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Random UV Mutagenesis Stimulated over Production of Citric Acid by Aspergillus niger

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Abstract- **The Aspergillus niger strain was isolated from soil sample and employed for the production of citric acid from easily available raw materials (citrus fruit wastes). A total of four substrates-lime peels, orange peels and banana peels and beet root peels were selected in this study. The citric acid fermentation was carried out upto 6 days at 300C. Fermentation process was carried out by using three types of inoculum i.e A. niger spores and mycelium and also mutated A.niger mycelium for citric acid production. Estimation of citric acid and sugars were done by every 48 hrs. Highest yield of citric acid (10g/100ml) was observed in the mutated mycelium than the spores and normal mycelium. Among the substrates used, orange peels were found to be more suitable for citric acid production. Greatest antimicrobial activity of synthesized citric acid was observed against E.coli and Staphylococcus aureus and also trail of Calcium Hydrogen Phosphate Dihydrate (CHPD) crystal dissolution was tested. As the increased concentration of citric acid, the dissolution of CHPD crystals was found to be high.**

Key words: citric acid, inoculums, mycelium., spores etc.

INTRODUCTION

Citric acid ($C_6H_8O_7$, 2-hydroxyl- 1, 2, 3-propane tricarboxylic acid), a natural constituent and common metabolite of plants and animals, is the most versatile compound. It is used as an acidifying agent, flavour enhancer, preservative, anti-oxidant, stabilizer in food, beverages and the pharmaceutical industries (Anwar et al., 2009). It is also used as an eco-friendly cleaning agent and acts as an antioxidant (Aysegul and Christian, 2003). Citric acid is recognized as safe for use in food by all major national and international food regulatory agencies (Ali et al., 2002).

In the total production of citric acid, 70% using for food industry, followed by about 12% for the pharmaceutical industry and 18% for other applications (Milsom, 1987). Although methods are well developed to synthesis citric acid using chemical means also, better successes were achieved by using microbial fermentations and over the period of time, this technique has become the method of ultimate choice for its commercial production, mainly due to economic advantage of biological production over chemical synthesis. Currently the global production of citric acid is estimated to be around736000 tones/year and entire production is done by the fermentation (Mattey, 1992).

The citric acid is produced mainly by submerged or solid state fermentations using Aspergillus niger from a variety of substrate such as molasses (Saad et al., 2003). Haq et al. (2004) reported that commercial production of citric acid is generally by submerged fermentation of sucrose or molasses using the filamentous fungi Aspergillus niger. Although high level of citric acid is currently achieved, the research for better yield is in progress in various laboratories on ways of improving the efficiency of the fermentation process. One of the best method for strain improvement by mutation (Haq et al., 2001). Mutation can be either spontaneous or induced. They arise because of changes in the base sequence of the nucleic acid of organisms. Among the physical mutagens, gamma-radiation (Gunde and Cimerman, 1986; Islam et al., 1986) and UV radiation (Pelechova et al., 1990) have often used. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, Ɣ-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, etc. (Parekh et al., 2000). Vaidya et al. (2003) reported that the Alcaligenes xylosoxydans mutant (UV28) which derived from ultraviolet radiation could produce chitinase enzyme at levels higher than the wild type by 2.3 fold. Streptomyces pseudogriseolus UV mutant, excreted over-production of xylanase (161% production improved) as compared to the wild type (Abdel-Aziz et al., 2011).

MATERIALS AND METHODS

SUBSTRATES:

Selected raw materials such as sweet lime peels, orange peels, banana peels, beetroot peels wastes were collected in local shop. Then they were cleaned, dried and used for the substrate of citric acid production.

ISOLATION AND SCREENING OF FUNGI:

The fungi were screened from waste dumping site soil by serial dilution techniques. The dilutions were inoculated by the spread plate technique on potato dextrose agar (PDA) and

incubated for 300 C for 48 hours. The fungal cultures obtained in this primary screening were plated on the selected media. The selected media for the citric acid producers were Czapeck dox agar with bromocresol as an indicator. The mycelial growth which showed yellow colour zones around them were selected as the citric acid producers (Sikander and Ali, 2005). The selected colonies were identified by observing conidial arrangement under the microscope and subculture for further uses.

