Plant Archives Vol. 20, Supplement 2, 2020 pp. 1710-1713 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

PRODUCTION OF CITRIC ACID USING PINEAPPLE PEELS AS THE SUBSTRATE BY LOCALLY ISOLATED AND MTCC 281 *ASPERGILLUS NIGER* STRAINS

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Abstract

Citric acid is a weak organic acid, used in many pharmaceutical and in other industrial food products. Increased demand for citric acid has led to the search for high yielding fermentable strains of microorganisms and cheaper fermentation substrate in many countries. The present study deals with the isolation and screening of citric acid producing *A. niger* and production of citric acid by *A. niger* (MTCC 281 and local isolates), using pineapple peels as the substrate. Citric acid production is carried out by simultaneous saccharification and fermentation (SiSSF). The fermentation process was carried out in two different sets, set 1 contains spore suspension as the inoculum and set 2 contains the intact mycelium as the inoculums, and the yield was compared. The fermentation process was carried for six days. Initially, immediately after autoclaving, after the additions of inoculums were added, the samples were collected and subjected to the initial estimation of sugar and citric acid. The samples were collected every 48 hours and processed for the estimation of leftover sugar and the citric acid produced. The amount of citric acid produced was estimated spectrophotometrically by pyridine acetic anhydride method and leftover sugar by 3, 5 DNS method. The highest yield was recorded by locally isolated intact mycelium than the spores and found more suitable than the intact mycelium.

***Keywords*:** Citric acid, submerged fermentation, *Aspergilus niger*, pineapple peels.

Introduction

Citric acid is a six-carbon molecule containing tricarboxylic acid, first isolated from lemon juice and was crystallized by Scheele in 1784. Citric acid is 2 hydroxyl propane, 2, 3, tricarboxylic acid ubiquitous in nature. Citric acid is an intervene product of metabolism (Dhillion *et al.,* 2011). It is solid at room temperature and melts at 153ºC (Ana Maria, 2011). Citric acid produced by microbial fermentation is considered as synthetic while that of the present in fruits is referred as natural. Citric acid is one of the most important organic acids produced by different fermentation techniques and also the most utilized biotechnological product (Dhillion *et al.,* 2011). It is a weak organic acid and a natural preservative and is also used to add an acidic or sour taste to foods and soft drinks. It is a versatile chemical widely used of which 70% is used in food, 12% for pharmaceutical and the remaining 18% by other industries. The worldwide demand for citric acid is $6x10^5$ tons per year.

Many microorganisms have been evaluated for the production of citric acid, including bacteria such as *Bacillus licheniformis*, *B. subtilis*, *Corynebacterium* spp. (Kapoor *et al.*, 1983), fungi such as *A. niger*, *A. awamori*, *A. foetidus*, *Penicillium restrictum* (Mattey and Allan, 1990; Kubicek, 1998). Yeast such as *Candida lipolytica*, *C. intermedia* and *Saccharomyces cerevisiae* (Crolla and Kennedy, 2001; Archer *et al.*, 2001; Kamzolova *et al.*, 2003). However, *A. niger* a filamentous fungus remained the organism of choice for citric acid production due to ease of handling, its ability to ferment a variety of cheap raw materials, and high yields (Schuster *et al.*, 2002). A cost reduction in citric acid production can be achieved by using cheap agricultural wastes such as apple and grape pomace, orange peel, kiwi fruit peel, cotton waste, okara soy-residue and cane molasses (Kiel *et al.*, 1981; Hang and Woodams, 1986; 1987; Khare *et al.*, 1995; Haq *et al.*, 2004). Pineapple peel is a by-product resulting from the processing of pineapple into slices and represents about 10% w/w of the weight of the original fruit. Current disposal of it poses considerable economic and environmental problems. The objective of this study was to adopt the use of pineapple peel as a cheap medium for the production of citric acid by *A. niger*.

Citric acid is recognized worldwide as GRAS, which was authorized by the Joint FAO/WHO Expert Committee on Food Additives (Ashish Kumar *et al.,* 2008). Therefore, it is not only used in the food industry and pharmaceutical industry but also used in biopolymers, for drug delivery and in a vast area of biomedical application (Ashish Kumar *et al.,* (2008).

