International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue3, 2014, pp346-352
http://www.bipublication.com

Comparative Study of Citric Acid Production from *Annona reticulata* and Its Peel with Effect of Alcohol as a Stimulant

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[Received-06/05/2014, Accepted-06/07/2014]

ABSTRACT

Citric acid i.e. 2-hydroxy propane,2,3-tricarboxylic acid (CH2COOH.COH.COOH.CH2COOH) is ubiquitous in nature. In the present study more focus was made on the economical production of citric acid from *Annona reticulate* and its peel, which was in turn compared with a citric acid production rate from rich carbohydrate source ie. Sucrose as a substrate. In order to fulfill the same *Aspergillus niger* MTCC 281 culture was used as a source of organisms. Wild sweetsop also called as Custard apple (*Annona reticulate)* and its peels which are dumped indiscriminately after using the edible portion, and this activity may lead to environmental pollution. So, this waste was considered for the citric acid production. In the second part of the work test for alcohols as a stimulant or as a inhibitor for citric acid production rate was done. Three different alcohols were used (methanol, ethanol and Butanol) to check the same.

Key words : Citric acid, *Annona reticulata*, peel, stimulants, substrate, Alcohols

INTRODUCTION

Citric acid is a 6-Carbon containing tricarboxylic acid which was first isolated from lemon juice and was crystallized by Scheele in 1784. Citric acid obtained through the microbial fermentation is considered synthetic while that of present in fruits is referred to as natural [6], [14]. Because of its high solubility, very low toxicity, ready assimilability and palatability, it can be used industrially for food and pharmaceuticals. Approximately, 75.0% commercial use of this acid is for food and 12.0% for pharmaceutical industries and many more [4],[5].

Many other uses have placed greater stress on increasing the citric acid production and search for more efficient processes [2],[7]. The demand for citric acid is increasing day by day world wide. To fulfill the requirement of Citric acid we have to produce it through fruit waste. With the increase in

production of processed fruit products, the amount of fruit wastes generated is increasing enormously. These wastes can be effectively disposed by manufacturing useful by products from them.

The main aim of the study is to study on the citric acid production on the economical grounds using fruit waste as a substrate which are considered as a municipal waste. The specific fruit that was selected is *Annona reticulata* (wild sweetsop) and its peel. *Aspergillus niger* (MTCC281) was selected for the production of citric acid.

The present study also deals with effect of alcohols as stimulants on citric acid production using fruit and its waste, so that we can get maximum amount of citric acid even from fruit waste which is considered as municipal waste.

MATERIALS AND METHODS

Organism used : *Aspergillus niger* MTCC281. The growth medium for the organism is Czapek Yeast Extract Agar medium (CYA).

Instruments : pH meter, Autoclave, Orbital shaking Incubator, Colorimeter, Water bath, Electronic weighing balance.

Substrates : *Annona reticulata* (Wild sweet sop) and its peel

METHODS

The critical parameters for citric acid production by *Aspergillu niger* were defined empirically, include high carbohydrate concentration but should not be more that 15 to 20 % [19], [21]. Normally strains of *A.niger* need a fairly higher initial sugar concentration in the medium at a range of 15-18%, w/v[1]. The higher sugar concentrations than the requirement lead to greater amounts of residual sugars making the process uneconomical [8]. In order to know the initial sugar concentration in the substrate Anthron's method was used.

The Anthrone method [9], [10]

This method is both quicker and more accurate and suites well for the determination of carbohydrates.

Anthrone Reagent:

Anthrone reagent is prepared by dissolving 2 gm. Anthrone in 1 L of 95 % sulphuric acid. This reagent has to be prepared fresh daily and was between 4 to 8 hours old. After this time gradual increase in colour occurred. After which it should not be used and has to be discarded .