INOCULUM PREPARATION:

Two types of inoculums were prepared,

a. Spore suspension

b. Intact mycelium

a. In the spore suspension method, Aspergillus niger strains were grown on potato dextrose agar (PDA) medium at a temperature of 300C for 5 days. After the incubation, the plates were washed with sterile 0.1% of Tween 80 solutions to collect the spores. 1ml of this suspension is used as the inoculums.

b. In the Intact mycelia method, Aspergillus niger strains were grown on the potato dextrose agar plates at a temperature of 300C for 5 days. From these agar plates, the agar plugs were inoculated into 250ml Ehrlenmeyer flasks containing potato dextrose broth and incubated for 72hrs at 300C till the mycelia mat develops and this mycelia mat was used as an inoculum.

SUBSTRATES:

The fermentation medium consists of distilled water and 5% of substrates. Selected raw materials such as sweet lime peels, orange peels, banana peels and beetroot peels were used for the production of citric acid. The amount and size of the substrates used in the fermentations given in the following Table:1.

Table 1 Substrate and their particle sizes used in the Saccharification process:

S.No	Substrate	Particle size of the substrate	Amount used
1.	Lemon peels	1×2 cm	5gm/100ml
2.	Orange peels	1×2 cm	5gm/100ml
3.	Banana peels	1×2 cm	5gm/100ml
4.	Beet root peels	1×2 cm	5gm/100ml

DETECTION OF CITRIC ACID:

Thin layer chromatography plates of silica gel G were used for detection of citric acid. The chromatogram was developed in solvent system such as n-Butanol:Formic acid:Water (40:20:40). 0.4% aqueous solution of bromophenol blue was used as locating agent. For citric acid production, spores from selected colony were inoculated into the fermentation broth and incubated at 300C for 3 days. After incubation, the mycelium was separated by filtration. The filtrate was assessed in presence of citric acid by TLC. Then the citric acid concentration was estimated by titration method.

6. PRODUCTION OF CITRIC ACID:

Submerged fermentation: The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. Fermentation experiments were performed in 250ml Ehrlenmeyer flasks containing 100ml distilled water and 5% of the substrate. Spore suspension: 1ml of spore suspension was inoculated into all the flasks and incubated in 300C.

Intact mycelium: One intact mycelium mat 7cm in diameter was inoculated in all the flasks and incubated at 300C. The fermentation process was performed for 6 days. Samples were collected from every 48hrs and subjected for estimation of citric acid by titrimetrically and the left over sugar by phenol sulphuric acid method spectrophotometrically.

7. CITRIC ACID DETERMINATION:

Citric acid was determined titrimetrically (AOAC, 1995) by using 0.1N NaOH and phenolphthalein as indicator and calculated as % according to the formula:

$$\%\text{citric acid} = \frac{\text{Normality} \times \text{volume of 0.1M NaOH} \times \text{quivalent wt of citric acid} \times \text{Dilution factor}}{\text{Weight of sample (g)}} \times 10$$

8. BIOMASS, RESIDUAL SUGARS AND PH DETERMINATION:

Biomass, sugar and pH values were determined according to AOAC (1995). To determine biomass the whole fungal culture was filtered with sterile filter paper and dried to a constant weight at 1050C. Results were expressed in g/Kg of peels. The sugar content was determined using a refractometer and pH was measured by Analog pH meter. Each analysis was conducted in triplicate.

9. ESTIMATION OF SUGAR BY PHENOL SULPHURIC ACID METHOD:

Total residual sugar of fruit peel wastes of four different fruits was estimated calorimetrically by phenol sulphuric acid method.

