes in or related to Krebs cycle, rendering them suitable for citric acid accumulation. The stimulating effect of methanol can be attributed to the inhibition of spore formation and its effect on the cell permeability level; it allows citrate to be excreted from the cell [(16, 17)].

This study was developed to study the effect of different carbon concentrations in the form of locally available sawdust acid hydrolysis. The effect of the initial pH value, different concentration of nitrogen in the form of ammonium sulphate and finally the effect of methanol concentration on citric acid production by *Aspergillus niger* isolates was also determined.

MATERIALS AND METHOD

The microorganism

Three isolates of the fungus *Aspergillus niger* (An1, An2 and An3) were used throughout this study which have been obtained from culture collection of mycology research laboratory department of biology, college of science, university of Duhok. They

were maintained and activated every four weeks using potato dextrose agar medium (PDA).

Acid-hydrolyzed sawdust

Acid-hydrolyzed sawdust was used as a basal medium and carbon source for the growth of the fungus *Aspergillus niger* strains and production of citric acid. It was prepared by addition of 10g to 400ml of 10% H_2SO_4 and heated at 10 ^{0}C in shaking water bath (shaking water bath, SBS 40) for one and half hour after reaching 100^{0}Ctemperature. After that the solution was left for cooling and the debris was separated from the sawdust syrup by filtration using filter papers (Qualitative filter paper low ash and hardened 15 cm). The sugar concentration of the hydrolyzed sawdust was measured spectrophotometrically at 490nm using Jenway 6305 spectrophotometer, U.K) to determine the suitable carbon concentration for the growth of the fungal strainsand production of citric acid.

Inoculation medium

This medium was used to prepare fungal isolates inoculum which contain the following chemical compounds: sucrose, 10%; $(NH_4)_2SO_4$ 0.4%; yeast extract, 0.1%; K_2HPO_4,0.1% at pH value of 3.5

Stock solution

This solution is of the following composition: $FeSO_4.H_2O$ 3.0%, $ZnSO_4.7H_2O$ 4.0%, $CuSO_4.7H_2O$ 4.0% and $MnSO_4$. $7H_2O$ 1.5%. It added to the fermentation medium at concentration of 1%. .

Fermentation technique

The fermentation media contained the required sugar concentration in the form of sawdust was prepared followed by the addition of ammonium sulphate as a nitrogen source and 1% of stock solution. The pH of the media was adjusted to 3.5 using pH meter (HANNA instrument, Taiwan) and the experiment was continued for 8days. Finally, the fermentation technique was achieved according to Haider, (2014)[18]

Analytical methods

Adjustment of initial and final pH value

The initial and final hydrogen ion concentration (pH) value of fermentation media was adjusted using hydrogen ion concentration instrument (pH HANNA instrument, Taiwan).

Determination of biomass dry weight

The described method of Haider, (2014)[18] was followed for determination of the fungal isolates dry weight.

Estimation of the citric acid concentration

The method mrntioned by Pearson (1973)[19] was used to determine the concentration of citric acid in fermentation media at the end of each specific interval time.

Estimation of sugar concentration

Estimation of the sugar concentration was according to a method explained by Dubois *et al.* (1956)[20]. The sugar concentration was calculated according to previously prepared standard curve using different concentrations of glucose (20, 40, 60, 80, and 100) µg/ml.

Experiments

1. The effect of different sugar concentrations (10, 12.5, 15, 17.5) % in the form of sawdust hydrolysis on citric acid production
2. The effect of the initial pH value (2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6) on citric acid production.
3. The effect of different concentrations of $(NH_4)_2SO_4$ (0.2, 0.3, 0.4, 0.5, 0.6)%as a nitrogen source on citric acid production
4. The effect of different concentrations of methanol (1, 2, 3, 4, 5)% on citric acid production

RESULTS

1. The effect of sugar concentration on citric acid production by local isolates of *A. niger*

This experiment has been carried out to find the effect of different sugar concentrations in the form of sawdust hydrolyzed on the production of citric acid by *A. niger* strains and the results are shown in table (1). As shown in presented data, the amount of citric acid increased directly with the increase in sugar concentration in the fermentation medium. The best sugar concentration for highest citric acid production was 15% in which the produced amount reached (14.35g/l, 67.06%) (14.63g/l, 60.38%) and (14.76g/l, 64.43%) for An1, An2 and An3 respectively, followed by the fermentation medium contains 12.5% sugar.

