The Pharma Innovation Journal 2018; 7(6): 141-146

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(6): 141-146

www.thepharmajournal.com
Received: 04-04-2018
Accepted: 06-05-2018

Vidya P
P.G & Reaserch Department of Microbiology, D.G. Vaishnav College, Chennai, Tamil Nadu India

Annapoorani AM
P.G & Reaserch Department of Microbiology, D.G. Vaishnav College, Chennai, Tamil Nadu India

Jalalugeen H
P.G & Reaserch Department of Microbiology, D.G. Vaishnav College, Chennai, Tamil Nadu India

Correspondence
Vidya P
P.G & Reaserch Department of Microbiology, D.G. Vaishnav College, Chennai, Tamil Nadu India

Optimization and utilisation of various fruit peel as substrate for citric acid production by *Aspergillus niger* isolated from orange and carrot

Vidya P, Annapoorani AM and Jalalugeen H

Abstract
Citric acid is one among the most adaptable organic acid and generally used in different industries including food, cosmetics, pharmacy, beverages and many others. Apart from its consumption as a food additive, citric acid is also considered as a vital component of various pharmaceuticals, synthetic detergents, cosmetics, and many other value-added products. It is predominantly produced by microbial fermentation of *Aspergillus niger*. The study was conduct to explore the potential of *Aspergillus niger* AsnO and AsnC (isolated from Orange and Carrot) for citric acid production. These two isolates were screened for citric acid production in Czapekdox agar incorporated with 1% Bromocerol green indicator and were subjected to citric acid production in Czapekdox broth. The effect of different pH, temperature, carbon source, nitrogen source, stimulators and incubation time on citric acid production by the isolate AsnO was analysed. The maximum amount of citric acid production was recorded in carbon source-Sucrose (21.43g/l) at10% concentration (22.78g/l), pH-5.0 (31.64g/l), temperature-30°C (20.33g/l), Nitrogen source-ammonium chloride-(21.83g /l) at 0.3% (28.32 g/l), stimulator- methanol at 1% concentration (22.81g/l) and incubation time 96 hours (45.37g/l). While processing various fruit peel as a substrate for citric acid production, maximum production (11.36 g / l) was obtained from orange peel media followed by pine apple peel media (11.06 g/l), lemon peel media (9.6 g / l) and sweet lime peel media (8.7 g / l).

Keywords: citric acid, *Aspergillus niger*, stimulators and fruit peels

Introduction
Citric acid is a weak organic acid belonging to the family of carboxylic acids, present naturally in citrus fruits like limes, lemons, oranges, berries, tangerines and grapes fruits and in many animal tissues and fluids. It is an important intervene product of the Kerb's cycle (TCA cycle) and therefore occurs in the metabolism of almost all aerobic organisms [1]. The name of this organic acid is originated from Latin word citrus, which denotes to trees of the genus citrus, including lemon trees. The chemical name of citric acid is 2-hydroxypropane-1,2,3-tricarboxylic acid ($C_6H_8O_7$) and in its pure from is readily soluble in water and colour less [2]. Citric acid executes a great multifariousness of functions and has important industrial applications. Apparently the prime application of citric acid is food industry because of its pleasant acid taste and its high solubility in water. It is globally accredited as "GRAS" (generally recognized as safe), approved by the Joint FAO/WHO Expert Committee on Food Additives [3]. The pharmaceutical and cosmetic industries grasp on to 10% of its consumption and the rest is used for different other purposes like, metal finishing, lubricants, chelating agents, animal feeds and plasticizers [4].

Citric acid can be procured from natural sources (e.g. lemon, lime and orange) or synthetic sources (mechanically, chemically and through fermentation). The mechanical and chemical production method is however, not reasonable [5]. Citric acid is noticeably produced by microorganisms through TCA cycle. This normal metabolic pathway is the generally convenient method for citric acid production on industrial level. Numerous microbial strains including fungi (*Aspergillus niger, A. carbonarius, A. aculeatus, A. awamori, A. fonsecaeus, A. foetidus, A. phoenicis* and *Penicillium janthinellum* and yeasts such as *Candida tropicalis, C. oleophila, C. guilliermondii, C. citroformans, Hansenula anamola* and *Yarrowia lipolytica*) and Bacteria (*Bacillus licheniformis, Arthrobacter paraffinens* and *Corynebacterium* ssp.,) have reported to produce citric acid [6,7]. But the majority of these strains, do not produce sufficient yields of citric acid. To date *Aspergillus niger* has retained its position in citric acid production as it has advantages over other microorganisms.

It is easy to handle, can ferment an extensive choice of low-cost raw materials and provides high yields [8].