10. RANDOM MUTAGENESIS OF A.niger WITH MUTAGENIC AGENTS:

The homogenous spore suspension of A.niger prepared in saline with Tween 80 was exposed to mutagenic agents. The numbers of spores was determined by microscopic counting

on hemocytometer. Number of spores in the central 25 chamber was counted. The solution was diluted to100 spores/ml. For treatment with UV radiations 0.1ml of A.niger spore suspension was spread of surface of Sabouraud's Dextrose Agar incorporated with bromophenol blue. Plates were then exposed to UV mutagenesis for different time interval 10mins, 15mins, 20mins. After the mutagenesis the fungal cultures were incubated at 300C for 3 days. After mutagenesis 0.1ml of spore suspension was taken out and spread on Sabouraud Dextrose Agar medium and incubated for 3 days at 300C. Then the highest yellow colour production fungi were selected for the citric acid production.

11. DETERMINATION OF CITRIC ACID USING HPLC:

a. Preparation of Mobile Phase:
A 50mM phosphate buffer solution to use as the mobile phase was prepared by dissolving 6.82 g of potassium phosphate monobasic (Fisher) in deionised water. The pH of the buffer was then adjusted to 2.80 by adding concentration phosphoric acid dropwise while monitoring with the calibrated pH meter. The solution was then diluted to 1.00L with deionized water. To obtain an HPLC grade mobile phase the solution was filtered through a 0.22-µm nylon membrane by vacuum filtration. HPLC grade methanol was used before and after the experiment to prevent phase collapse.

b. Standard Preparation:
Citric acid standards of 100, 200, 300, 500 and 1000ppm were prepared from granular citric acid (Fisher) and deionized water. These were then transferred to auto sampler vials for HPLC analysis.

c. Preparation of Samples:
Four samples were used. Four were orange peels, lemon peels, banana peels and beet root peels was also used.
From each bottled sample 10.00ml aliquot was transferred to a 50ml volumetric flask. The sample which had to be dissolved was prepared by dissolving 1.01g into a 50ml volumetric flask. This mass was equivalent to a 10ml sample of Tang prepared according to the instructions on the label. The navel orange sample was prepared by juicing an orange and then pipetting 10ml of the liquid into a 50ml volumetric flask. All 50ml flasks were diluted to volume with deionized water. All dilute samples were filtered through a 0.45-µm filter membrane before HPLC analysis.
After an initial analysis by HPLC, all of the juices were further diluted by transferring 10ml of the once diluted samples into new 50ml volumetric flasks and diluting to volume with deionized water. This provided peaks that would be more accurately analysed than the more highly concentrated samples except for juicy juice, which was kept at the higher concentration level for final analysis.

d. Instrument Preparation:
An isocratic elution was carried out on a Varian ProStar HPLC system with 50mm× 4.6mm Kinetex XB-C18 column (Phenomenex) with a 2.6µm particle size and 100Å pore size. The mobile phase was 100% 50mM phosphate buffer at pH 2.80. The HPLC system operated at a low rate of 0.5ml/min, the injection volume was 5µl and the external temperature control column oven was set at 35C. The detection wavelength was 214nm.

12. ANTIMICROBIAL ACTIVITY:

The solution of bacterial concentration was determined and adjusted to 1-3×105 CFU/ml prior to the inoculation and quantitative measurement of antimicrobial activity was made using turbidity and MIC assays. The MIC of the citric acid was determined by the broth dilution method. MIC is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism. After overnight incubation, MIC is the lowest concentration of the substance that will kill the microorganisms. To determine the MIC, the citric acid ranges from 1ml, 1.5ml, 2ml, 2.5ml, and 3ml were taken in separate test tubes. After that 0.1ml of E.coli (urinary tract pathogen) culture was added to each of the test tubes. The culture was incubated for 24hrs. After the incubation, the bacterial growth was observed in the test tubes. The turbidity formed in the test tube showed bacterial growth. After measuring their turbidity spectrophotometrically, 0.1ml of culture was inoculated on nutrient agar plates.

13. PRESERVATION OF TOMATO USING CITRIC ACID SOAKED CLOTH:

Two tomatoes were taken and were labelled as experiment and control. The experiment tomato was wrapped with citric acid soaked cloth and the control tomato was unwrapped. Both tomatoes were kept in room temperatures and observed for 10days.

