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Research Article

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Effect of Different Alcohols at Various Concentrations on Citric Acid Fermentation Using *Manilkara zapota* and its Peels as a Carbohydrate Source

Sashi Prabha .M[1]* and Subba Rangaiah .G [2]

[1]Department of Microbiology, Dr. Lankapalli Bullayya P.G. college and Research center, Visakhapatnam, Andhra Pradesh, India

[2]Department of Microbiology, Andhra university, Visakhapatnam, Andhra Pradesh, India

*Corresponding Author E-mail: shashi23581@rediffmail.com

ABSTRACT

Citric acid is one of the most important bulk produced organic acids. Citric acid is a 6-Carbon containing tricarboxylic acid (CH2COOH.COH.COOH.CH2COOH) which was first isolated from lemon juice and was crystallized by Scheele in 1784. In the present study more focus was made on the economical production of citric acid from Manilkara zapota and its peel, which was in turn compared with the rate of citric acid produced from sucrose as a substrate. Aspergillus niger MTCC 281 is the choice of the organism for the present study. Sapodilla (Manilkara zapota) is a seasonal fruit and its peel will be dumped indiscriminately after using the edible portion, and this activity may lead to environmental pollution. This environmental waste was considered for the present study as a substrate for citric acid production. The rate of production from fruit and its peel was in turn compared with the citric acid rate of production from sucrose. Three different alcohols were used (methanol, ethanol and Butanol) to check the inhibitory or the stimulatory action of alcohol on citric acid production, and was compared.

Keywords : *Citric acid, Manilkara zapota , stimulants, substrate, peels, Alcohols.*

INTRODUCTION

Citric acid i.e. 2-hydroxy propane,2,3-tricarboxylic acid is ubiquitous in nature. Citric acid obtained through fruits is referred to as natural while it can be produced from microbes i.e. through microbial fermentation then it is called as synthetic[14,30]. Citric acid is having many uses, 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[10,13] there are many other uses of citric acid. These and many other uses have placed greater stress on increasing the citric acid production and search for more efficient processes[3,15]. The worldwide demand for citric acid is about 1,70,000 metric tons per year.

All chemical methods for citric acid production have so far been proved uncompetitive or unsuitable, mainly on economic grounds, with starting material worth more than the end product[31,36] . The effects of various cultural conditions and the rates of citric acid production by surface[4] submerged[9,12,16] and solid-state surface culturing is still being used, most of the newly built citric acid plants have adopted submerged fermentation, a more sophisticated technology[2,38,43]. A submerged process appears to be highly desirable and many articles and patents have appeared in the literature[8,11,28,40].

Many microorganisms have been evaluated for the citric acid production including bacteria, fungi and yeasts. However, *Aspergillus niger*, a filamentous fungus remained the organism of choice for citric acid production[1,19,36]. Some 400,000 tons are produced per year largely by process involving *Aspergillus niger*[21].

Citric aid production using waste has become a great interest this is partly because it has lower energy requirements and produces less waste water and partly because of environmental concern regarding the disposal of solid wastes[29].

A variety of solids have been reported as substrate for the citric acid bioproduction, including kiwifruit peel[7], apple pomace, grape pomace[5], Wheat bran[37], sugar cane baggage, concentrated liquor of pineapple waste[42], Sweet potato[20,44] and carrot[34,39].

The main aim of the present study is production of citric acid production on the economical grounds using *Manilkara zapota* fruit waste as a substrate which are considered as a municipal waste, using submerged citric acid fermentation method. The specific fruit that was selected was *Manilkara zapota* (Sapodilla) and its peel. *Aspergillus niger* (MTCC281) was selected for the production of citric acid.

The present study also deals with effect of alcohols as a stimulant 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

MATERIALS

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 : *Manilkara zapota* (Sapodilla) and its peel

METHODS

One of the critical parameter for citric acid production by *A.niger* were defined empirically i.e. it require high carbohydrate concentration but should not be more than 15- 20 % [46]. The higher sugar concentrations lead to greater amounts of residual sugars making the process uneconomical[18]. So, in order to fulfill the same, the carbohydrate content of fruits and peels were estimated using anthrone method.

