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Saw Dust Powder Hydrolysis as a Carbon Source for the Production of Citric Acid by Three Isolates of the Fungus *Aspergillus niger*

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Abstract: *Three isolates of the fungus Aspergillus niger were used in this study to determine their abilities for citric acid production using acid hydrolysis of the sawdust as a basal medium and carbon source at concentration of 15% sugar. The results showed that the accumulation of citric acid was increased with an increase in fermentation time and the highest yield was obtained at 8 days of incubation,(8.37g/l, 63.22%) for An1, (8.94g/l, 44.04%) for An2 and (9.77g/l, 50.26%)for An3, respectively. The results also indicated that citric acid production was affected by the nitrogen source presents in the fermentation medium and most superior one was ammonium sulphate at concentration of 0.55% and the best yield (16.88g/l, 83.32%) was achieved using this source in the case of fungal isolate An1. The effect of the addition of calcium chloride and ethanol to fermentation medium on citric acid production was also investigated and the data demonstrated that the highest accumulation of the acid was obtained in medium containing 0.03% calcium chloride and 2% ethanol in the tested isolate An2, (37.29g/l, 153.08%).*

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I. Introduction

Biotechnology is the largest entrance to the industrial microbial applications for the conversion of waste to availed products. The microorganism releases a various number of substances such as simple compounds (e.g. Lower alcohol, acids, etc), complex compounds (e.g. Natural product and cellulose), or as preliminary products and compounds produced from secondary metabolism (Angumeenal and Venkappayya, 2013).

In last three decades, the production of citric acid has been significantly increased from 350,000 tons in 1986 to 1.1 million tons in 2006 (Anca and Alexandru, 2010). More than 70% of citric acid production is used in food and fermentation stabilizers, flavor enhancers and chelators, soaps and detergents 20% (Anca and Alexandru, 2010), and less than 10% is used in cosmetic and pharmaceutical industries (Maharani *et al.,* 2014).

Citric acid is one of the most versatile industrial organic acids because of its high solubility and low toxicity (Anca and Alexandru, 2010). It is a primary intermediate product of metabolism formed in krebs cycle (Tricarboxylic cycle - TCA) (Belen *et al.,* 2010). The role of *Aspergillus niger* in citric acid accumulation by *A. niger* led to rapid development of fermentation process because it is considered as important and largest part of production in global scale (Karaffa *et al.,* 2001). Refined sugars such as glucose and sucrose are the most commonly substrates used for the production of citric acid by fermentation processes, however, owing to their high cost 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 used as a carbon source for fungal growth and production of citric acid (Bakhiet and Al-Mokhtar (2015); Shetty (2015); Addo *et al.* (2016). Moreover, raw agrowaste, known as sawdust, possesses high carbon content, approximately 60%, render it as an important, inexpensive carbon source for citric production (Bachir and Halima, 2012).

Citric acid has been commercially produced by submerged fermentation of starch or sucrose based media using *Aspergillus niger* (Lofty *et al.,* 2007; Barrington and Kim, 2008). Different microorganisms such as yeasts, molds and bacteria are capable of converting carbohydrate to organic acids, *e.g.* citric acid (Papagianni 2007; Kuforiji *et al.,* 2010). Industrial production of citric acid is carried out by fermentation process in the presence of filamentous fungi which are used for commercial production of citric acid. *Aspergillus niger* is the most effective strains due to its ability to produce more citric acid per unit time and ferment different inexpensive raw materials (Luciana *et al.,* 1999).

This study is designed to use cheap substances as a carbon source and basal medium (sawdust acid hydrolysis) for the production of economically important citric acid through the activity of locally isolated strains of the fungus *Asperigillus niger.* The study also involves using different chemicals and physical parameters at optimum conditions to stimulate the highest production of citric acid by the used fungal isolates.

II. Materials and Methods

The microorganism

Three stock cultures of ***Aspergillus niger*** isolates (An1, An2 and An3) were obtained from the culture collection of mycology research laboratory, department of biology, college of science, university of Duhok which have been previously identified by Asst. Prof. Dr. Asia Saadullah used in the present study. The strains were kept at 4^0Cand cultured on potato dextrose agar (PDA) slants for 4-5 weeks according to Aftab *et al.*, (2010).

Growth media

Potato-dextrose-agar (PDA) Medium

PDA was the culture medium for the fungal isolates in this current study. Briefly, the medium was prepared by dissolving 80g of PDA into 200ml D.H_2O. The hydrogen ion concentration (pH) of the medium was adjusted to 4.5 and sterilized by autoclave at 1.5 lb /in^2 and 121ºC for 15 minute. Subsequently, the sterilized media were divided into Petri dishes of adequate size and stored in refrigerator at 4^0C.