14. ARTIFICIAL KIDNEY STONES DISSOLVED USING CITRIC ACID:

Glass test tubes of 2.5cm diameter and 15cm length were used for growing the crystals. Sodium meta-silicate solution of specific gravity 1.06 was acidified by adding appropriate amount of ortho phosphoric acid so that 4.0 and 5.0 pH could be obtained for the mixture, which was subsequently transferred into different test tubes. After gelation took place, 10ml, 1M aqueous solution of calcium chloride was carefully poured on the set gels. Crystals were found to be growing rapidly within two days from pouring the supernatant solutions. The molar concentration of 0.4 M citric acid in different concentration (2ml, 4ml, and 6ml) was added into the experimental crystal and their effect was observed after 24hrs.

RESULTS:

During the primary screening, the fungi showed highest yellow colour zone around them was selected for the citric acid production. The isolated fungi was stained and identified under the microscope. The black sporulation arrangement of conidia and mycelial structure confirmed the isolated fungus was Aspergillus niger. This fungus has the ability to produce

the organic acid after the incubation period of 48hrs on Czapeck dox agar medium.

During the fermentation, the sugar content of the medium was reduced gradually by A.niger and the amount of citric acid production increased in proportion with the sugar utilization. The result of thin layer chromatography of fermented broth developed spots of organic acid of Rf value 0.70. that is equal to Rf value of standard citric acid spot. This proved the presence of citric acid in the fermented broth.

In the spore suspension, after the incubation of 2nd day of fermented broth, germination of the fungal spores and the growth of mycelium was observed in all the flasks containing the peels (orange peels, lemon peels, banana peels, beet root peels) and negligible amount of citric acid production was recorded. From the 3rd day of fermentation the citric acid production was observed gradually increasing. Highest yield of citric acid production was recorded on the 6th day of fermentation in all the flasks, among the four raw materials, after the 6th day of fermentation, orange peels produced highest citric acid (8.3%) than compared to others (lemon peels 6.4%, banana peels 6%, beet root peels 5.6% (Figure:1).

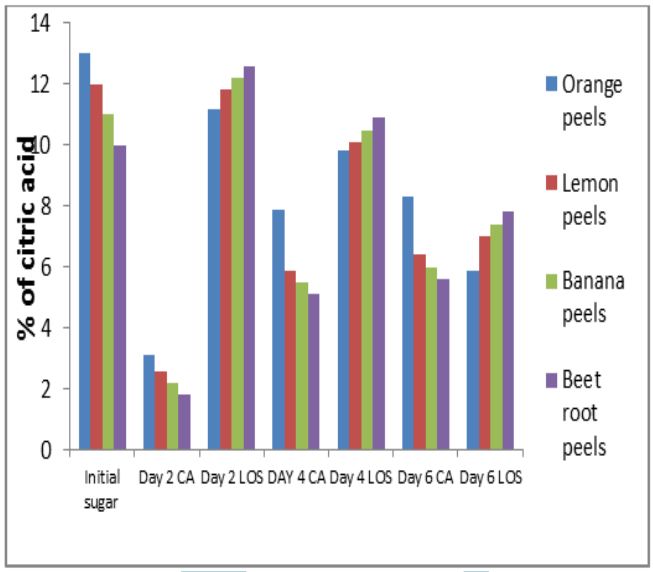

Figure :1 Citric acid production by spore suspension of A. niger

CA- Citric acid production LOS-Loss of sugar

In the mycelia inoculation, biomass production was gradually increased and also increased the production of citric acid (Figure: 2), the highest production of citric acid was observed after the 6th day of fermentation. In the mycelia inoculation, 2 folds increased the production of citric acid than the spore suspension. Among the 4 raw materials, after the 6th day fermentation orange peels produced highest citric acid (9.2%) than compared to others (lemon peels8.4%, banana peels 8%, beet root peels 7.6%).

