IJST (2014) 38A2: 193-197
Iranian Journal of Science & Technology

http://ijsts.shirazu.ac.ir

High performance liquid chromatography analysis, production and brief comparative study of citric acid producing microorganisms from spoiled onions in and around Vellore district

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Abstract

India is one of the largest producers of onion in the world producing over 13,920,000 metric tons. Storage is a major problem which leads to spoilage, and microbes play an important role in the spoilage. In this present study, biotechnologically important strains have been isolated from the spoiled onions by using appropriate enriched media. Two potential strains were isolated and a brief Comparative study was done for fungal and bacterial strains in terms of citric acid production and the production of citric acid was also checked by using the designed media named as PAPS. HPLC analysis was performed to confirm the presence of citric acid in the culture medium. Partial recovery was done by filtration and precipitating oxalic acid as calcium oxalate. Optimization was done for various physical and nutritional parameters such as carbon source, temperature, pH and incubation period in order to recover maximum amount of citric acid.

Keywords: Citric acid; HPLC; partial recovery; optimization; physical and nutritional parameters

1. Introduction

A large range of microbes including bacteria, fungi and yeasts are utilized to produce citric acid. Most of them, however, are not inclined to turn out commercially acceptable yields (Kumar et al. 2008; Max et al. 2010). The chief mechanism operating behind bio-synthesis of citric acid is Kreb's cycle or TCA cycle, which is a nearly universal central catabolic pathway where compounds, derived from the breakdown of carbohydrates, fats, and proteins are oxidized and converted to carbon dioxide, and the energy of oxidation is held by the electron carriers $FADH_2$ and NADH (Mehaia et al. 1991; Sankpal et al. 2000; Papagianni et al. 2007). During aerobic respiration, the electrons are transferred to oxygen and the energy of these electrons are trapped as ATP molecules. Acetyl-CoA enters the citric acid cycle (in the mitochondria of eukaryotes, the cytosol of bacteria) where it condensates with oxaloacetate to form citrate (Dhillon et al. 2011).

Many microorganisms are evaluated for the citric acid production including Bacillus licheniformis, Bacillus subtilis and Cornynebacterium spp., Aspergillus niger, A. foetidus, A. awamori, Trichoderma viride, Penicillium restrictum, Mucor pyriformis and yeast like Candida lipolytica and also Saccharomyces cerevisiae (Alagarsamy et al. 2010).

Received: 25 June 2013 / Accepted: 16 April 2014

The world's demand for citric acid is more than 1.4 million tons annually. Presently, most of the citric acid employed in food industries comes from the fermentations processes especially especially with *Aspergillus niger* (Papanikolaou et al. 2010). Although chemical synthesis of this organic acid is feasible, so far there is no alternative synthetic method developed which is superior to biological fermentation (Yuogo et al. 1999; Suneetha et al. 2010). There are three varieties of citric acid, namely monohydrate citric acid, anhydrate citric acid and sodium citrate (Arzumanov et al. 2000; Bayraktar et al. 2000). So a great deal of information is available with the biochemical activities of microbes, on the other hand, these clusters of organisms have not been vigorously exploited for the synthesis of citric acid (Mattey et al. 1992; Haq et al. 2001; Suneetha et al. 2013). The main objective of this study was to screen a potential strain from the spoiled onions that had the ability to produce citric acid.

2. Materials and methods

2.1. Sample collection and screening of microorganisms

All the media and chemicals used for this study have been purchased from Hi Media, Mumbai. For the screening of the potential microbial strains,

spoiled onion samples were collected in polypropylene bags aseptically and transported to laboratory for further studies. Two different media like LB and SDA were used in order to screen the bacterial and fungal isolate (Pazouki et al. 2002).

