

Effect of Alcohols as Stimulants on Citric Acid Production using *Musa sapientum* and its Peel

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Citric acid is one of the most important bulk produced organic acids. In the present study more focus is on the economical grounds of citric acid production for which, different fruits and fruit peels were selected as a substrates for the production, which can be proved economical. In order to fulfill the same *Aspergillus niger* MTCC 281 culture was used as a source of organisms, Banana (*Musa sapientum*) and its peels which are dumped indiscriminately after extracting the edible portion and this uncontrolled activity leads to environmental pollution with the resultant health hazard to the populace. So, these municipal wastes were considered for the citric acid production. The production of citric acid using fruit peels were compared with the production using the fruits, which are in turn considered as a rich carbohydrate source. The whole productions i.e. fruit yield and fruit peel yield were compared with citric acid yield from sucrose as a source of substrate. Different alcohols (Methanol, Ethanol and Butanol) as a stimulants at different concentration s were used to enhance the citric acid production.

Key words : Citric acid, *Musa sapientum*, Stimulants, Substrate, Alcohols.

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 ($\text{CH}_2\text{COOH.COH.COOH.CH}_2\text{COOH}$) is ubiquitous in nature and exists as an intermediate in the tricarboxylic acid cycle¹. Citric acid is one of the most important bulk-produced organic acids², having wide range of applications. The food and pharmaceutical industries utilize citric acid

extensively because of its high solubility, pleasant sour taste. Very low toxicity and ready assimilability. Citric acid also find applications in some cosmetic preparations, in metal and chemical cleaning, electro pickling and copper plating, secondary oil recovery and other industries³.

There are many other uses of citric acids which placed greater stress on increasing the citric acid production and search for more efficient processes.

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 *Musa sapientum* (Banana) 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

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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

Musa sapientum (Banana) and its peel

Methods

The initial sugar concentration in fruit and its peel has been found to determine the amount of citric acid. Normally strains of *A.niger* need a fairly higher initial sugar concentration in the medium at a range of 15-18%, w/v⁴. The higher sugar concentrations than the requirement lead to greater amounts of residual sugars making the process uneconomical⁵. In order to know the initial sugar concentration in the substrate Anthrone's method was used.

The Anthrone method^{6&7}

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.

The amounts of carbohydrate present in the substrate was determined i.e. the amount of carbohydrates present in the Banana 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 °C. They were then pulverized and stored in dark bottles. This enables to obtain a homogenous sample and to analyze aliquots repeatedly^{8,9}. 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 inoculum was 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 inoculum

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/in² pressure (121 °C) for 15 minutes. One milliliter of the *A.niger* conidial suspension (1.2 × 10⁶ culture per ml) was used for inoculation. The flask was incubated at 30 °C in a rotary shaking incubator at 200 rpm for 24 hour.

Fermentation technique

vegetative inoculum was transferred into the sterile fermentation medium at a level of 4.0 % (v/v). The incubation temperature was kept at 30 °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 Banana 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 than 15 to 20%. So, in order to fulfill the requirement the concentration of carbohydrates in Banana 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 Banana and its wastes i.e. peel in shake flasks. The amount of sugar consumed, dry

Table 1. Estimation of carbohydrates in fruits

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.	Banana	1	20.81	20.81	4	0.2
2	Banana peel	1	11.44	11.44	4	0.11

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*

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	Banana	7.92±0.04	92.26±1.37	22.98±0.57
3	Banana peel	8.73 ±0.10	74.99±1.15	15.63±0.31

* Fermentation period 168 h, Sugar concentration 150 g/l, Initial pH 2.5, incubation temperature 30°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* MTCC281 using Sucrose salt medium in shake flasks*

S. No	Sample	Alcohol	Conc. %	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 °C, and initial pH 2.5.

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

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 Banana and its peel

Table 4. Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the *Aspergillus niger* MTCC281 using Banana as a substrate in shake flasks*