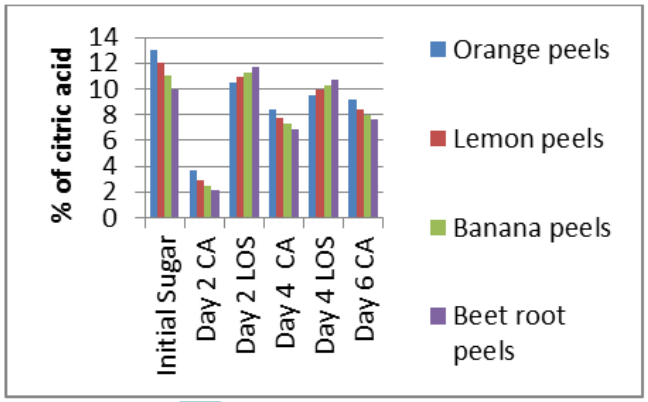

Figure: 2 Citric acid production by intact mycelium of A. niger

CA- Citric acid production LOS-Loss of sugar

High yielding strain was obtained by mutated the strain repeatedly. This procedure produced stable mutant fungi for citric acid production. The UV light was a potent mutagen, the exposure to UV for 15minute have severely damaged the spore as well as the mycelium. The mutated strain showed increased citric acid production than the wild type (Figure:3). Mutated strain (mutated for 5 times) was selected for citric acid production. Among the 4 raw materials , after the 6th day of fermentation, orange peels produced highest citric acid (10%) than compared to others (lemon peels 9.6%, banana peels 9.2%, beet root peels 8.8%).

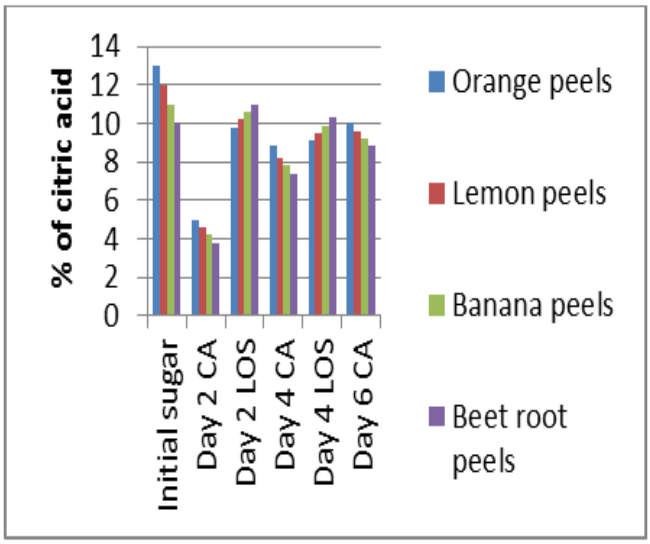

Figure: 3 Citric acid production by UV mutated mycelium of A. niger

CA- Citric acid production LOS-Loss of sugar

Citric acid production, after the 6th day of fermentation in the raw material of orange peels showed that highest yield in UV mutant strain of fungi produced 10gm/100ml of citric acid production than the wild type fungi produced 9.2% and spore suspension of fungi produced 8.3% of citric acid production. The standard chromatogram of citric acid, which eluted from the column at 2.16 min. Based on the integrated peak areas from the HPLC chromatogram showed the peak at 2.16 min was citric acid but, HPLC of citric acid from orange 1 showed 2.4 min as highest peak. The concentration of citric acid was

determined the sample by integrating the citric acid peak and comparing that value to calculation were then made to determine the original citric acid concentration in the undiluted sample was 290ppm of citric acid. Lemon 1 chromatogram showed the citric acid peak at 2.16min. The concentration of citric acid was determined in the sample by integrating the citric acid peak and comparing that value to calculations were then made to determine the original citric acid concentration in the undiluted sample is 270ppm of citric acid(Figure 4&5).

