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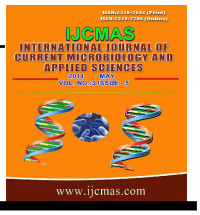

Original Research Article

Citric Acid Production Using *Ananas comosus* and its Waste with the Effect of Alcohols

M.Sashi prabha[1*] and G.Subba Rangaiah[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

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A B S T R A C T

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 Ananas comosus and its peel, which was in turn compared with a citric acid production rate from rich carbohydrate source. In order to fulfill the same, Aspergillus niger MTCC 281 culture was used as a source of organisms. Pine apple (Ananas comosus) 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 Pineapple and its waste was considered for the citric acid production in the present study. 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.

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 i.e. 2-hydroxy propane,2,3-tricarboxylic acid (CH2 COOH.COH.COOH.CH2COOH) is ubiquitous in nature, can be extracted from the citrus fruit juices by adding calcium oxide to form calcium citrate followed by recovery through the addition of sulphuric acid (Bizek et al., 1992; Juang and Chang, 1995; Karklins et al., 1996). Citric acid can be produced by the fermentation of glucose with aid of *Aspergillus niger*.

Citric acid obtained through the microbial fermentation is considered synthetic while that of present in fruits is referred to as natural (Ranya et al., 1999; Karklins et al., 2001).

Citric acid is one of the most important bulk-produced organic acids (Wieizorek and Brauer,1998; Berovic, 1999; Vanags and Viesturs, 2001). Approximately, 75.0% commercial use of this acid is for food and 12.0% for pharmaceutical industries (Haq et al., 2001; Johnson 2003). Many microorganisms have been

evaluated for the citric acid production including bacteria, fungi and yeast. However, *Aspergillus niger*, a filamentous fungus remained the organism of choice for citric acid production (Maddox and Brooks, 1998; Arzumanov et al.,2000; Schuster et al., 2002).

Citric aid production using waste has become a great interest, this is partly because of environmental concern regarding the disposal of solid wastes. The main aim of the study is to study on the citric acid production on the economical grounds using *Ananas comosus* fruit waste as a substrate which are considered as a municipal waste, using submerged citric acid fermentation method. The specific fruit that was selected is *Ananas comosus* (Pine apple) and its peel. *Aspergillus niger* (MTCC281) was selected for the production of citric acid. The present study also deals with the 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 peel which is considered as municipal waste.

Materials and Methods

Aspergillus niger MTCC281 was grown in the Czapek Yeast Extract Agar medium (CYA) and the substrate *Ananas comosus* (Pine apple) and its peel were used.

The initial sugar concentration has been found to determine the amount of citric acid in the culture broth (Honecker et al., 1989; Iwaki et al., 1990; Navaratnam et al., 1998). Normally strains of *A.niger* need a fairly higher initial sugar concentration (15-18%, w/v) in the medium (Ali et al., 2002). The higher sugar concentrations lead to greater amounts of residual sugars making the process uneconomical (Kubicek, 1988). In order to know the initial sugar concentration in the substrate Anthron's method was used.

The Anthrone method for the determination of carbohydrates

Morse, E.E.,(1947) & Morris, D.L., (1948), 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.

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 (Ludwig & Goldberg, 1956).

Using the above stated method the amount of carbohydrate present in the *Ananas comosus* (Pine apple)and its peel was determined, in order to do so, for the sample preparation the pine apple 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 (R.D.Williams et al., 1940; Roukas, 1998, 1999). 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
Microorganism used

The microorganism used was the

Aspergillus niger MTCC 281, received from Microbial Type Culture Collection and Gene Bank.

Shake flask studies

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

Preparation of conidial inoculum

Conidial inoculum was used in the present study. The spores from 4-6 days old slant cultures of PDA medium were used for the inoculation. Inoculation is carried out using spores of *A.niger.*

Preparation of vegetative inoculum

To 100 ml of sterile fermentation media in a 1 liter conical flask, 1ml 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.

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.

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 *Aspergillu niger* MTCC*281*, using *Ananas comosus* fruits 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) in each case i.e. with fruit and its peel, the same was performed with the standard production medium and was compared.

Results and Discussion

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 *Ananas comosus*(Pine apple) and its peel was estimated and calculated (table 1). Basing on table 1, 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 Pine apple and its peels.

Table 2 has shown the data regarding the production of citric acid with *Aspergillus nige*r MTCC 281 using Pine apple 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 is 52.96±0.56 g/l using sucrose as a substrate, where as with Pine apple and its peel the yield obtained is 20.08±0.04 g/l and 8.58±0.31 g/l (table 2) respectively. The rate of yield from Pine apple and its peel were compared with control yield.

The effect of alcohols as stimulants at various concentrations were also tested on all the three samples, alcohols used were Methanol (Table 3), Ethanol (Table 4) and Butanol (Table 5). Various concentrations of alcohols that were used are 0.5, 1.0, 1.5, 2.0 and 2.5 % (v/v). Alcohols were added into the fermentation medium at the time of inoculation.