Carbon source

hydrolysis of Sawdust acid was used as a carbon source and based medium for the growth of the fungus *Aspergillus niger* strains and production of citric acid. It was prepared by addition of 5g to 200ml of 10% H_2SO_4 and heated at 100 ^{0}C in water bath (shaking water bath, SBS 40) for one hour after reaching 100 C^0 temperature. After cooling down the solution, 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 to determine the suitable concentration for the growth of the fungus *A. niger* and production of citric acid.

Standard medium

This medium was used to prepare the inocula of the following chemical composition: 10% sugar sucrose; 0.4% $(NH_4)_3SO_4$; 0.1% yeast extract; 0.1% K_2HPO_4, and pH kept at 3.5.

Stock solution

It was prepared and added to the fermentation medium for growth of *A. niger* strains at concentration of 1%. It contains the following chemical substances: $FeSO_4.H_2O$ 3.0%, $ZnSO_4.7H_2O$ 4.0%, $CuSO_4.7H_2O$ 4.0%, $MnSO_4$. $7H_2O$ 1.5%.

Cultural conditions

The fermentation medium contained 10% sugar which is suitable as a carbon source. It was prepared by addition 120ml hydrolyzed sawdust acid to 500ml of distilled water containing 3% nitrogen source in the form of sodium nitrate, followed by addition 8ml stock solution. The pH of the media was adjusted to 3.5. Finally, the volume completed to 800ml by distilled water. The fermentation medium was distributed in 250 ml conical flasks in triplicate samples in which 50ml of broth medium was added to each one. Thereafter, the flasks were plugged and covered by aluminum foil before being autoclaved for 15 minutes at 121ºC. After cooling the culture medium flasks were inoculated with fungal mycelium using 10mm diameter cork borer. The inoculation culture flasks were incubated in shaking incubator (150RPM) for sufficient time at 30 ^{0}C. Samples in three replicates were taken at different intervals after every treatment for further analysis.

Analytical methods

Measurement of initial and final pH value

The adjustment of the initial and final hydrogen ion concentration (pH) value of fermentation media was obtained by using hydrogen ion concentration instrument.The pH instrument calibrated by a buffer solution, absolute HCL and 1N NaOH which were used to adjust pH for the required solution.

Determination of biomass dry weight

To determine the biomass dry weight of the fungal culture, it was filtered with sterile filter paper and dried overnight at 60-65 ^{0}C. The filtrated fermentation medium was collected for citric acid and residual sugar determination. The fungal mycelia were dried at 70 ^{0}C. Thereafter, the biomass dry weight was measured accurately.

Estimation of the citric acid concentration

The method of Pearson (1973) was used to determine the citric acid concentration in fermentation media at the end of each specific incubation period.

Estimation of sugar concentration

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

Experiments

1. Determination of sugar concentration of the sawdust acid hydrolysis
2. Effect of different incubation periods (4, 6, 8, 10,and 12)days on citric acid production.
3. Effect of different nitrogen sources on citric acid production namely $(NH_4)_2$ SO_4, 0.23 %, $(NH_4)_2$ HPO_4, 0.5%, Peptone, 0.29 % and Urea, 0.13 % were prepared each of them contained the same amount of nitrogen presented in 0.3% of $NaNO_3$
4. Effect of different concentrations of $CaCL_2$ (0.03, 0.06, 0.09, 0.12 and 0.15) % on citric acid production.

III. Results

1. The percent of sugar content in sawdust powder

The total amount of sugar content in sawdust powder was estimated after treatment of the sawdust powder with 10% H_2SO_4 at100 ^{0}C for 60 minutes and the results showed that the amount of liberated sugar in the form of monosaccharide equal 70%.

2.The effect of incubation periods on citric acid production by local isolates of *A. niger*

The effect of different incubation periods on the production of citric acid was studied to determine the most suitable time for a highest yield. The sawdust hydrolyzed medium was used and citric acid was determined at interval times of (4, 6, 8, 10 and 12) days and the results are given in table (1).

As shown, the amount of citric acid increased with the increases in the incubation period time until 8 days of incubation of the tested isolates . It is also clear that the fungal isolate An3 was superior than other strains with respect to citric acid production and the total amounts of the obtained acid after eight days were (8.37g/l,(63.22%) for An1, (8.94g/l, (44.04%) for An2 and (9.77g/l, 50.26%) for An3, respectively.