The amounts of carbohydrate present in the substrate was determined i.e. the amount of carbohydrates present in the Wilds sweet sop and its peel was estimated using anthrone method. For this purpose, the fruits and its peel was collected and were then macerated or sliced separately, together with the expressed juice dried in a hot air oven at less than 60 ^{0} C. They were then pulverized and stored in dark bottles. This enables to obtain a homogenous sample and to analyze aliquots repeatedly [16] [17]. Aliquots of ½ to 2 gm. Pulverized material were used for analysis (William, 1940) and followed the Morris anthrone method. The amount of carbohydrate in the test sample was estimated from a standard curve.

Citric acid Production

Shake flask studies:

The *Aspergillus niger* cultures were used for citric acid production in 250 ml Erlenmeyer flasks.

Preparation of conidial inoculum:

Conidial inoculums were used in the present study. The spores from 4-6 days old slant cultures of PDA medium were used for the inoculation.

Preparation of vegetative inoculums:

One hundred milliliters of the fermentation medium was added into a 1.0 L conical flask. The flask was cotton plugged sterilized at 15.0 lbs/in^2 pressure (121 ^{0}C) for 15 minutes. One milliliter of the *A.niger* conidial suspension (1.2 ×10^6 culture per ml) was used for inoculation. The flask was incubated at 30 ^{0}C in a rotary shaking incubator at 200 rpm for 24 hour.

Fermentation technique:

Vegetative inoculums were transferred into the sterile fermentation medium at a level of 4.0 % (v/v). The incubation temperature was kept at 30 ^{0}C throughout the fermentation period of 144

hours. The shaking speed of the orbital shaker was adjusted to 160 rpm. The pH of fermentation medium was adjusted to 3.5 by 0.1N NaoH/ HCl before autoclaving.

After the incubation period the ingredients of the flasks were filtered and the filtrate was used for the estimation of citric acid produced and residual sugar content. The dry cell mass was also calculated.

Effect of different alcohols at various concentrations:

The effect of different alcohols such as methanol, ethanol and butanol at varying concentrations on citric acid fermentation by the strain *Aspergillus niger* MTCC281, using Wild sweetsop and its peels as a carbohydrate substrate in shake flasks, was carried out. The concentration of alcohols varied from 0.5 to 2.5 %, (v/v). The same was performed with the standard production medium and was compared.

RESULTS:

The critical parameters for citric acid production by *Aspergillus niger* were defined empirically, include high carbohydrate concentration but should not be more that 15 to 20 %. So, in order to fulfill the requirement the concentration of carbohydrates in Wild sweetsop and its peel was estimated and calculated (table 1). So, 15 g/100 ml concentration of each fruit and its peel were calculated and were used for the present study of citric acid production using fruits and its peels.

Table 2 has shown the data regarding the production of citric acid with *Aspergillus niger* MTCC 281 using Wild sweetsop and its wastes i.e. peel in shake flasks. The amount of sugar consumed, dry cell mass and citric acid produced was estimated (Table 2). . According to the table 2, the amount of citric acid obtained with control is 52.96±0.56 g/l, using sucrose as a substrate, where as with Wild sweetsop and its peel the yield obtained is 19.11±0.83g/l(Table 2) and 1.86±0.28g/l (Table 2) respectively. The rate of yield from Wild sweetsop and its peel were compared with that of the control yield.

The effect of alcohols as stimulants at various concentrations were also tested, alcohols used were Methanol (Table 3), Ethanol (Table 4) and Butanol (Table 5). After using different concentrations of different alcohols as stimulants on all the three substrates i.e. sucrose, Wild sweet sop and its peel we got 61.98±0.03 g/l (Table 3**)** of citric acid with sucrose as a substrate at 1.0% Methanol as a stimulant, for Wild sweetsop and its peel, the highest amount of citric acid obtained is 26.65±1.82g/l and 7.92±0.17g/l respectively (Table 4 and 5)**.** In all the three cases 1.0 % Methanol is acting as a good stimulants in compared to that of Ethanol and Butanol and other concentrations of methanol.