Nowadays, the cost of microbial metabolites production is overpriced as the cost of substrate and medium used is very expensive as a result; blooming of new processes to boost the yield with decrement the production cost is highly appreciable from the mercantile view point. To achieve these objectives, during the recent years, efforts have been focussed to trim down the production costs through improving the yield, and the use of either cost free of low cost feed stocks or agricultural by products as substrates. Assorted inexpensive and readily available raw materials like Cane molasses, beet and blackstrap molasses, whey, semolina, areca husk, maize, wheat bran, rice, pumpkin v [9], sweet orange pulp, sweet orange peel, sweet lime pulp, sweet lime peel, pineapple waste, wet corn distiller grains, apple pomace, cassava bagasse, carob pod extract, date syrup, olive oil, palm oil, coffee husk, corncob, grape pomace, kiwifruit peel, okara, glycerol, date syrup and coffee husk [10, 11, 12] are used for industrial production of citric acid [10, 11, 12].

By considering the demand & application of citric acid the present investigation was therefore, undertaken to find out the possibility of using various fruit peels for citric acid production using *Aspergillus niger* AsnO and AsnC isolated from Orange and Carrot and optimization of fermentation conditions.

Materials and Methods

Microorganisms

Aspergillus niger isolates AsnO and AsnC (isolated from Orange and Carrot) were used for this study.

Qualitative Screening for Citric Acid Production

The fungal isolates (AsnO and AsnC) were processed for qualitative assay for acid production using acid indicator medium containing Bromocerol green at pH 6 [13]. A loopful of fungal spore was inoculated on Czapek-Dox broth medium contained (g/l): Sodium nitrate 2.0, Dipotassium hydrogen phosphate 1.0, Magnesium sulphate 0.5, Potasssium chloride 0.5, Ferrous sulphate 0.01, sucrose 30, Bromocresol green dye 40.0 ml (1.0 %, w/v), in distilled water and incubated for five days for the formation of yellow zone around the mycelial growth.

Quantitative Assessment of citric acid production In Czapek-Dox broth

The *A. niger* strains AsnO and AsnC were further tested for citric acid production by submerged fermentation technique in 250 ml Erlenmeyer flasks using Czapek-Dox broth.

Inoculum Preparation

The conidial inoculum was used in the present study. *A. niger* AsnO and AsnC culture was reactivated by streaking a loop full of the culture on potato dextrose agar (PDA) plates and incubated at30ºC for 5-7 days. After the incubation, 10 ml of 0.1% Tween 80 were added to each plate to harvest spores. Diluted spore suspensions of 1.2 x 10^6 spore/ml were counted using haemoeytometer.

Fermentation

50 ml of this medium were inoculated with 1.0 ml of *A. niger* isolates conidial suspension (1.2 x 10^6 spore/ml).The inoculated flasks (triplicates) were incubated at 30ºC in rotary shaking incubator for 9 days. At regular intervals the pH of the fermentation broth was measured and recorded. The highest decrease in the medium pH was taken as criteria for efficient citric acid production. This was based on the assumption that citric acid is the only acid produced during fermentation [14]. After 9 days incubation, the broth was then processed for determination of citric acid.

Optimization of citric acid production

To optimize the various constraint (carbon and nitrogen sources and concentrations, temperature, pH, stimulators and incubation period) the Czapek Dox medium was used. The parameters and their ranges were listed below.

1. Carbon sources- Glucose, fructose, maltose, sucrose and starch (50 g/l)
2. Initial sucrose concentrations- (2.5, 5, 7.5, 10, 12.5 and 15 %)
3. Initial medium pH- pH values of 2, 3, 4, 5, 6, 7, 8 and 9
4. Temperature - 20, 30, 40, 50 and 60ºC
5. Nitrogen sources- urea, yeast extract, peptone, ammonium sulphate, ammonium chloride and sodium nitrate.
6. Different concentrations of ammonium sulphate (0.1, 0.2, 0.3, 0.4 and 0.5 %)
7. Stimulators- methanol and ethanol (0.5, 1.0, 1.5, 2.0 and 2.5 %)
8. Fermentation period- 24, 48, 72, 96 and 120 hours

Determination of citric acid concentration:

The citric acid estimation was done gravimetrically by pyridine-acetic anhydride method [15]. 1.30 ml of pyridine was added to one ml of the diluted culture filtrate in a test tube. The tube was swirled carefully and 570 ml of acetic anhydride was added. The test tube was placed in a water bath at 32±0.5ºC for 30 min. The optical density was measured at 420 nm using a spectrophotometer. The citric acid concentration of the sample was estimated with the help of standard curve constructed by using different concentrations of citric acid.