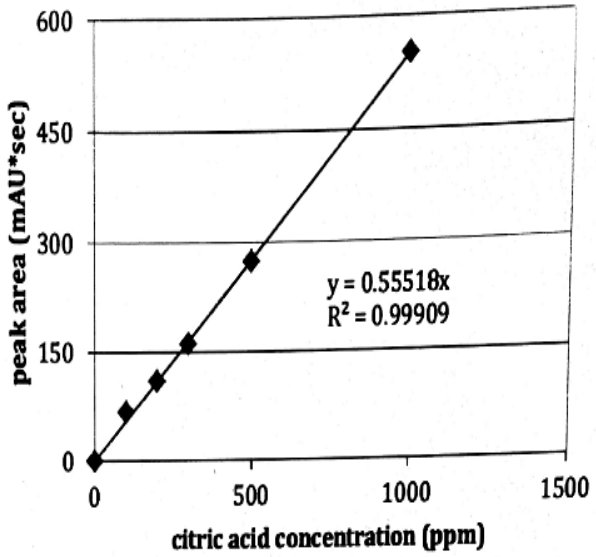

Figure 5: Standard calibration for citric acid using chromatogram

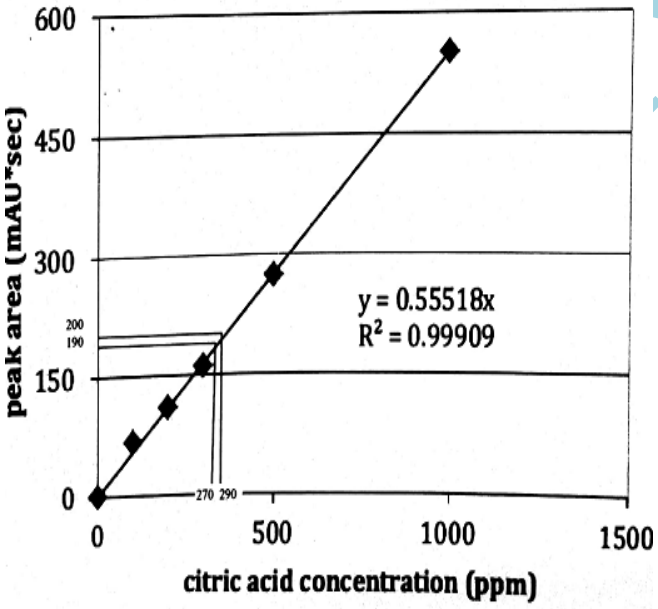

Figure 6: Calibration plot for orange I, lemon I.

Figure :4 Calibration plot of citric acid production from Orange and Lemon peel

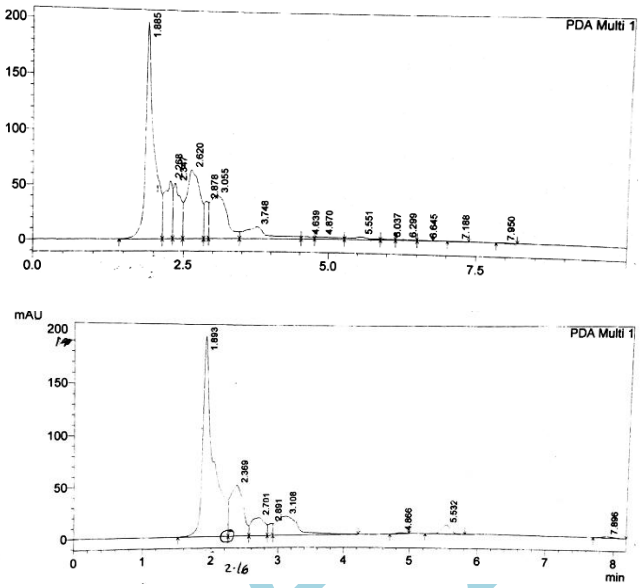

Figure 5 HPLC analysis report of Orange and Lemon

The E.coli organism was isolated from urinary tract infected urine sample. Antibacterial activity of citric acid against E.coli and Staphylococcus aureus was tested, they showed 1ml citric acid was enough for killing of 0.1ml culture of E.coli in the 5ml broth. After 24hrs incubation, highest amount of citric acid concentration (2.5ml) showed no growth on the medium. But 1.5ml of citric acid greatly reduced the bacterial growth. Above 2ml of citric acid added broth showed decreased optical density (Table 2&3). MIC of citric acid against Escherichia coli was 2.5ml/5ml and Staphylococcus aureus was also showed 2.5 ml/5ml.