The highest biomass dry weight of the tested fungal strains was also obtained in the fermentation medium containing 15% sugar (24.23g/l) in the case of the fungal isolate An2, but fermentation medium containing 17.5% sugar was the best for An1 and An3 and the amount of the obtained biomass dry weight was (23.6g/l) for An1 and (25.2g/l) for An3 strain.

It was also demonstrated that highest value of consumed sugar was achieved in the fermentation medium containing15% of sugar which reduced to (1.49%), (1.42%) for An1 and An3 respectively, while for An2 it was reduced to (1.38%) in fermentation medium containing 12.5% sugar.

Table 1. The effect of sugar concentrations on citric acid production by local isolates of *A. niger*

Sugar %	*A. niger* isolates	Mean dry weight g/L	Critic acid g/L	Critic acid %	Residual sugar %
10%	**An1**	16.51	7.79	47.18	1.19
	An2	18.10	9.77	53.98	1.19
	An3	17.43	9.98	57.26	1.27
12.5%	**An1**	19.3	10.35	53.63	1.38
	An2	21.6	12.48	57.78	1.38
	An3	22.6	13.95	61.73	0.85
15%	**An1**	21.4	14.35	67.06	1.49
	An2	24.23	14.63	60.38	1.13
	An3	22.91	14.76	64.43	1.42
17.5%	**An1**	23.6	11.11	47.08	1.25
	An2	23.5	12.04	51.23	1.08
	An3	25.2	12.53	49.72	1.13

2. The effect of the initial pH value on citric acid production by local isolates of *A. niger*

The initial pH of the fermentation medium is the most important parameter which impact on the amount of accumulated citric acid by the fungal strains .The amount of citric acid has been determined at different initial pH values which varied from 2.5 up to 6 after eight days of incubation time at 30^0C(table 2). In general the maximum amount of citric acid for An1 and An2 isolates (12.14g/l, 77.72%) and (13.11g/l, 86.02%) respectively was achieved at pH of 3.5, whereas for An3the initial pH 4.0 is the most favorable for high production in which 13.12g/l of the acid was produced. This amount is equivalent to 84.97% of the biomass dry weight. In the other hand, the biomasses dry weight is increased with the increase in initial pH value and the highest rate of growth for An1, An2 and An3 reached (26.96g/l, 27.45g/l and 30.14g/l) respectively in fermentation medium of initial pH of 6.0. Moreover, the lowest rates of residual sugar for An, An2 and An3 were (0.49%, 0.3% and 0.25%) respectively which obtained in fermentation media of 3.5 initial pH value.

Table 2. The effect of initial pH value on citric acid productionby local isolates of *A. niger*

pH	*A. niger* isolates	Mean of dry weight g/L	Critic acid g/L	Critic acid %	Residual sugar %
2.5	**An1**	6.2	4.67	75.32	1.61
	An2	6.23	4.62	74.16	1.42
	An3	7.14	5.17	72.41	1.42
3.0	**An1**	10.68	7.65	71.63	1.61
	An2	9.85	6.98	70.86	0.18
	An3	10.03	6.86	68.39	1.61
3.5	**An1**	15.62	12.14	77.72	0.49
	An2	15.24	13.11	86.02	0.3
	An3	15.08	10.77	71.42	0.25
4.0	**An1**	18.43	10.95	59.41	1.42
	An2	19.12	10.19	53.29	1.27
	An3	15.44	13.12	84.97	1.19
4.5	**An1**	19.98	10.15	50.80	1.01
	An2	20.12	10.01	49.75	1.19
	An3	24.44	11.14	45.58	1.01
5.0	**An1**	24.13	9.63	39.91	1.48
	An2	25.54	8.91	34.89	1.43
	An3	18.23	10.46	57.38	0.35
5.5	**An1**	26.45	8.94	33.80	1.61
	An2	25.43	6.94	27.29	1.1
	An3	28.94	9.57	33.07	0.84
6.0	**An1**	26.96	4.17	15.47	1.48
	An2	27.45	4.07	14.83	0.6
	An3	30.14	5.02	16.66	1.19