Materials and Methods

Microorganisms

Two cultures were employed in the present work for the production of citric acid. Few strains were locally isolated from soil samples while one was s procured from the Microbial type culture collection centre and gene bank (MTCC) Chandigarh.

Isolation and Identification of the Local isolate : The locally isolated sample was screened from the soil sample by serial dilution technique. The soil sample was collected from different areas like chevella, Raichoor and Mumtaz College campus Hyyderabad. The serially diluted soil sample was inoculated by spread plate technique on potato dextrose agar plates and incubated for 30˚C for 48 hours. The fungal cultures obtained in this primary screening were identified by observing under the microscope by referring Prof. Manoharachary's Handbook of Fungi. These fungal cultures were subcultured on selective media. The selective media used was Czapeck's dox agar with bromothymol blue as the indicator (Sikander Ali, Ph.D. Thesis, 2005). The citric acid producers showed yellow colour zones around them. The mycelial growth which showed the larger zone was selected and given to zeal biological laboratory for 16srRNA sequencing. After the confirmation report from the lab that it's *A.niger*, this strain was used for the production of citric acid.

Another strain of *A. niger* was procured fro microbial type culture collection centre and gene bank Chandigarh. The strain was revived and preserved on Potato dextrose agar slants.

Inoculums: In this work, two different types of inoculums were used one was the

i) **Spore Suspension:** The microbial strains (MTCC & local isolates) were preserved on the potato dextrose slants. These cultures were subcultured on PDA plates at 30˚C for five days. After incubation of five days, the plates were washed with sterile 0.1% of tween 80 solution to collect the spores. The numbers of spores were counted using a haemocytometer or Neubauer chamber. 10^7 to 10^8 spores were counted and collected in sterile tubes. This suspension was preserved and 1 ml was used as the inoculum.

ii) **Intact Myecelium:** The isolated *Aspergillus niger* strains were grown on potato dextrose agar at 30 ºC for five days. From these plates the discs were inoculated in potato dextrose broth in 250 ml Erlenmeyer flasks an incubated at 30 ºC for 70 hours, ill the complete mycelial mat grows. This fully grown mycelial mat was used as an inoculum.

Substrate: In the present study, pineapple peels were used as the substrate or the production of citric acid. One hexagon of the peel was separated and used.

Saccharification and Production Media:
Simultaneous saccharification and fermentation (SiSSF) was adapted:

Autoclaving/Sterilization (Hydrolysis): The fermentation process was carried out 250 ml Ehrlenmeyer flasks containing 100ml double distilled water and 5% of the substrate. These flasks with distilled water and a substrate were sterilized at 15 lbs of pressure for 15 minutes at 121˚C.

After autoclaving, the initial sugar was estimated by 3, 5 DNS method (Miller, 1959). Sterilisation process was done for two purposes, (i) eradication of microbial contamination and (ii) mild hydrolysis.

To check the efficiency of each, the spore suspension and the intact mycelium, the fermentation process was carried out in two different sets, set-I and set-II. Set-I was carried out with spore suspension and set with intact mycelium as the inoculums.

Results

In this, the fermentation, the sugar content of the medium was reduced and the amount of citric acid production increased in proportion with sugar utilization. The results obtained are presented in the following tables:

Table 1: Set-I Spore Suspension as Inoculum:

			Citric acid produced and leftover sugar gm/100ml					
Strains employed	**Initial sugar gm/100ml**	**Initial citric acid gm/100ml**	**Day 2 CA**	**Day2 LOS**	**Day 4 CA**	**Day 4 LOS**	**Day 6 CA**	**Day LOS**
MTCC	24	3.0	4.9	21	5.2	19	5.3	18
Sample S1	24	3.0	5.0	20	5.3	18.3	5.4	17.2
Sample S2	24	3.0	5.1	19.8	5.2	18.5	5.4	17.3
Sample S3	24	3.0	5.3	18.5	5.4	17.3	5.5	18
Sample S4	24	3.0	5.2	18.5	5.4	17.3	5.3	18
Sample S5	24	3.0	5.4	17.3	5.6	17	5.4	17.9