Citric acid production from fruits peel

For this study orange fruit peel, sweet lime fruit peel, lemon fruit peel and pineapple peel were used. All the fruit peels were washed with tap water and cut in to small pieces and dried in oven at 60°C for overnight. The substrate was powdered and used for further process. The production medium was prepared by using 10g of above mentioned fruit peel powder. The production medium was also supplemented with 10% glucose; 0.35 ammonium chloride and sterilized at 121 °C for 15 min. After cooling, 1% methanol was added. All flasks were then inoculated with 1.0 ml of conidial suspension and incubated at 30ºC, for 96 h. At the end of the fermentation period, culture broth was filtered and citric acid concentration was determined in g/l.

Results and Discussion

Citric acid is one of the most familiar product which has a everlasting insist in the global market. It plays a essential role of an acidulant in food and beverage industries. Citric acid fermentation is one of the ancient fermentations but still its production is going on rising with passage of time using different types of microorganisms. *Aspergillus niger* is the most generally used species for the production of citric acid. In this study two isolates of *A. niger* AsnO and AsnC (Fig 1) were tested for their ability to produce citric acid from defined & crude sources.

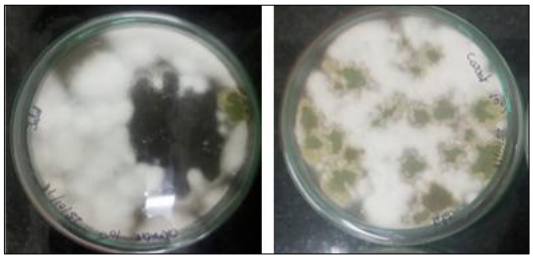

Fig 1: *Aspergillus niger* isolates AsnO and AsnC

Qualitative Screening for Citric Acid Production

The isolates were screened for citric acid production by plate method on Czapek dox agar. The presences of the yellow zone (Fig 2) authenticate the citric acid production**.**

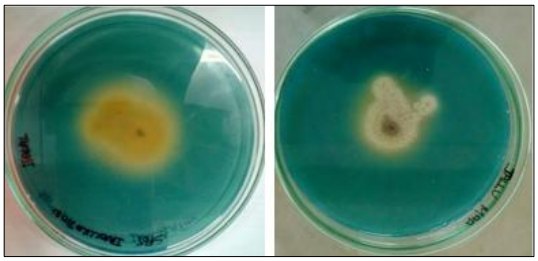

Fig 2: Primary screening on Czapek dox medium in the presence of Bromocerol green

Quantitative Assessment of Citric Acid Production in Czapek-Dox Broth

The two *A. niger* isolates were tested for citric acid production in Czapek-Dox broth. After four days, the medium was filtered and citric acid concentration was determined and recorded (Fig 3). Several investigations have been carried out for scrutinizing *A. niger* isolates for citric acid production via different sources [16, 17, 18]. The maximum citric acid production was noticed among 16 isolates of *A. niger* which ranged between 2.63-47.50 g/l [16].

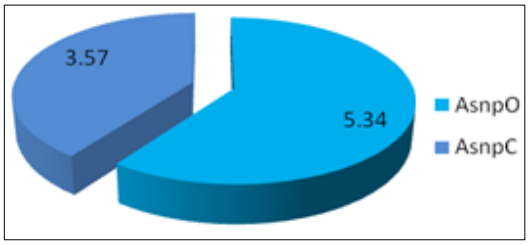

Fig 3: Quantitative Assessment of citric acid production (g/l) in Czapek-Dox broth

The isolate AsnO produced more citric acid (5.34 g / l) than other isolate and used for subsequent studies.

Optimization of Some Key Parameters for Citric Acid Production

Some key parameters were optimized for citric acid production by AsnO isolate. These were: carbon source, initial sucrose concentration, initial pH, incubation temperature, nitrogen source and concentration, concentration of some citric acid stimulators and fermentation period.

Effect of different carbon sources

The effect of different carbon sources on the citric acid production were studied using Czapek Dox as a basal fermentation medium, results are shown in Fig 4