2.2. Fermentative production, Filtration and recovery process for citric acid

The designed medium (designated as PAPS medium) for the production of citric acid was prepared with different constituents with mentioned amount (Table 1). Eight different PAPS media were prepared. Three of each PAPS media were inoculated by bacterial culture and fungal culture. Two separate production medium were kept as standard. The pH was maintained at 4.0 using 0.1 N HCl or 0.1 N NaOH and was incubated at room temperature for 7 days (Vandenberghe et al. 2000).

Table 1. Composition of PAPS medium for the production of citric acid

Serial No	Components	Amount
1	Diammonium Sulphate	0.25 g
2	Potassium biphosphate	0.15 g
3	Magnesium Sulphate	0.25 g
4	Sucrose	14.00 g
5	Distilled Water	100 mL

Culture broths were then filtered by using Whatman filter paper no. 1. The dry cell mass was detected by filtering the culture broth. The cell mass obtained after filtration was dried in an oven at approximately 80°C for 1 hour (Walid et al. 2007). The filtrate was used for further analysis.

2.3. HPLC Analysis of culture filtrate

The broth was harvested after 6 days of incubation following centrifugation at 8,000 rpm for 10 min. After that the culture filtrate was subjected to HPLC (Waters, model: 1525). The supernatants were filtered through 0.22 lm Millipore filter and the filtrate was injected to a Waters 1525 binary HPLC pump equipped with C18 column (150 mm 9 4.5 lm) with waters 2487 dual λ absorbance detector. The chromatograms were developed using a mobile phase consisting of HPLC-Grade water moving at a constant flow rate of 0.5 mL min^{-1} in isocratic mode. Retention time of each signal was recorded at a wavelength of 210 nm.

2.4. Quantitative analysis of Citric acid

Citric acid concentration in the filtrate was estimated spectrophotometrically, using pyridine–acetic anhydride method as suggested by Marrier and Boulet (1958). The concentration of the citric acid was determined by the following formula
Citric acid = Citric acid/Sugar used × 100

2.5. Optimisation of the process parameter for the maximum yield

2.5.1. Carbon Source

Carbon source plays a vital role in citric acid production and the production cost mainly depends upon the use of the carbon source. Effect of different carbon sources (glucose, sucrose, fructose and lactose) for maximum yield of citric acid were analyzed.

2.5.2. Incubation temperature

Culture filtrate was kept at different temperatures (26°C, 28°C, 30°C, 32°C, 34°C and 36 °C) in order to find the optimized incubation temperature for maximum yield. Temperature between 25-30°C is usually considered optimum for fungi. However a temperature above 36°C inhibits mycelia growth and favors oxalic acid accumulation (Anastassiadis et al. 2006).

2.5.3. pH

pH is the major regulator of enzyme activity which is responsible for metabolic activity and in this way regulates citric acid production (Tran et al. 1998). To find optimum pH level for citric acid production different range of pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5) were maintained to find the maximum yield of citric acid.

2.5.4. Incubation time period

Citric acid production varies according to incubation period. To find the optimum incubation period, fungal and bacterial culture filtrate were kept at different incubation period (1-7 days).

3. Results and Discussion

3.1. Sample collection and screening of microorganisms

For the isolation of the citric acid producing micro-organism, spoiled onion samples were collected from different places in and around Vellore market area (Fig. 1a and Fig. 1b). Out of 20 different strains two potential strains (designated as PAPS 3B, a bacterial strain and PAPS 4F, a fungal strain) was screened and checked for the production of citric acid. The morphological characterisation of

PAPS 4F and PAPS 3B were performed with lactophenol cotton blue mounting and Gram's staining respectively (Fig. 2a and Fig. 2b).