The Anthrone method for the determination of carbohydrates

Morse, E.E.[23] & Morris, D.L.[22] have described the use of anthrone for the quantitative estimation of carbohydrates. This method is both quicker and more accurate and suites well for the determination of carbohydrates. To obtain this degree of accuracy, it was found necessary to heat the mixture of the carbohydrate sample and the anthrone reagent at 100 ^{0} C. for 5 to 10 minutes after mixing.

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 *Manilkara zapota* (Sapodilla) and its peel was determined used the above mentioned method. For the sample preparation the Sapodilla and its peel was collected separately and macerated, 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[27,32,33]. Aliquots of ½ to 2 gm. Pulverized material were used for analysis and followed the Morris anthrone method. The amount of carbohydrate in the test sample was estimated from a standard curve.

Production of citric acid

Shake flask studies:

The *Aspergillus niger* MTCC 281 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 were used at varying concentrations on citric acid fermentation by the strain *Aspergillus niger* MTCC*281*, using Sapodilla 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 to know whether the respective alcohols are working as a stimulator or an inhibitor, if it is a stimulator at which concentration it is stimulating the production rate. The production rate of Sapodilla and its peel after exposing to the alcohols were compared with the rate of production of control.

Recovery:

Partial citric acid recovery was accomplished by the precipitation method (Kristiansen *et al.*,1999). After fermentation was completed fermentation broth was filtered completely. The filtrate was boiled with equivalent amount of lime and tri-calcium citrate, this involves precipitation method. The calcium citrate was filtered off and then treated with sulphuric acid (60-70 %, v/v) to obtain citric acid and precipitate of calcium sulphate.

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 sapodilla 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 Sapodilla and its waste.

Table 2 has shown the data regarding the production of citric acid with *Aspergillus nige*r MTCC 281 using Sapodilla and its wastes in shake flask method. 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 Sapodilla and its waste the yield obtained is 14.65±0.16 g/l (Table 2) and 8.69±0.34 g/l (Table 2) respectively. The rate of yield from Sapodilla and its waste 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, Sapodilla and its waste we got highest of 61.98±0.03 g/l (Table 3**)** of citric acid with sucrose as a substrate at 1.0% Methanol as a stimulant, for Sapodilla and its waste, the highest amount of citric acid obtained is 20.41±1.30g/l and 14.05±0.51g/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 Sapodilla 14.65±0.16 g/l (Table 4) and its peel 8.69±0.34 g/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 Methanol as a stimulants, for Sapodilla fruit and its peels we got 20.41±1.30g/l and 14.05±0.51g/l, 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.

Finally, even though the amount of citric acid obtained by Sapodilla (20.08±0.04 g/l) and its peel (8.69±0.34 g/l) is very less than the amount of citric acid produced by control (52.96±0.56 g/l) i.e. sucrose as a substrate, but the value is not negligible. The rate of citric acid produced in all the three cases has increased with the addition of 1% (v/v) Methanol. Similarly, by considering all other parameters we can improve the rate of production even with Sapodilla and its waste, which leads to economical production of citric acid.

DISCUSSION

Citric acid production was studied and compared from all the three samples i.e. with Sapodilla, Sapodilla peel and the sucrose as a substrate (Table 2). A variety of solids have been reported as substrate for the citric acid bioproduction, including kiwifruit peel[7], apple pomace, grape pomace[5], along with concentrated liquor of Pineapple waste[42].

In order to check the effect of alcohols on the rate of production, three different alcohols were used i.e. methanol, ethanol and butanol at different concentrations, the addition of alcohols increased the rate of citric acid with Methanol were as the butanol is showing adverse effect on the rate of production.(table 3,4and 5). Zulay *et al.*,[47] proved the use of methanol as a stimulant and butanol had adverse affect on the rate of citric acid fermentation. This might be due to the methanol presence increased the permeability of cell membrane, which resulted in a better citric acid excretion from mycelia cells. In addition, methanol markedly depressed cell proteins in the early stages of cultivation[24] and also in creased the enzymatic metabolic activity[26]. In addition, the addition of low molecular weight alcohols to the medium increases fungal tolerance to trace metals during fermentation[35,45]. 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. Similar, type of work has also been carried out by Hang and woodams[6] and Navaratnam *et al.*,[25].