Even though the amount of citric acid obtained with Wild sweetsop 19.11±0.83g/l (Table 4) and its peel 1.86±0.28g/l (Table 5) is less than the citric acid obtained from sucrose 52.96±0.56 g/l as a substrate, but the amount produced from fruit and its peel were not negligible, which has enhanced after the addition of stimulants 26.65±1.82g/l and 7.92±0.17g/l , for Wild sweetsop and its peel respectively. The point to be noted here is that the Ethanol and Butanol were not acting as a stimulant, in turn it is decreasing and inhibiting the rate of production in both the cases i.e. with fruit and its peel.

DISCUSSION:

Citric acid produced from Wild sweetsop and its peel were compared with sucrose as a substrate for citric acid production (Table 2). In order to increase the yield, alcohols as a stimulants were added, as expected the addition of methanol has increased the yield (table 3, 4 and 5). The explanation for how the methanol is acting as a stimulants is, addition of low molecular weight alcohols to the medium increases fungal tolerance to trace metals during fermentation [18], [21]. Methanol presence increased the permeability of cell membrane, which resulted in a better citric

acid excretion from mycelia cells. In addition, methanol markedly depressed the synthesis of cell proteins in the early stage of cultivation [12] and also increased the metabolic activity of enzyme citrate synthase. When methanol concentration was further increased, it resulted in the decreased citric acid production (Table 3, 4&5) because of the disturbance in fungal metabolism. Methanol has also some role in conditioning the mycelia without impairing their metabolism. Zulay et al., 1995, proved the use of methanol as a stimulant and butanol had adverse affect on the rate of citric acid fermentation. Similar, type of work has also been carried out by [3], [13].

Thus, yield of citric acid can be enhanced more by considering all other physical and chemical parameters. By doing so we can produce one of the important bulk producing organic acid i.e. citric acid economically using a municipal waste, wild sweetsop and its peel.

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Table 1: Estimation of carbohydrates in fruits

Sl.No.	Name of the sample	Vol. of sample[1] (ml)	Conc. of sample for 0.1 mg (µg)[2]	Conc. of sample for 100 gm (gm)	Vol. of Anthrone (ml)	O.D. at 620 nm
1	Wild sweetsop	1	11.44	11.44	4	0.11
2	Wild sweetsop peel	1	7.28	7.28	4	0.07

Note:

1. 1ml of volume of the sample = 0.1 mg of dried powder of the fruit/ sample
2. Concentration of sample was determined from the standard graph

Table: 2: Using of *A.niger* MTCC281 for citric acid fermentation, using fruits in shake flask*

Sl.No	Sample	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Sucrose (Control)	15.97±0.49	97.99±0.56	52.96±0.56
2	Wild sweetsop	9.89±0.41	98.83±1.93	19.11±0.83
3	Wild sweetsop peel	10.55±0.19	60.33±0.30	1.86±0.28

Note:

* Fermentation period 168 h, Sugar concentration 150 g/l, Initial pH 2.5, incubation temperature 30 ^{0}C.

± Indicate standard error mean (SEM) of the mean.

Table 3: Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the *Aspergillus niger* MTCC*281* using Sucrose salt medium in shake flasks*

Sl. No	Sample	Alcohol	Conce-ntration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Sucrose-Control	-	-	15.97±0.49	97.99±0.56	52.96±0.56
2	Sucrose	Methanol	0.5	16.02±0.42	95.31±0.29	56.60±1.29
			1.0	**15.69±0.50**	**96.74±0.07**	**61.98±0.03**
			1.5	15.33±0.06	95.87±0.29	61.66±0.38
			2.0	14.92±0.53	94.92±0.38	57.79±0.39
			2.5	16.43±0.73	95.24±0.33	53.45±0.18
3	Sucrose	Ethanol	0.5	16.51±0.37	100.40±0.35	49.60±1.29
			1.0	**16.93±0.26**	**101.44±0.74**	**53.98±0.03**
			1.5	16.96±0.03	101.92±0.88	53.66±0.38
			2.0	16.48±0.51	102.70±1.31	50.79±0.39
			2.5	16.75±0.38	101.26±0.59	46.45±0.18
3	Sucrose	Butanol	0.5	13.98±0.39	101.29±0.25	38.93±0.57
			1.0	**13.68±0.49**	**102.76±0.06**	**42.31±0.87**
			1.5	13.35±0.06	101.86±0.28	39.66±0.38
			2.0	12.90±0.50	100.93±0.38	36.46±0.28
			2.5	14.42±0.70	101.26±0.33	32.79±0.31

