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

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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 conducted 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 (22.78g/l) at 10% 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 pineapple 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 (C₆H₈O₇) and in its pure form 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. fujisaeicaus, A. foetidus, A. phoenicus and Penciclillum 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 spp.,) 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 \cite{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 molasses, savava, 10 ml of different types of microorganisms. The most familiar production is 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 \cite{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, Potassium 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 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.

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 60°C
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 \cite{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±0.5°C 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 30°C, 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 an everlasting insist in the global market. It plays a essential role in the production. This was based on the assumption that citric acid is the only acid produced during fermentation \cite{14}, After 9 days incubation, the broth was then processed for determination of citric acid.
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.

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].

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. 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 AsnO 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.

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.
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[26]. 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.

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 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).

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].

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

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

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