By considering all the other required parameters we may get very good amount of citric acid. So, by this we can say that even by using municipal waste i.e. fruit peels we get good amount of citric acid economically which is very useful to the society.

Table 1: Estimation of carbohydrates in *Ananas comosus* and its peel

S. 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	Sapodilla	1	17.69	17.69	4	0.17
2	Sapodilla peel	1	5.20	5.20	4	0.05

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: Comparative study of citric acid production in shake flask using *A.niger* MTCC281*

S. 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	Sapodilla	8.34±0.27	74.79±0.42	14.65±0.16
3	Sapodilla peel	9.19±0.03	76.50±0.28	8.69±0.34

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 & Butanol at various concentration on citric acid fermentation by the *Aspergillus niger* MTCC*281* using Sucrose salt medium in shake flasks*

S. 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

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30^0C, 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 & Butanol at various concentration on citric acid fermentation by the *Aspergillus niger 281* using Sapodilla as a substrate in shake flasks*

S. No.	Sample	Alcohol	Conce-ntration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Sapodilla-Control	-	-	8.34±0.27	74.79±0.42	14.65±0.16
2	Sapodilla	Methanol	0.5	7.13±0.06	74.53±0.68	16.46±0.37
			1.0	8.68±0.09	73.43±0.42	20.41±1.30
			1.5	7.55±0.15	73.16±0.05	17.57±0.45
			2.0	8.46±0.26	73.63±0.05	14.63±0.03
			2.5	7.86±0.34	73.60±0.31	14.06±0.40
3	Sapodilla	Ethanol	0.5	8.14±0.06	80.51±0.68	10.51±0.38
			1.0	9.69±0.12	79.41±0.42	13.76±0.47
			1.5	8.53±0.15	79.17±0.08	12.25±0.39
			2.0	9.44±0.26	79.68±0.59	10.94±0.30
			2.5	8.84±0.34	79.62±0.30	9.04±0.21
3	Sapodilla	Butanol	0.5	5.46±0.32	77.59±0.66	3.13±0.06
			1.0	6.13±0.54	76.37±0.56	7.41±0.15
			1.5	5.63±0.17	76.23±0.12	4.57±0.13
			2.0	6.34±0.44	76.88±0.72	1.63±0.03
			2.5	5.83±0.36	76.59±0.32	0.00

Note:
* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30^0C, 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 Sapodilla peel as a substrate in shake flasks*

S. No.	Sample	Alcohol	Conce-ntration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Sapodillapeel- Control	-	-	9.19±0.03	76.50±0.28	8.69±0.34
2	Sapodilla peel	Methanol	0.5	7.39±0.07	72.49±0.35	12.10±0.30
			1.0	7.98±0.50	71.73±0.42	14.05±0.51
			1.5	7.85±0.58	71.46±0.05	13.21±0.53
			2.0	8.43±0.14	71.93±0.61	10.59±0.36
			2.5	8.16±0.34	70.90±0.29	7.03±0.12
3	Sapodilla peel	Ethanol	0.5	10.74±0.06	80.44±0.64	4.78±0.06
			1.0	9.69±0.12	79.41±0.46	7.69±0.20
			1.5	10.46±0.17	79.77±0.08	6.19±0.12
			2.0	9.44±0.26	80.01±0.96	3.54±0.36
			2.5	8.84±0.34	79.95±0.49	1.31±0.12
3	Sapodilla peel	Butanol	0.5	5.56±0.32	79.68±1.08	Nil
			1.0	6.23±0.54	80.15±0.86	Nil
			1.5	5.73±0.17	79.96±0.55	Nil
			2.0	6.10±0.33	79.37±0.28	Nil
			2.5	5.93±0.36	81.04±0.39	Nil

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

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

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