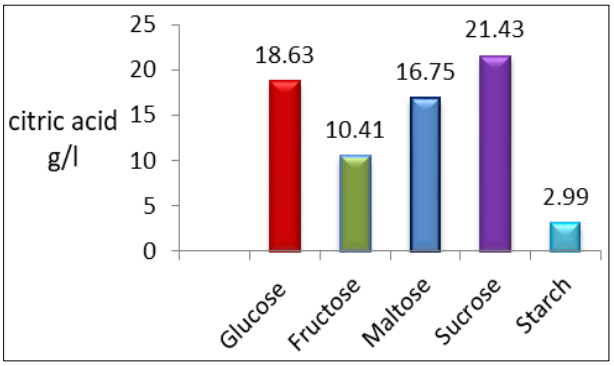

Fig 4: Effect of different carbon sources

From the results it is observed that, the highest citric acid concentration (21.43g/l) was obtained when sucrose was used as a carbon source. It is also clear that glucose was a good carbon source for citric acid production yielding 18.63 g/l.This was followed by maltose (16.75 g/l) and fructose (10.41g/l). The least amount of citric acid (2.99 g/l) was produced when starch was used as a carbon source. These results are in concord with Matty (1992), who delineated that only sugars that are hastily taken up by the fungus permit high yield of citric acid [19]. Supremacy of sucrose over glucose and fructose was also confirmed by earlier studies [20, 21, 22].

Effect of initial sucrose concentration

A.niger AspnO was grown on Czapek Dox broth with different sucrose concentrations (2.5, 5, 7.5, 10, 12.5 and 15 %), results are shown in Fig 5.

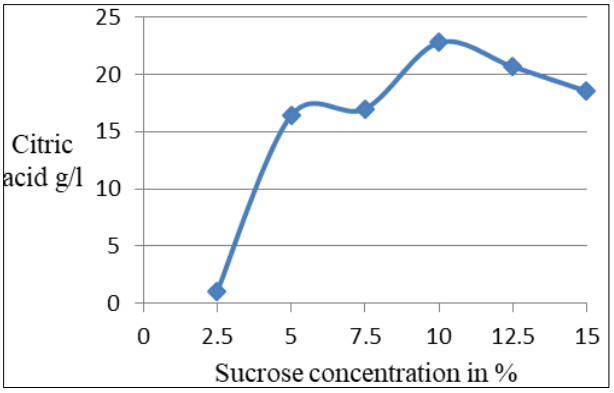

Fig 5: Effect of initial sucrose concentration

Maximum citric acid (22.78g/l) was obtained with initial 10% sucrose concentration. It is also explicable that citric acid accumulation increased as sucrose concentration increased up to 10%. At higher sucrose concentrations, citric acid production decreased sharply. Low level of citric acid accumulation at lower and higher sucrose concentrations might be due to the formation of oxalic acid and polyalcohols [23, 24, 25].

Effect of initial pH

The effect of different initial pH values (2 – 9) on citric acid production was studied using Czapek Dox broth and the results are shown in Fig.6. From the results it is observed that concentration of citric acid increased with pH up to a maximum at a pH of 5 after which it declined. Maximum amount of citric acid (31.64 g /l) was observed at pH 5. At pH values higher than 5, there has been a gradual decrease in citric acid production.

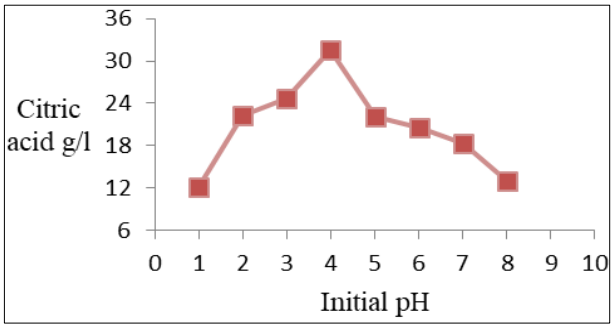

Fig 6: Effect of initial pH

Similar results were reported by, Al-Shehri and Mostafa (2006) and Afifi (2011) that the initial pH of 5.5 was optimum for citric acid production [26, 27]. Ali *et al.*, (2002) reported a comparable pH value of 6 for maximum citric acid production [28]. A conflicting result was observed by El-Hussein *et al.*, (2009) who reported a lower pH (3.5) for maximum citric acid production [29]. The pH of the medium is significant for two aspects. Firstly, for germination of spore at pH of 5 and above and secondly, protons are released to the media when ammonia is taken up by germinating spores. This causes a release of hydrogen ions thus lowering the pH of the medium. The low pH has the effect of improving citric acid production [7]. Thus initial pH must be very well defined and optimized for each microorganism strain, substrate and production technique [30].

Effect of incubation temperature

Among the various temperatures tested, maximum citric acid production by AspnO was recorded in 30°C (20.33g/l).On the other hand, minimum amount of citric acid production was recorded in 60°C (9.43g/l) (Fig. 7).The temperature of fermentation medium is one of the decisive factors that have a strong effect on citric acid production.