Note: * Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C and initial pH 2.5.

Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicates.

Table 4: Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the *Aspergillus niger* MTCC*281* using Wild Sweetsop as a substrate in shake flasks*

Sl. no	Sample	Alcohol	Conce-ntration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Wild sweetsop-Control	-	-	9.89±0.41	98.83±1.93	19.11±0.83
2	Wild sweetsop	Methanol	0.5	9.47±0.51	94.31±0.84	21.88±0.77
			1.0	**9.72±0.74**	**93.54±1.60**	**26.65±1.82**
			1.5	9.01±0.76	94.07±0.73	23.10±0.78
			2.0	10.26±0.47	94.63±1.29	19.85±0.47
			2.5	8.89±0.50	93.15±0.99	18.94±0.52
3	Wild sweetsop	Ethanol	0.5	8.46±0.52	106.98±0.48	14.85±0.42
			1.0	**8.72±0.42**	**106.54±0.92**	**17.00±0.41**
			1.5	8.00±0.47	107.73±0.42	16.07±0.44
			2.0	9.26±0.30	106.29±0.71	11.20±0.45
			2.5	7.76±0.32	107.82±0.56	10.28±0.43
3	Wild sweetsop	Butanol	0.5	6.47±0.46	100.64±0.48	8.84±0.48
			1.0	**7.05±0.43**	**99.87±0.92**	**12.92±0.49**
			1.5	6.31±0.47	100.36±0.15	9.69±0.15
			2.0	6.89±0.47	100.89±0.71	5.82±0.18
			2.5	5.82±0.47	99.12±0.39	1.93±0.17

Note:

*Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C, initial pH 2.5.

Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicates.

Table 5: Effect of Methanol, Ethanol & Butanol at various concentration on citric acid fermentation by the *Aspergillus niger 281* using Wild sweetsop peel as a substrate in shake flasks*

Sl. no	Sample	Alcohol	Conce-ntration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Wild sweetsop peel- Control	-	-	10.55±0.19	60.33±0.30	1.86±0.28
2	Wild sweetsop peel	Methanol	0.5	8.40±0.18	56.25±0.76	5.81±0.18
			1.0	**7.69±0.27**	**56.14±0.92**	**7.92±0.17**
			1.5	7.94±0.44	56.33±0.27	7.03±0.16
			2.0	8.20±0.44	57.23±0.74	4.45±0.30
			2.5	8.19±0.60	55.09±0.25	1.54±0.50

3	Wild sweetsop peel	Ethanol	0.5	10.56±0.31	67.08±1.01	0.00
			1.0	10.82±0.42	66.64±0.38	0.00
			1.5	10.43±0.15	67.83±0.76	0.00
			2.0	11.36±0.27	66.39±0.50	0.00
			2.5	10.53±0.24	67.59±0.50	0.00
3	Wild sweetsop peel	Butanol	0.5	6.31±0.15	67.52±0.66	0.00
			1.0	6.91±0.37	66.64±0.57	0.00
			1.5	7.09±0.30	66.49±0.22	0.00
			2.0	7.59±0.73	66.68±0.81	0.00
			2.5	7.04±0.20	66.86±0.52	0.00

Note:

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 ^{0}C, initial pH 2.5.
Each value is an average of three parallel replicates. ± Indicates standard error mean among the replicates.