Fig. 1. Sites of dumped spoiled onions in and around Vellore market (a and b)

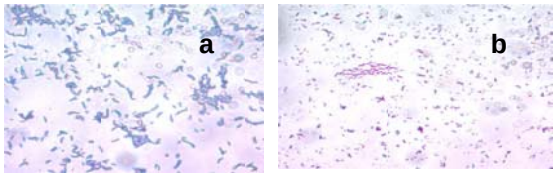

Fig. 2. Lacto phenol cotton blue mounting (a) and Gram's staining of screened microbes (b) with the magnification of 100x

3.2. Fermentative production and recovery of Citric acid

The maximum citric acid was produced by PAPS 4F strain which generated 2.31 g/L citric acid and bacterial PAPS 3B strain which yielded 0.56 g/L citric acid (Fig. 3a and Fig. 3b). A comparative study was done with these two strains for the various process parameters in order to get the maximum yield.

Fig. 3. Quantitative determination by Titration (a) and Recovery of Citric acid (b)

3.3. HPLC Analysis of the Culture filtrate

From the HPLC analysis, it was found that there are two different peaks in the chromatogram of the analytes. After comparision with the standard's chromatogram it was found that some amount of citric acid was produced in the culture filtrate (Fig. 4a, Fig. 4b and Fig. 4 c). It is possible that another product was released metabolic byproduct during the fermentation process which was indicated by the other peaks in the sample's chromatogram.

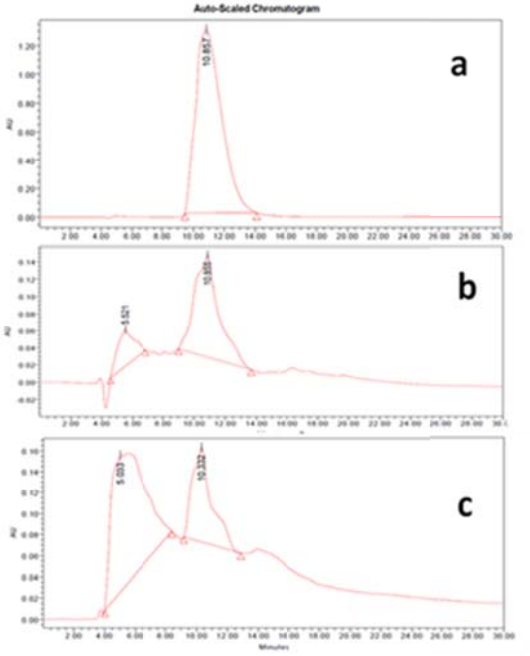

Fig. 4. HPLC chromatogram of standard Citric acid (a) and Culture filtrates (b,c).(Note:- b: Culture filtrates of PAPS 4F and c: Culture filtrates of PAPS 3B strain)

3.4. Optimisation of the physical paramers

3.4.1. Effect of Carbon Sources

The carbon source used in the designed media, is converted into citric acid. So the nature and types of the carbon source play a major role in citric acid production. Four different carbon sources were taken (glucose, sucrose, fructose and lactose). Sucrose was found with maximum productivity for both fungal (PAPS 4F) and bacterial (PAPS 3B) strains (Fig. 5).

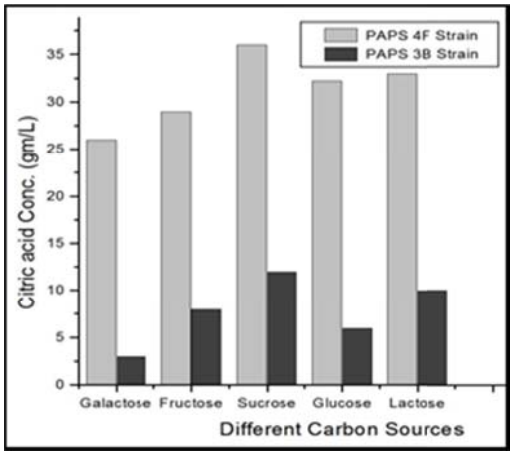

Fig. 5. Effect of different carbon sources on Citric acid production (Graph have been plotted by taking the mean value of the 3-Experiments)

3.4.2. Temperature

Citric acid was synthesized due to metabolic activities of microbes and these activities largely depend upon the incubation time (Walid et al. 2007).