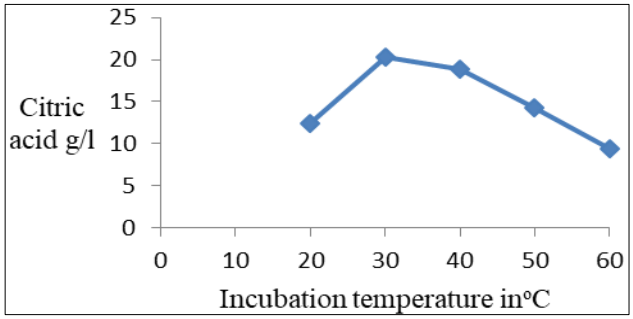

Fig 7: Effect of incubation temperature

In the present study 30°C temperature was found to be optimum for citric acid production. According to Steel *et al.*, (1955) incubation temperature should be in the range of 28 to 32°C, [31] while Gerhardt *et al.*,(1947) found at 30°C was the optimum temperature for citric acid production [32]. An increase or a decrease in the incubation temperature beyond 30ºC has been found to decrease citric acid yield due to denaturation of the enzyme citrate synthase and activation of oxalic acid synthesis pathway [33].

Effect of different nitrogen sources

Nitrogen constituent has an intense effect on citric acid production because nitrogen is not only important for metabolic activities in the cells but it is also the fundamental part of cell proteins.

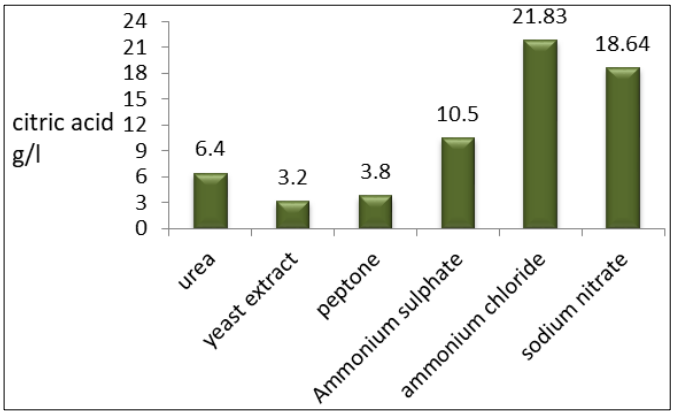

Fig 8: Effect of different nitrogen sources

Maximum (21.83g /l) citric acid production was observed when ammonium chloride used as nitrogen source followed by sodium nitrate (18.64g/l). Least citric acid production was observed in yeast extract (3.2g/l) (Fig.8).

Effect of different concentrations of nitrogen source (ammonium chloride)

Effect of different concentrations of ammonium chloride on citric acid production was given in Fig 9. Ammonium chloride at a level of 0.3 % is found to be the best for citric acid production (28.32 g/l).

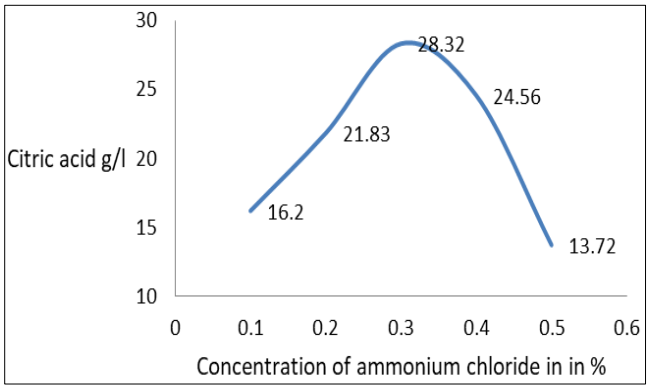

Fig 9: Effect of different concentrations of nitrogen source (ammonium chloride)

Any increase or decrease beyond this level, resulted in the disturbance of fungal growth and citric acid production. This result is contradictory with the results of Haq *et al.*, (2005) Who used ammonium nitrate as a nitrogen source [34]. On the other hand, Kubicek & Röhr (1986) and Yokoya (1992) reported a range of 0.1-0.4 g/l nitrogen for optimal citric acid production [35, 36].

Effect of stimulators

The effect of addition of some stimulators like lower alcohols (methanol& ethanol) was studied**.**

Effect of different alcohols

The effect of different alcohols (ethanol and methanol) at varying concentrations on citric acid fermentation by the strain *A.niger* AspnO was given in Fig 10. The concentration of alcohols varied from 0.5 to 2.5 %,(v/v). While comparing the results, methanol is acting as a good stimulant, compared to that of Ethanol. Zulay *et al.*, (1995) proved the use of methanol as a stimulant [37]. Hang and Woodams (1998)

reported that the increase in citric acid production due to methanol resulted from a decrease in mycelia growth, indicating an efficient use of carbohydrates for citric acid production rather than for cell growth [38]. In addition, methanol is known to increase the permeability of the cell membrane thus, improving citric acid secretion [39].