Moreover, the growth of the fungal isolates was also increased gradually with the increase in incubation time, but the best growth of the tested isolates was achieved at different periods of incubation and the obtained biomass dry weight for An1 was (14.22g/l) after ten days, (20.30g/l) for An2 after eight days and (20.94g/l) for An3 after twelve days of incubation.

It is also clear that the lowest rate of residual sugar in fungal fermentation medium was achieved after twelve days of incubation (0.28%) for An1 and (0.31%) for An3 and after six days of incubation (0.23%) in the case of An2.

Table (1) Effect of incubation periods on citric acid production by local isolates of *A. niger*

A. niger strains	Incubation periods days	Mean dry weight g/L	Critic acid g/l	Critic acid %	Residual sugar %
An1	4	7.47	4.67	62.52	0.39
	6	13.13	8.07	61.46	0.65
	8	13.24	8.37	63.22	0.44
	10	14.22	8.01	56.33	0.32
	12	13.85	7.73	55.81	0.28
An2	4	18.42	5.24	28.45	0.48
	6	20.04	6.82	34.03	0.23
	8	20.30	8.94	44.04	0.38
	10	17.72	8.70	49.10	0.38
	12	17.01	6.79	39.92	0.42
An3	4	14.61	4.35	29.77	0.85
	6	19.24	8.78	45.63	0.67
	8	19.44	9.77	50.26	0.58
	10	20.01	9.33	46.63	0.41
	12	20.94	7.39	35.29	0.31

3. Effect of different nitrogen sources on citric acid production by local isolates of *A. niger*

This experiment was performed to find out the most favorable nitrogen source for highest citric acid production. Therefore, different nitrogen sources containing equivalent amount of nitrogen present in 0.3% of $NaNO_3$ were applied and the amount of yielded citric acid was calculated. It is clear from the results presented in table (2) that the fermentation medium containing ammonium sulphate as a nitrogen was superior over other tested nitrogen sources with respect to citric acid production. Moreover, citric acid production was stimulated by An1 isolate more than other two fungal isolates and the amount of produced citric acid of the three solates was (16.88g/l, 83.32%) for An1, (14.63g/l, 79.81%) for An2 and (14.98g/l, 82.17%) for An3. Additionally fermentation medium containing peptone as a nitrogen source has shown to have inhibitory effect on citric acid production by the three tested fungal isolates, but it has promoting effect on fungal growth. The biomass dry weight of fungal isolates in the peptone containing fermentation medium was (25.4g/l) for An1, (22.3g/l) for An2 and (26.4g/l) for An3 respectively. Also the highest sugar utilization was obtained in the fermentation media promoted high production of citric acid by the fungal isolates.

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

Nitrogen sources	*A. niger* strains	Mean dry weight g/L	Critic acid g/L	Critic acid %	Residual Sugar %
$(NH_4)_2SO_4$	An1	20.26	16.88	83.32	0.09
	An2	18.33	14.63	79.81	0.09
	An3	18.23	14.98	82.17	0.15
$(NH_4)_2HPO_4$	An1	19.54	11.39	58.29	1.61
	An2	23.2	13.77	59.35	0.25
	An3	21	12.60	60.0	0.18
Urea	An1	20.0	13.66	68.3	0.25
	An2	18.4	12.96	70.43	0.18
	An3	21.6	10.34	47.87	0.18
peptone	An1	25.4	10.68	42.83	0.09
	An2	22.3	10.94	49.05	0.18
	An3	26.4	9.67	36.62	0.15
$NaNO_3$	An1	19.0	12.95	68.16	1.35
	An2	18.2	12.39	68.08	1.61
	An3	19.4	4.89	25.21	0.6

4. Effect of different concentration of $CaCL_2$ on citric acid production by local isolates of *A. niger*

This experiment was applied to study the effect of different concentrations of $CaCl_2$ on citric acid production by *A. niger* strains and to explore the best on. As indicated in the results (table 3) that the amount of yielded citric acid was enhanced in fermentation medium containing 0.03% calcium chloride by the tested fungal isolates comparing to the previous experiment and other used concentrations of $CaCl_2$. The produced amount increased to 25.99g/l, (121.34%)for An1 23.26g/l,(106.02%) for An2 and21.21g/l,(99.95%) for An3.

Moreover, the same concentration of $CaCl_2$ (0.03%) favored the best fungal growth comparing to other used $CaCl_2$ concentrations throughout this experiment .The obtained biomass dry weight reached (21.42g/l) for An1, (21.94g/l) for An2 and (21.22g/l) for An3. Additionally, the lowest amount of residual sugar was obtained in fermentation medium stimulated the fungal growth and increased the production of citric acid.