3. The effect of different concentrations of $(NH_4)_2SO_4$ on citric acid production by local isolates of *A. niger*.

This experiment was designed to determine the best concentration of $(NH_4)_2SO_4$ that highly enhanced citric acid production in sawdust hydrolyzed medium by *A. niger*isolates.The results presented in table (3) shows that 0.3% is the best selected concentration studied in the present investigation with respect to citric acid production. Also, the An1 strainwas superior over other two strains. The amount of the citric acid accumulated by these strains in fermentation medium containing 0.3% $(NH_4)_2SO_4$ was (18.86g/l, 94.54%) for An1 , (17.05g/l, 83.58%) for An2 and (16.24g/l, 83.84%) for An3. It is clear that the high growth of the tested strains was obtained in fermentation medium that contain high concentration of ammonium sulphate (0.6%). The total amount of biomass dry weight was (27.86g/l) for An1, (25.22g/l) for An2 and (27.45g/l) for An3.The results also indicated that the high utilization of sugar was obtained in fermentation medium promoted high production of citric acid by the fungal isolates.

Table 3. The effect of different concentrations of $(NH_4)_2SO_4$ on citric acid production by local isolates of *A. niger*.

$(NH_4)_2SO_4$ %	*A. niger* isolates	Mean dry weight g/L	Critic acid g/L	Critic acid %	Residual Sugar %
0.2	**An1**	18.85	15.52	82.33	1.49
	An2	17.61	13.98	79.39	0.51
	An3	19.36	14.29	73.81	0.78
0.3	**An1**	19.95	18.86	94.54	0.14
	An2	20.40	17.05	83.58	0.07
	An3	19.37	16.24	83.84	0.22
0.4	**An1**	20.05	17.95	89.53	0.42
	An2	20.83	16.98	81.52	0.60
	An3	19.74	15.78	79.94	0.38
0.5	**An1**	22.75	13.93	61.23	1.01
	An2	24.15	14.12	58.47	0.6
	An3	26.32	13.77	52.32	0.69
0.6	**An1**	27.86	11.74	42.14	0.6
	An2	25.22	10.15	40.25	0.68
	An3	27.45	10.47	38.14	0.38

4. The effect of different concentrations of methanol on citric acid production by local isolates of *A. niger* .

This experiment was designed to determine the most suitable concentrations of methanol to be added to the fermentation medium for higher citric acid production by *A. niger*isolatess.

The results in table (4) points out that the addition of (1%) methanol to the fermentation medium enhanced the accumulation of citric acid and the growth of fungal strains comparing to other used concentrations of methanol . Furthermore, as in experiment (3) the fungal isolates An1 was highly activated for production of citric acid comparing to An2 and An3.The obtained amount of citric acid was (42.57g/l, 198.46%) for An1 , (35.47g/l, 172.02%) for An2 and (35.11g/l, 164.53%) for An3 respectively. The growth of fungal strains and amount of citric acid was declined gradually by gradual increasing of amount of methanol added to the fermentation media. Additionally, high utilization of carbon source by the fungal strains was occurred in fermentation medium containing 1% methanol.

Table 4.The effect of different concentration of methanol on citric acid production by local isolates of *A. niger*.