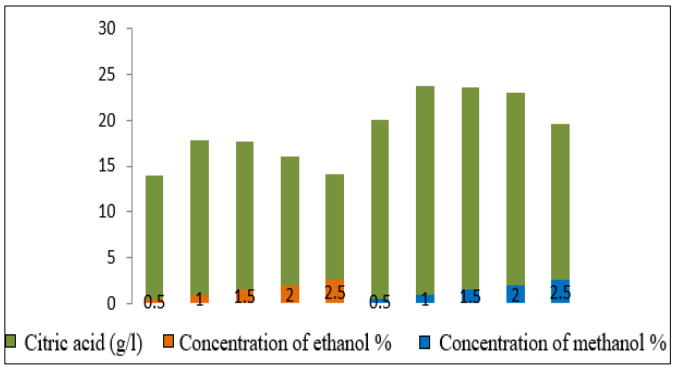

Fig 10: Effect of different alcohols on citric acid production

Effect of fermentation period

The effect of different kinds of incubation time was tested on citric acid production (Fig. 11).

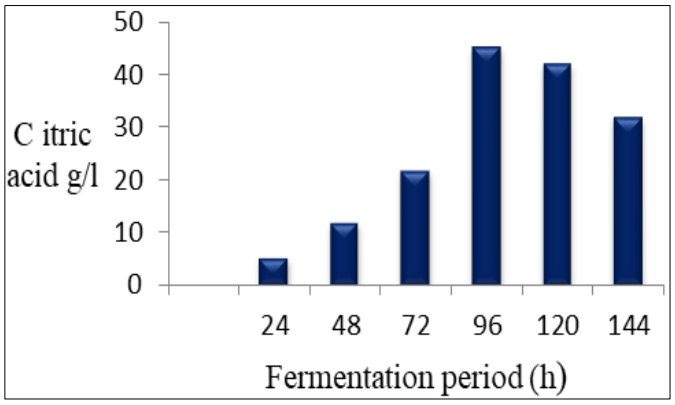

Fig 11: Effect of fermentation period

Maximum amount of citric acid production was observed in 96 hours incubation time (45.37g/l). Further increase in the incubation period resulted in a insignificant decrease in citric acid concentration.

Citric acid production from fruits peel

Due to increase in the production cost of citric acid there is a huge attention in investigating a variety of cheap sources substrates for the production of citric acid which may contribute in reducing the production cost. In the present study four different fruit peel such as orange fruit peel, sweet lime fruit peel, lemon fruit peel and pineapple peel were investigated for the production of citric acid (Fig. 12).

The fruit processing industry yearly generate, tonnes of residues, including peel and sequent membrane from the extraction of juice in industrial plants. These wastes, which produce odour and soil pollution represents a major predicament for the food industry [40]. These wastes are rich in moisture, carbohydrates and other compounds depending upon their origin. Apart from moisture and carbohydrates, they also contain considerable quantities of proteins, fats, natural colorants and in some cases, antioxidants and other bioactive compounds [41]. So, they can be used as substrates for fermentation to produce value added products.

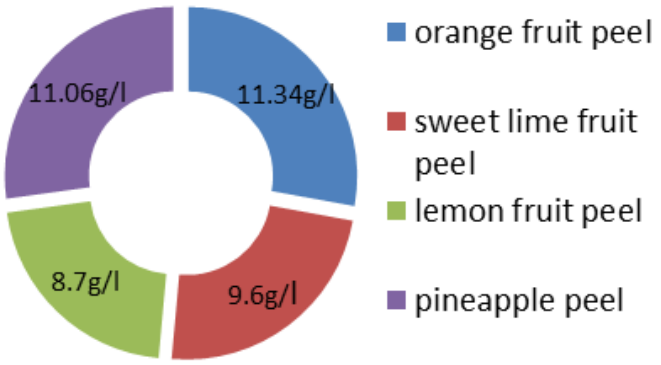

Fig 12: Effect of fruit peels on citric acid production

The maximum citric acid production was found in orange fruit peel (11.34 g/l) and next to that pine apple peel supplemented medium (11.0 6g/l). The maximum production in orange peel media might be due to its constituent- sugars (16.9% wt), starch (3.75% wt), fibre (cellulose, 9.21% wt and pectin, 42.5% wt), ashes (3.50% wt); fats, (1.95% wt) and proteins (6.5% wt) [42].

Similar studies were also reported by Ma *et al*., 1993 and Rivas *et al*., 2009 who have utilized orange peel as a cheap source for the production of citric acid [40, 42].