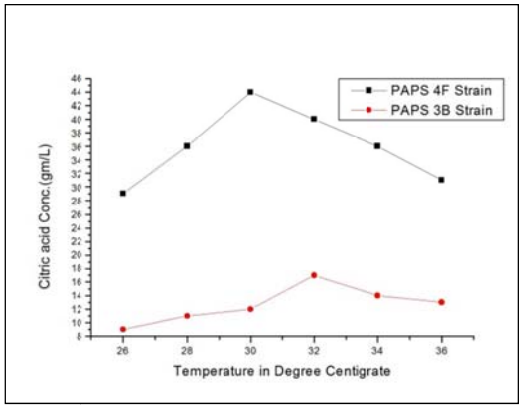

Fig. 6. Effect of different Temperature on the production of Citric acid (Graph has been plotted by taking the mean value of the 3-Experiments)

From the experiment it was found that 30°C was the optimum temperature for PAPS 4F strain and 32°C was the optimum temperature for the PAPS 3B strain (Fig. 6).

3.4.3. pH

pH is the major regulator of enzyme activity which is responsible for metabolic activity and in this way regulates citric acid production very closely. Optimum pH level for citric acid production for PAPS 4F strain and PAPS 3B strain was found to be 6 and 5.5 respectively (Fig. 7).

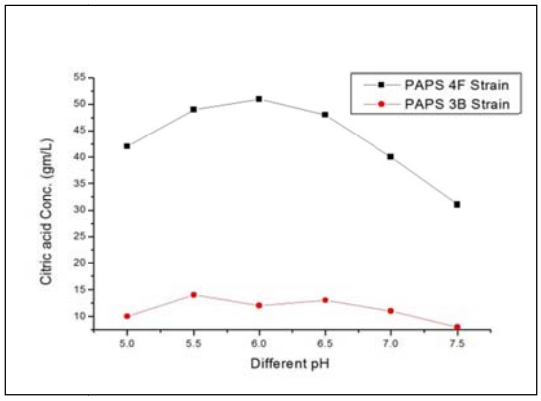

Fig. 7. Effect of different pH range on the production of Citric acid (Graph has been plotted by taking the mean value of the 3-Experiments)

3.4.4. Incubation Time

Citric acid production varies according to incubation period. Optimum incubation was found out by keeping the culture filtrate at different incubation period. It was found that optimum incubation period for PAPS 4F and PAPS 3B was 5 days (120 hours) and 6 days (144 hours) respectively (Fig. 8).

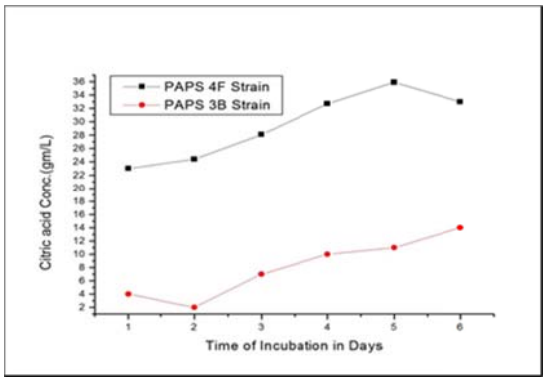

Fig. 8. Effect of incubation time for the production of Citric acid (Graph has been plotted by taking the mean value of the 3-Experiments)

4. Conclusion

In the modern biotechnology world, waste generation and its remediation is an extremely critical issue. The current emphasis is on biological conversion of agricultural wastes for the development of the value-added products. This research aimed at using spoiled onion to produce an economically viable product, citric acid by using the microbes present. The various process parameters were optimized for the production process. Future aspect like standardization of the downstream processing for the maximum yields is in progress.

Acknowledgement

The author wants to express her sincere gratitude to Dr. G. Viswanathan, Chancellor of VIT for constant support and encouragement and DST for financial assistance.

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