After using different concentrations of different alcohols as stimulants on all the three substrates i.e. sucrose, Pine apple 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 Pine apple and its peel, the amount of citric acid obtained is 30.18±0.67 g/l and 14.71±0.12 **g/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.

Coming to the effect of ethanol and butanol, the presence of ethanol and butanol is decreasing the production of citric acid in compared to the control. Table 4 and 5 clearly shows that unlike methanol, the ethanol and butanol were not working as a stimulant for citric acid production.

Finally, even though the amount of citric acid obtained by pine apple (20.08±0.04 **g/l)** and its peel (8.58±0.31 **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 pine apple waste, which leads to economical production of citric acid.

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

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	Pine apple	1	14.57	14.57	4	0.14
2	Pine apple 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 Citric acid production in shake flask using *A.niger* MTCC281*

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	Pineapple	8.85±0.11	105.84±0.44	20.08±0.04
3	Pine apple peel	8.90±0.06	80.46±0.23	8.58±0.31

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

Sl. No	Sample	Alcohol	Concentration %	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 ^{0}C, and initial pH 2.5.

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

Table.4.15 Effect of alcohols at various concentration on citric acid fermentation by the *Aspergillus niger 281* using Pine apple as a substrate in shake flasks*

Sl. no	Sample	Alcohol	Concentration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Pine apple-Control	-	-	8.85±0.11	105.84±0.44	20.08±0.04
2	Pine apple	Methanol	0.5	7.77±0.41	105.18±0.54	24.12±0.74
			1.0	7.55±0.35	104.83±0.40	30.18±0.67
			1.5	7.75±0.31	104.65±0.37	26.53±0.93
			2.0	7.86±0.25	105.02±0.27	20.81±0.23
			2.5	7.77±0.22	105.38±0.48	20.32±0.09
3	Pine apple	Ethanol	0.5	8.74±0.36	111.96±0.96	16.51±0.23
			1.0	8.42±0.30	111.93±0.31	20.21±0.48
			1.5	8.48±0.31	112.88±0.28	18.36±0.06
			2.0	8.50±0.29	112.99±0.97	15.43±0.28
			2.5	8.89±0.44	111.89±0.67	14.62±0.21
3	Pine apple	Butanol	0.5	4.73±0.34	108.84±0.42	7.76±0.36
			1.0	4.25±0.58	108.53±0.29	11.81±0.44
			1.5	5.02±0.13	108.71±0.25	9.00±0.46
			2.0	4.63±0.30	108.98±0.45	4.81±0.34
			2.5	4.74±0.14	109.18±0.38	1.16±0.09

Table.4.29 Effect of alcohols at various concentration on citric acid fermentation by the *Aspergillus niger 281* using Pine apple peel as a substrate in shake flasks*

Sl. no	Sample	Alcohol	Concentration %	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid (g/l)
1	Pine apple peel-Control	-	-	8.90±0.06	80.46±0.23	8.58±0.31
2	Pine apple peel	Methanol	0.5	5.95±0.49	76.79±0.40	12.68±0.10
			1.0	6.96±0.50	76.09±0.54	14.71±0.12
			1.5	6.96±0.44	77.71±0.34	13.52±0.39
			2.0	6.63±0.71	76.16±0.47	10.27±0.39
			2.5	5.99±0.48	76.72±0.61	7.12±0.36
3	Pine apple peel	Ethanol	0.5	9.24±0.36	85.39±0.78	5.38±1.08
			1.0	8.75±0.32	85.43±0.18	7.41±0.12
			1.5	8.85±0.41	85.71±0.34	6.56±0.06
			2.0	9.13±0.42	85.93±0.37	3.97±0.39
			2.5	9.16±0.21	85.29±0.48	2.15±0.50
3	Pine apple peel	Butanol	0.5	5.03±0.35	84.16±0.43	Nil
			1.0	5.21±0.37	83.83±0.32	Nil
			1.5	4.98±0.41	83.71±0.31	Nil
			2.0	4.59±0.42	83.61±0.33	Nil
			2.5	5.36±0.27	84.51±0.38	Nil

* 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.

Citric acid production was studied and compared from all the three samples i.e. with Pine apple, Pine apple 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 (Hang et al., 1987), apple pomace, grape pomace (Hang and Woodams, 1985), along with concentrated liquor of Pineapple waste (Wang and Liu, 1996).

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., 1995, 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 and also in creased the enzymatic metabolic activity (Pazouki et al., 2000). In addition, the addition of low molecular weight alcohols to the medium increases fungal tolerance to trace metals during fermentation (Zakowska, Z. and Joloka, 1984) (Sanjay, K. and Sharma, P, 1994). 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.

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 which is very useful to the society.

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