Conclusion

In conclusion, the current approach put forward the use of various fruit wastes for production of citric acid signifying an efficient outlook of minimizing waste disposal problems, indirectly reducing the health hazards faced due to haphazard dumping of the waste and concurrently producing organic acids of valuable importance for food and pharmaceutical industries. *A.niger* AsnO used in the present study, showed extensive potential to utilize various substrates as a cost-effective growth supported media for citric acid production.

Acknowledgement

The authors are thankful to the Management, D.G. Vaishnav College, Chennai, India for providing necessary facilities for the successful execution of this research work.

References

1. Pallavi Singh, Sadiya Draboo, Aishwarya Singh, Swapnil Chaturvedi, Swati Sharma, Pooja Verma. Citric acid production from different sources under submerged conditions using *Aspergillus niger*. Int. J Curr. Microbiol. App. Sci. 2016; 5(5):483-492.
2. Angumeenal A, Kamalakannan P, Prabhu HJ, Venkappayya D. Effect of transition metal cations on the production of citric acid using mixed cultures of *Aspergillus niger* and Candida gulliermondii. J Indian Chem Soc. 2003; 80:903-906.
3. Pandey A, Soccol CR, Rodriguez-León JA, Nigam P. Production of organic acids by solid-state fermentation. In: Solid-state fermentation in biotechnology-fundamentals and applications. New Delhi: Asiatech Publishers, 2001, 113-126.
4. Bauweleers HMK, Groeseneken DR, Van Peij NNME. Genes useful for the industrial production of citric acid. In: Google Patents, 2014.
5. Abbas N, Safdar W, Ali S, Choudhry S, Ellahi S. Citric acid production from *Aspergillus niger* using banana peel. IJSER. 2016; 7(1):1580-1583.
6. Svetlana V Kamzolova, Igor G Morgunov, Andreas