Table (3) Effect of different concentration of $CaCL_2$ on citric acid production by local isolates of *A. niger*

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$CaCL_2$ %	*A. niger* strains	Mean dryu weight g/L	Critic acid g/L	Critic acid %	Residual sugar %
0.03	An1	21.42	25.99	121.34	0.04
	An2	21.94	23.26	106.02	0.04
	An3	21.22	23.18	112.58	0.07
0.06	An1	20.59	23.18	112.58	0.51
	An2	20.67	21.11	102.13	0.43
	An3	21.01	21.18	100.81	0.51
0.09	An1	20.58	20.83	101.21	0.78
	An2	20.61	20.49	99.42	0.85
	An3	20.48	18.69	91.26	0.85
0.12	An1	19.01	16.99	89.37	0.38
	An2	19.46	17.00	87.36	0.24
	An3	18.81	14.21	75.54	0.92
0.15	An1	15.11	10.93	72.34	0.24
	An2	14.88	11.62	78.09	0.85
	An3	15.25	9.97	65.38	0.85

5. Effect of different concentrations of ethanol on citric acid production by local isolates of *A*. *niger*

This experiment has been carried out to find out the effect of the addition of different ethanol concentrations to sawdust hydrolyzed medium on the production of citric acid by *A. niger* isolatess. The results in table (4) showed that the maximum amount of citric acid accumulation by the An2 isolates 37.29g/l, (153.08%) and An3 isoates 37.15g/l, (154.28%) was achieved in fermentation medium containing 1% ethanol. Addition of 2% ethanol to the fermentation medium highly promoted the accumulation of citric acid by the fungal isolate An1 39.96g/l, (189.205) comparing to other isolates. Moreover, addition of 1% ethanol to the sawdust medium is favorable for the growth of the used isolates and the amount of biomass dry weight was (22.45g/l) for An1, (24.36g/l) for An2 and (24.08g/l) for An3. The highest utilization of the sugar by the fungal isolates was obtained in the fermentation medium promoted high production of citric acid (table 4-8).

Table (4) Effect of different concentrations of ethanol on citric acid production by local isolates of *A. niger*

Ethanol %	*A. niger* strains	Mean dry weight g/L	Critic acid g/L	Critic acid %	Residual Sugar %
1.00	An1	22.45	37.37	166.28	0.05
	An2	24.36	37.29	153.08	0.05
	An2	24.08	37.15	154.28	0.09
2.00	An1	21.12	39.96	189.20	0.03
	An2	22.08	32.73	148.23	0.91
	An3	22.94	32.76	142.81	0.88
3.00	An1	19.98	35.24	176.38	0.85
	An2	20.36	29.32	144.01	0.43
	An3	20.37	27.72	136.08	0.92
4.00	An1	16.08	24.12	150.00	1.01
	An2	19.21	26.91	140.08	0.92
	An3	19.80	25.98	131.21	0.38
5.00	An1	14.41	18.48	128.24	0.85
	An2	16.94	22.61	133.47	0.78
	An3	16.04	20.93	130.49	0.26

IV. Discussion

It has been recorded that citric acid production by microorganisms, specially *A. niger* is affected by the chemical and physical properties of the fermentation media and also by the type of the fungal strains (Papagianni, *et al* ., 1998; Jianlong, 2000 ; Haq, *et al.,* 2003). Therefore , different experiments were designated throughout this study to determine the effect of these factors , using three locally isolated strains of the fungus *A. niger* .

The first experiment was applied to determinate the suitable incubation period for the highest yields of citric acid by *A. niger* isolates. The results showed that the highest accumulation of citric acid occurred after 8 days of incubation. Similar results were confirmed by many investigators who stated that the optimal incubation period for the highest production of citric acid was after eight days (Mazhar *et al.,* 2003; Iqbal *et al.,*2015; Nazz *et al*.,2016. On the other hand, Ali *et al.,* (2002), El-hashmy, (2004) and Jamal *et al.,* (2005) demonstrated that the best incubation period to obtain a maximum yield of citric acid was after six days. On the contrary, Helen *et al.,* (2014) found that the five days of incubation is perfect for the production of citric acid and explained that the highest accumulation of citric acid depends on the fermentation medium, the type of microorganism and production of inhibitors by fungus it's self .

Our results also indicated that the increase of the incubation period more than eight days the production of citric acid began to decline when attributed to the exhaustion of sugar and nitrogen in the fermentation medium, or inhibition in the activity of some enzymes involved in the biosynthesis of citric acid. The depletion of the activity of these enzymes comes from the change of initial pH value of the fermentation medium due to the metabolic activity of the fungal isolates (Kristiansen and Sinclair,1979; Roukas, 1998).