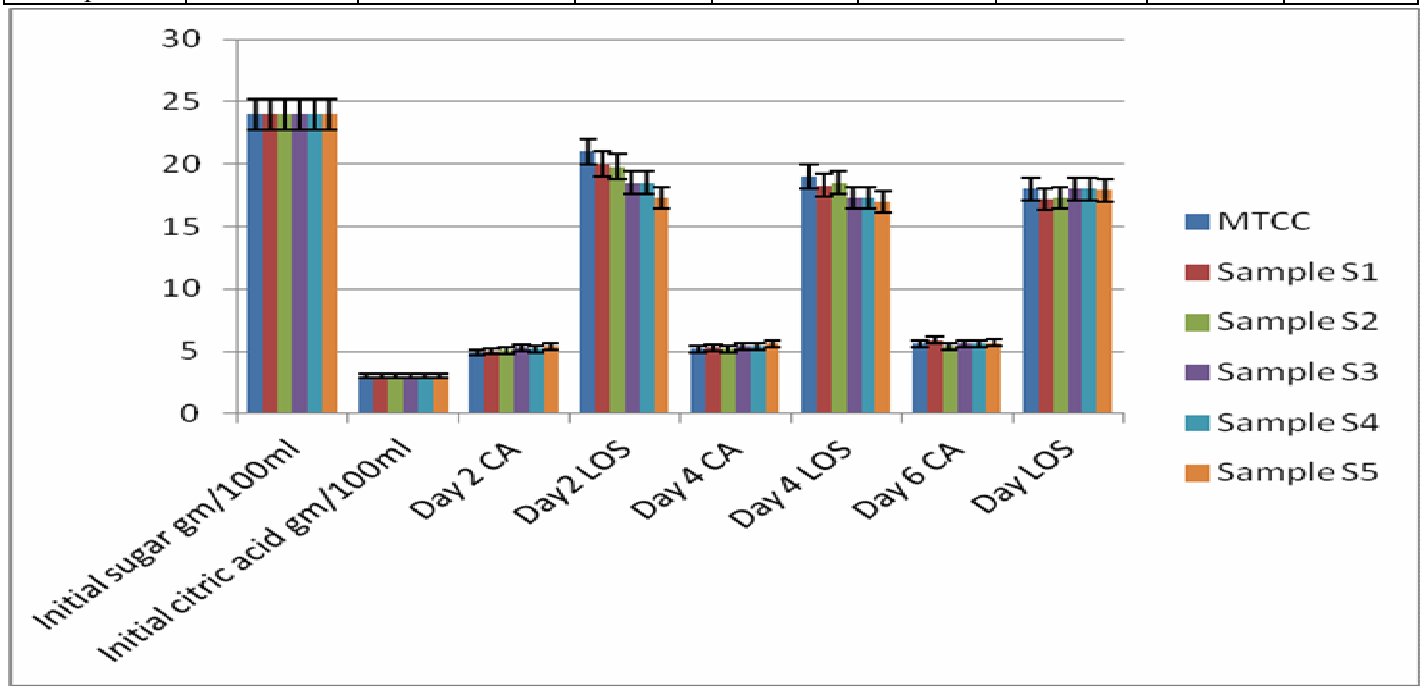
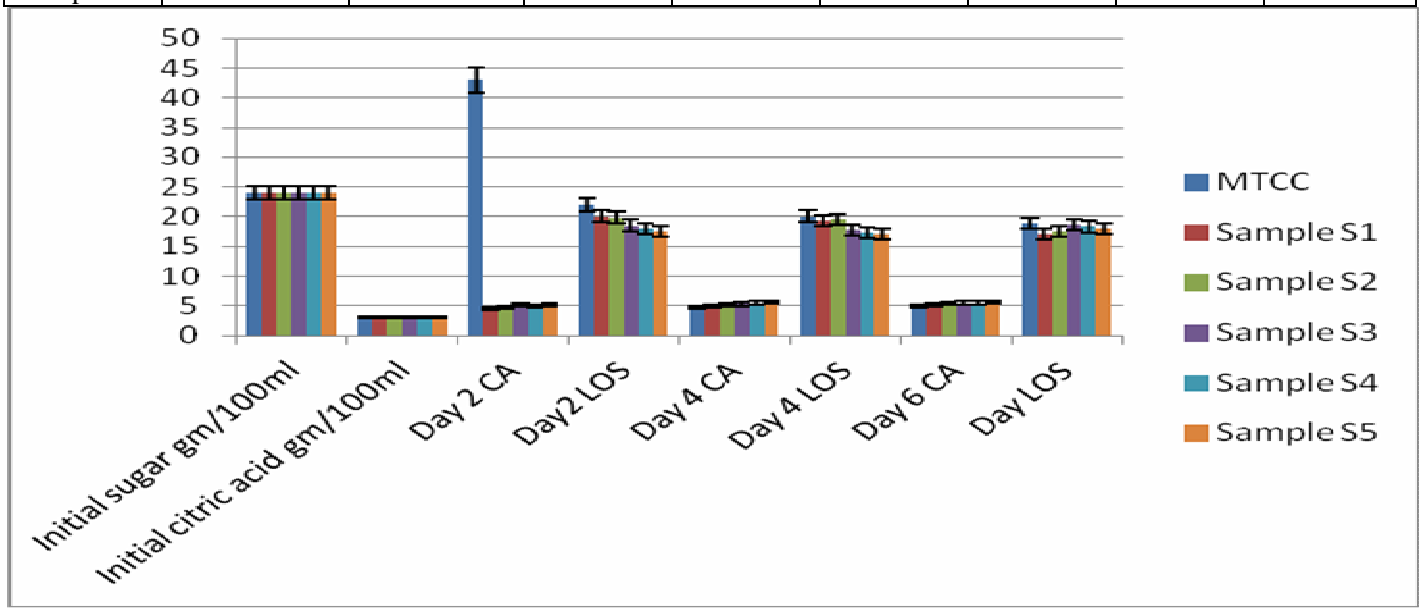