Aurich, Oksana A Perevoznikova, Nadezda V Shishkanova, Ulrich Stottmeister, *et al*. Lipase Secretion and Citric Acid Production in *Yarrowia lipolytica* Yeast Grown on Animal and Vegetable Fat. Food Technol. Biotechnol. 2005; 43(2):113-122.
7. Papagianni M. Advances in citric acid fermentation by *A. niger*: Biochemical aspects, membrane transport and modelling. Biotechnol. Adv. 2007; 25:244-263.
8. Themelis DG, Tzanavaras PD. Reagent-injection spectrophotometric determination of citric acid in beverages and pharmaceutical formulations based on its inhibitory effect on the iron (III) catalytic oxidation of 2,4-diaminophenol by hydrogen peroxide. Anal Chem Acta. 2001; 428:23-30.
9. Majumder L, Khalil I, Munshi MK, Alam K, Rashid H, Be gum R, Alam N. Citric acid production by *Aspergillus niger* using molasses and pumpkin as substrates. European Journal of Biological Science. 2010; 2:01-08.
10. Ali S, Haq I, Qadeer MA, Iqbal J. Biosynthesis of citric acid by locally isolated *A. niger* using sucrose salt media. Online J Biol. Sci. 2001; 1(4):178-181.
11. Haq I, Rehman A, Ali S. Biosynthesis of citric acid by *A. niger* using cane molasses as basal substrate. Pak. J Biologic. Sci. 2001; 4(6):601-602.
12. Pradoz1 FC, Vandenberghe1 LPS, Woiciechowski1 AL, Rodrígues-León JA, Soccol CR. Citric acid production by solid-state-fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. Brazilian Journal of Chemical Engineering. 2005; 2(04): 547-555.
13. Helen Shnada Auta, Khadijat Toyin Abidoye, Hauwa Tahir, Aliyu Dabai Ibrahim, Sesan Abiodun Aransiola. Citric acid production by *Aspergillus niger* cultivated on parkia biglobosa fruit pulp. International Scholarly Research Notices. 2014; 21(20):8-19.
14. Sakurai A, Imai H, Ejiri T, Endoh K, Usami S. Citric acid production by surface culture using *Aspergillus niger*: kinetics and simulation. J Ferment Bioeng. 1991; 72:15-19.
15. Marrier JR, Boulet M. Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. J Dairysci. 1958; 41:1683.
16. Ali S, Haq I, Qadeer MA, Iqbal J. Biosynthesis of citric acid by locally isolated *A. niger* using sucrose salt media. Online J Biol. Sci. 2001; 1(4):178-181.
17. Rehman A, Ali S, Haq I. Selection of fermentation for citric acid in bioreactor. Biotechnology. 2003; 2(3):178-184.
18. Kareem S, Akpan I, Alebiowu O. Production of citric acid by *Aspergillus niger* using pineapple waste. Malaysian Journal of Microbiology. 2010; 6:161-165.
19. Mattey M. The production of organic acids. Crit Rev Biotechnol. 1992; 12:87-132.
20. Gupta JK, Heding LG, Jorgensen OB. Effect of sugars, hydrogen ion concentration and ammonium nitrate on the formation of citric acid by *A. niger*. Acta Microbiol. Acad. Sci. Hung. 1976; 23(1):63-67.
21. Hossain M, Brooks JD, Maddox IS. The effect of the sugar source on citric acid production by *A. niger*. Appl. Microbiol. Biotechnol. 1984; 19:393-397.
22. Xu BD, Madrit C, Röhr M, Kubicek CP. The influence of type and concentration of the carbon source on the production of citric acid by *A. niger*. Appl. Microbial. Biotechn. 1989; 30:9-15.
23. Honecker S, Bisping B, Yang Z, Rehm HJ. Influence of sucrose concentration and phosphate limitation on citric acid production by immobilized cells of *A. niger*. Appl. Microbiol. Biotechnol. 1989; 31:17-24.
24. Gutierrez-Rozas M, Cordova J, Auria R, Revah S, Favela-Torres E. Citric acid and polyols production by *A. niger* at high glucose concentration in solid state fermentation on inert support. Biotechnol. Lett. 1995; 17:219-224.
25. Haq I, Ali S, Qadeer MA, Iqbal J. Stimulatory effect of alcohols (methanol & ethanol) on citric acid productivity by a 2-deoxy D-glucose resistant culture of *A. niger* GCB-47. Bioresource Technol. 2003; 86:227-233.
26. Al-Shehri AM, Mostafa SY. Citric acid production from date syrup using immobilized cells of *A. niger*. Biotechnology. 2006; 5:461-465.
27. Afifi MM. Naturally occurring microorganisms of industrial wastes for citric acid production by solid-state fermentation. J Envir. Sci. Technol. 2011; 4(4):377-386.
28. Ali Haq I, Qadeer M, Iqbal J. Production of citric acid by A. niger using cane molasses in a stirred fermentor. Electronic J Biotechnolo. 2002; 5(3):258-271.
29. El-Hussein AA, Tawfig SA, Mohammed SG, El-siddig MA, Siddig MAM. Citric acid production from Kenana cane molasses by *A. niger* in submerged fermentation. J Gen. Eng. Biotech. 2009; 7(1):1-6.
30. Soccol CR, Vandenberghe LPS, Rodrigues C, Pandey A. New perspectives for citric acid production and application. Food Technol Biotechnol. 2006; 44:141-149.
31. Steel R, Lentz CP, Martin SM. A standard inoculum for citric acid production in submerged culture. Can J Microbiol. 1955; 1:299.
32. Gerhardt P, Dorrell WW, Baldwin IL. Citric acid fermentation of beet molasses. J Bact. 1947; 52:555-564.
33. Asad-ur-Rehman, Sikander Ali, Ikram-ul-Haq. Temperature Optima for Citric Acid Accumulation by *Aspergillus niger*. Biotechnology. 2002; 1:108-110.
34. Haq I, Ali S, Qadeer CMA, Iqbal J. Optimization of nitrogen for enhanced citric acid productivity by a 2-deoxy D-glucose resistant culture of A. nigerNGd-280. Bioresource Technology. 2005; 96:645-648.
35. Kubicek CP, Rohr M. Citric acid fermentation. Crit. Rev. Biotechnol. 1986; 3:331-373.
36. Yokoya F, Fermentação cítrica, Série Fermentações Industriais. Campinas, São Paulo. 1992; 1:82.
37. Zulay MP, Gisela P, Ferrer JR. Effect of methanol and ethanol on citric acid production by Submerged fermentation. Rev. Technol. 1995; 18(3):321-332.
38. Hang YD, Woodams EE. Production of citric acid from corn cobs by *A. niger*. Bioresourse Technol. 1998; 65(3):251-253.
39. El-Holi MA, Al-Delaimy KS. Citric acid production from whey with sugars and additives by *A. niger*. Afri. J Biotechnol. 2003; 2(10):356-359.
40. Ma E, Cervera Q, Mejía Sánchez GM. Integrated utilization of orange peel. Bioresour. Technol. 1993; 44:61-63.
41. Hilde H Wijngaard, Christian Roßle, Nigel Brunton. A survey of Irish fruit and vegetable waste and by-products as a source of polyphenolic antioxidants. Food Chemistry. 2009; 116(1):202-207.
42. Rivas B, Torrado A, Torre P, Converti A, Domínguez JM. Submerged citric acid fermentation on orange peel autohydrolysate. J Agric. Food Chem. 2008; 56:2380-2387.