Another experiment was developed to determine the most suitable nitrogen source that stimulates citric acid production by the fungal isolates. Therefore, different sources were used all containing the same amount of nitrogen and the results showed that ammonium sulphate was superior to other nitrogen sources with respect to citric acid production. While the results obtained by several investigators (Kubicek & Roher, 1985 ; Chen, 1996) showed that ammonium dihgdrogen phosphale was superior that other nitrogen source for the highest production of citric acid. In the study of Nazz *et al.* (2016), it was recorded that ,there was no difference between ammonim nitrate and ammonium sulphate in the production of citric acid by *A. niger* strain, while Xu, *et al*.,(1989) reported that nitrogen source in the form of urea greatly enhanced citric acid production compared with other nitrogen sources. This finding might be due to the composition of fermentation media or to the nature of fungal strains. Shetty (2015) 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.

Additionally, the production of citric acid was induced when 0.3% of ammonium sulphate added as a nitrogen to the fermentation media. This is because at this concentration the building up of fungal cells was stimulated specially at the early stage of growth (log phase) and led to increasing the yield of citic acid at the end of this phase or at the beginning of stationary growth phase. Chen, (1996) and Haq, *et al.,*(2002) who reported that 0.2 % of ammonium sulphate was the best concentration that was added to the fermentation medium and resulted in an increase in the citric acid production by *A. niger*. Therefore , the nitrogen concentration contributed to citric acid production and had two effects , one of them was negative , since in excess of nitrogen stimulated a bigger growth and consequently diverted the source of carbon toward energy and biomass production . The other effect was positive , because a moderate input of nitrogen contributed to the maintenance of citric acid production (Saha *et al*., 1999 ; Jianlong, 2000 ; Haider & Al-bargathy, 2003).

It has been demonstrated that the presence of calcium ion in the fermentation media of the fungus *A. niger* plays an important role in tricarboxylic acid cycle (citric acid cycle) due to the activation of pyruvate dehydrogenase activity which is responsible for the production of Co-enzyme A as a substrate for the building up of citric acid (Voet & Voet , 1995). Therefore, different concentrations of calcium chloride were added to the fermentation medium and the results revealed that 0.03% of $CaCl_2$ highly stimulated citric acid production. Mazhar *et al*., (2003) showed that 1.5% of $CaCl_2$ is better concentration for higher citric acid production. Moreover, El-hashmy, (2004) noted that the addition of 0.05 % $CaCl_2$ to the acid hydrolysis of sawdust powder fermentation medium greatly increased citric acid accumulation by improved strain of *A. niger*. Haider, (2014) reported that the maximum amount of citric acid is produced when 0.05% of calcium chloride is added to the fermentation medium.

Pera & Callieri, (1997) explained that the addition of $CaCl_2$ to the fermentation medium reduced cell building and increased the absorption of phosphate and carbon by the fungus *A. niger* and enhanced citric acid accumulation. Moreover, Pera & Callieri , (1999) revealed that the positive effect of calcium may be related to the decrease of fungal cell size and increase of the mycelial branching level which probably stimulate citric acid diffusion out of the cell to the fermentation medium.

The effect of adding different concentrations of ethanol to the sawdust hydrolysis medium was also studied. It was shown that ethanol inhibits fungal growth and increases the biosynthesis of citric acid specially in fermentation medium containing 2 % ethanol .

These results are consistent with other studies (Haq & Deng, 1995; Haider, 2014) who recorded that the maximum yield of citric acid by the fungus *A. niger* was achieved when the concentration of ethanol in the fermentation media ranged between 1-3 % . Suha, (2005) revealed that 4% of ethanol enhanced citric acid production by *A. niger* strains.

This result is probably related to the difference in the metabolic process of the fungi which lead to the production of the acid. This metabolic process includes an increase in citric acid synthase activity which is responsible for the synthesis of citric acid and decreases aconitase activity as a result of the addition of ethanol (Manonmani & Sreekantiah, 1989; Robert *et al.*,(2003).

Furthermore, Bhat *et al*., (1980) stated that ethanol could be converted to acetyl-Co A which is required for citric acid formation. Ethanol also acts as a carbon source which increases the inflow of carbon through citric acid cycle (Roukas, 1998).)

V. Conclusion

The study demonstrated that the acid hydrolysis of saw dust powder is a good carbon source for the growth of the fungal isolates and production of citric. Moreover, optimization of the chemical factors of the growth media highly stimulated the production of citric acid by the treated fungal isolates.

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