Table 2 : Set II - Intact Mycelium as Inoculum:

			Citric acid produced and leftover sugar gm/100ml					
Strains employed	**Initial sugar gm/100ml**	**Initial citric acid gm/100ml**	**Day 2 CA**	**Day 2 LOS**	**Day 4 CA**	**Day 4 LOS**	**Day 6 CA**	**Day LOS**
MTCC	24	3.0	43	22	4.8	20	5.0	18.8
Sample S1	24	3.0	4.5	20	4.9	19.3	5.2	17
Sample S2	24	3.0	4.8	19.8	5.2	19.5	5.4	17.5
Sample S3	24	3.0	5.1	18.5	5.3	17.7	5.5	18.6
Sample S4	24	3.0	4.9	18	5.5	17.3	5.5	18.3
Sample S5	24	3.0	5.2	17.5	5.6	17	5.6	18



In this study, a parallel relationship between the citric acid production and consumption of sugar was observed.

Set-I: (Spore Suspension as Inoculum):

Till the second day the germination of spores and gradual development of mycelium was observed in all the flasks, negligible amount of citric acid was recorded. From the third day gradual development in the growth of the mycelium and increase in the production of citric acid was observed. Highest yield was recorded on 6th day. (Table: 1).

Set -II: (Intact Mycelium as Inoculum):

In set II there s a similar trend as set I except in the development of biomass and yield. The biomass produced was comparatively more and also the citric acid production was more.

The results in the above tables shows that efficient fermentation took place in set II where intact mycelium was used as the inoculums with S5 culture. From the above results it was observed that the intact mycelium present in the media, only utilized consumed sugars in the production of citric acid where as in set-I flasks which have spore suspension as the inoculum utilized consumed sugar for two purposes i.e. for growth (into mycelium) and for fermentation of citric acid. Hence, comparatively less sugar available for citric acid fermentation as a result low yield of citric acid was recorded in set I.

Discussion

In the present work it has been observed that there are two phases in citric acid production, in the first phase of citric acid (till 2nd day of fermentation), we have observed that the sugars which are readily available in the medium were released by mild hydrolysis (by autoclaving of the pineapple peels) provides to be sufficient to yield mycelial growth. With is observation it is noted that more easily available sugars which are present in the media were consumed for mycelial production without the need of breaking down cellulosic materials enzymatically. As a result negligible amount of citric acid was produced in both the sets I and II (table 1 and 2).

In the second phase of citric acid production gradual increase in citric acid production was observed in set-I flasks while rapid production was observed in set II flasks. This phase was considered as the production phase. Further we observed that the citric acid production increased in proportion with sugar utilization.

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