Methanol %	*A. niger* isolates	Mean dry weight g/L	Critic acid g/L	Critic acid %	Residual Sugar %
1.00	**An1**	21.45	42.57	198.46	0.04
	An2	20.62	35.47	172.02	0.046
	An3	21.34	35.11	164.53	0.044
2.00	**An1**	20.26	37.89	187.02	0.081
	An2	20.00	31.98	159.9	0.094
	An3	21.21	32.48	153.14	0.091
3.00	**An1**	18.45	31.81	172.41	0.42
	An2	18.26	28.30	154.98	0.63
	An3	18.18	26.54	145.98	0.71
4.00	**An1**	16.21	25.68	158.42	1.03
	An2	16.22	23.12	142.54	1.12
	An3	16.04	21.17	131.98	1.15
5.00	**An1**	14.34	18.95	132.15	1.16
	An2	14.21	17.18	120.90	1.20
	An3	14.96	17.54	117.25	1.29

DISCUSSION

The recorded data indicated that the citric acid production by the fungus was highly stimulated by gradual increasing the initial sugar concentration in fermentation media. The maximum yield of citric acid was achieved in fermentation medium of15% sugar, which is in agreement with the results obtained by Sarangbin & Watanapokasin [21] and Jernjc & Legisa [22] who stated that the best sugar concentration in fermentation medium for the stimulation of citric acid production by *A. niger* was 14%. On other hand, Rugsaseel , *et .al.*, [15] and Kirimura *et .al.,* [23] found that 12 % sugar in fermentation media was the best. Citric acid production by fungal strains was gradually decreased by the gradual increasing of sugar content in the fermentation media more than 15%. Similar results were previousley described [24,7] who stated that the presence of initial sugar more than 15 % in fermentation media slightly inhibited citric acid accumulation but stimulated fungal growth, due to the conversion of the sugar to biomass rather than citric acid.

Naaz *et al.* [25] indicated that the presence of the high level of sugar in the fermentation medium reduced the activity of citrate synthase which is responsible for condensation oxaloacetate with acetyl Co-A to produce citric acid as a first intermediate product of Krebs cycle. It has been postulated that the initial pH value have profound

effect on citric acid production by the fungus *A. niger* [26]. Our results indicated that the best initial pH value of the fermentation media for the highest production of citric acid ranged from 3.5 to 4.0. Similar results were obtained by Maharani *et al.,* [27]. The recorded results of this experiment were in agreement with the results reported by Papagianni *et al.,* [28] in which they found that the optimum initial pH value of the fermentation medium was not more than 4.0. The Initial pH value above 4.5 decreased citric acid productions. This might be due to the accumulation of Na ion's in the fermentation media which reduced the production of citric acid by the fungal cells [29], or due to the accumulation of the waste product which is considered a toxic substrate to the fungal strain, which reduced the productivity of the *A. niger* for citric acid production, or it might even inhibit the production process [30]

Another experiment was developed to determine the most suitable concentration of ammonium sulphate at nitrogen source that stimulates citric acid production by the fungal isolates. The results showed that ammonium sulphate at concentration of 3% was superior to other concentration with respect to citric acid production. Bominathan *et al.*, [31] and Patil and Patil [32] demonstrated that ammonium sulphate is a perfect nitrogen source for the production of more citric acid. This may be because acid ammonium compounds have a positive effect on citric acid production by decreasing and maintaining pH values in the first days of fermentation [33]. The result indicated that the high accumulation of the citric acid was achieved in the presence of 1% methanol. Similar results were observed by Prabha and Rangaiah [34] who demonstrated that 1% is the optimal methanol concentration for the higher production of citric acid in which the addition of methanol leads to inhibiting the fungal growth and delays spore formation but enhances the accumulation of citric acid. This is putatively because the effect of methanol on the permeability of cell membrane without affecting metabolic pathway of citric acid accumulation and it allows citrate to excrete from the cell [13]. Also, it stimulates the activity of the enzyme citrate synthase which, in turns, catalyzes the condensation between acetyl Co-A and oxaloacetate resulting in production of citric acid [26].

CONCLUSION

The optimization of some physiological factors of sawdust medium, highly stimulated the biosynthesis of citric acid by the tested *A. niger* isolates, in which the amount of the produced citric was highly increased when these factors were added to the sawdust fermentation medium at optimum concentration. Economically, the agriculture waste was a favorable substrate for the production of citric acid because it is easily available in the nature.

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