S. No	Sample	Alcohol	Concentration%	Dry cell mass(g/l)	Sugar consumed(g/l)	Citric acid(g/l)
1	Banana-Control	-	-	7.92 ± 0.04	92.26 ± 1.37	22.98 ± 0.57
2	Banana	Methanol	0.5	7.45 ± 0.24	85.82 ± 0.20	27.68 ± 0.15
			1.0	6.32 ± 0.07	87.46 ± 0.25	30.21 ± 0.34
			1.5	7.04 ± 0.43	86.55 ± 0.27	28.90 ± 0.09
			2.0	7.50 ± 0.30	86.36 ± 0.61	23.59 ± 0.21
			2.5	7.47 ± 0.28	88.23 ± 0.42	22.58 ± 0.29
3	Banana	Ethanol	0.5	8.45 ± 0.24	92.81 ± 0.20	18.98 ± 0.34
			1.0	7.32 ± 0.07	94.43 ± 0.25	21.25 ± 0.38
			1.5	8.04 ± 0.43	93.54 ± 0.27	20.26 ± 0.17
			2.0	8.50 ± 0.30	93.35 ± 0.61	17.56 ± 0.27
			2.5	8.47 ± 0.28	95.22 ± 0.42	16.54 ± 0.22
3	Banana	Butanol	0.5	5.42 ± 0.16	95.78 ± 0.31	8.98 ± 0.34
			1.0	4.62 ± 0.07	97.39 ± 0.16	12.25 ± 0.39
			1.5	4.67 ± 0.24	96.84 ± 0.32	9.93 ± 0.15
			2.0	4.80 ± 0.30	96.98 ± 0.68	5.89 ± 0.34
			2.5	5.43 ± 0.14	98.18 ± 0.25	3.88 ± 0.11

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 °C, and initial pH 2.5. Each value is an average of three parallel replicates. \pm Indicates standard error mean among the replicates.

Table 5. Effect of methanol, ethanol and butanol at various concentration on citric acid fermentation by the *Aspergillus niger* MTCC281 using Banana peel as a substrate in shake flasks*

S. No	Sample	Alcohol	Concentration%	Dry cell mass(g/l)	Sugar consumed(g/l)	Citric acid(g/l)
1	Banana peel-Control	-	-	8.73 ± 0.10	74.99 ± 1.15	15.63 ± 0.31
2	Banana peel	Methanol	0.5	6.67 ± 0.62	70.44 ± 0.38	19.11 ± 0.36
			1.0	6.46 ± 0.31	70.56 ± 0.42	22.03 ± 0.54
			1.5	6.49 ± 0.28	71.07 ± 0.26	20.24 ± 0.16
			2.0	6.52 ± 0.47	70.78 ± 0.35	16.97 ± 0.37
			2.5	6.55 ± 0.28	72.26 ± 0.32	14.00 ± 0.52
3	Banana peel	Ethanol	0.5	9.62 ± 0.16	77.98 ± 0.31	11.48 ± 0.24
			1.0	8.82 ± 0.07	79.59 ± 0.16	13.41 ± 0.95
			1.5	8.87 ± 0.24	78.37 ± 0.08	13.76 ± 0.43
			2.0	9.00 ± 0.30	78.85 ± 0.61	11.06 ± 0.39
			2.5	9.63 ± 0.14	80.38 ± 0.25	10.04 ± 0.42
3	Banana peel	Butanol	0.5	5.50 ± 0.19	75.89 ± 0.31	5.39 ± 0.15
			1.0	4.69 ± 0.10	77.50 ± 0.16	9.32 ± 0.19
			1.5	4.75 ± 0.26	76.95 ± 0.32	6.31 ± 0.21
			2.0	4.84 ± 0.32	77.09 ± 0.68	4.33 ± 0.06
			2.5	5.51 ± 0.16	78.29 ± 0.25	1.65 ± 0.22

* Initial sugar concentration 150g/l, Fermentation period of 168 h, incubation, 30 °C, and initial pH 2.5. Each value is an average of three parallel replicates. \pm Indicates standard error mean among the replicates.

the yield obtained is 22.98 ± 0.57 g/l (table 4) and 15.63 ± 0.31 g/l (table 5) respectively. The rate of yield from Banana and its peel were compared with 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, Banana 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 Banana and its peel, the amount of citric acid obtained is 30.21 ± 0.34 g/l and 22.03 ± 0.54 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.

Even though the amount of citric acid obtained with Banana 22.98 ± 0.57 g/l (table 4) and its peel 15.63 ± 0.31 g/l (table 5) is less than the citric acid obtained from sucrose 52.96 ± 0.56 g/l as a substrate, the amount produced from fruit and its peel are not negligible, which has enhanced after the addition of stimulants 30.21 ± 0.34 g/l and 22.03 ± 0.54 g/l, for Banana and its peel respectively.

DISCUSSION

Citric acid produced from Banana 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 stimulants has increased the yield (table 3, 4 and 5). The explanation for how the alcohols were acting as stimulants is, addition of low molecular weight alcohols to the medium increases fungal tolerance to trace metals during fermentation^{10,11}. In addition, methanol markedly depressed the synthesis of cell proteins in the early stage of cultivation¹² 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. Similar, type of work has also been carried out by Hang and Woodams (1986) and Navaratnam *et al.*, (1998).

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

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