Table: 2 Anti microbial activity (plate count method)

Organism	Citric acid volume				
	1 ml	1.5 ml	2 ml	2.5 ml	3 ml
Escherichia coli	180	105	50	_	_
Staphylococcus aureus	200	160	102	_	_

Citric acid	Absorption value (OD value)
1 ml	0.404
1.5 ml	0.333
2 ml	0.286
2.5 ml	0.105
3 ml	0.012

Table 3: Turbidity measures in spectrophotometrically (E.coli growth):

Vegetable preservation by using the citric acid soaked cloth wrapped around the tomatoes were observed after 10 days, they showed no signs of spoilage in the experimental tomatoes, but control tomatoes showed spoilage. The results showed citric acid soaked cloth prevented the growth of spoilage organisms and extended shelf life of tomatoes (Table 4).

Table 4 Preservation of tomatoes by wrapping citric acid soaked cloth

S.No	Wrapping citric acid soaked cloth (10cm)	Number of days (After)	Sign of spoilage
1.	2ml	5	+
		10	+++
2	4ml	5	+
		10	++
3	6ml	5	-
		10	-

+ Sign of spoilage ,++ Heavy Spoilage, +++Heavy Spoilage,- No spoilage

Another application of citric acid was dissolution of artificially made crystals (calcium hydrogen phosphate dihydrate crystals). They showed elongated platelet type and star shaped. The size of the experimental crystal was 0.3mm. By adding 2ml, 4ml, 6ml of 0.4M citric acid solution in to the experimental crystal. After 5 days, it was found that the size of the crystal was decreased gradually according to the concentration of citric acid. The highest concentration of (6ml) citric acid was dissolved the crystal completely faster than the others (Table 5).

Table 5 Dissolution of artificially made crystals by citric acid

S.No	Concentration of citric acid	CHPD crystal (0.3mm)
1.	2ml	0.3mm
2	4ml	0.2mm
3	6ml	0.0mm

DISCUSSION:

Citric acid is a weak organic acid, which is useful in many pharmaceutical and industrial food products. Increased demand for the citric acid has led to search for high yielding strains of citric acid producers. In my work, substrates like lemon peels, orange peels, banana peels, beet root peels were used for citric acid production. Cane molasses were also used for citric acid production reported by Adil (2009). At present a variety of agro-industrial waste residues and by-products are used as substrate for the production of citric acid such as Cassava bagasse (Prado et al., 2005), coffee husks(Selvi et al.,2011), wheat bran , apple pomace (Vanderberghe et al.,2004), pineapple waste (Shojaosadati and Babaeipour, 2002), kiwi fruit peel (Tran and Mitchell, 1995), corn waste banana peels, grape pomace (Hang, Luh and Woodams, 1987). Microbial production of citric acid is advantageous over the chemical synthesis in that way we can utilize raw materials such as fruit waste and produced citric acid in an economic way.

In the present study, citric acid production was carried out by two phases. In the first phase, noticed the growth of A. niger and in the second phase noticed the production of citric acid. A. niger spore inoculum does not directly ferment citrus fruit wastes peels into citric acid, these waste products at initial stage served as a potential carbon source for their growth. On the 2nd day of fermentation observed that the sugars were readily available in the medium, which providesd sufficient mycelial growth. This suggested that more easily available sugars which are present in the media were consumed for mycelia production without the need of breaking down the cellulosic materials enzymatically. As a result substantial growth of biomass and negligible amount of citric acid production was observed on the 2nd day of fermentation in both sets.

In citric acid production upto 6th day of fermentation exhibited that gradual production of citric acid was noticed in spore suspension containing flask, while rapid production was observed in intact mycelium, mutated intact mycelium inoculated flasks. The results that indicated A.niger hydrolyses cellulose present in the substrate to simple sugars by saccharification process and these simple sugars undergo hydrolysis to produce citric acid by fermentation process. Further we observed in fermentation, the citric acid production increased in proportion with sugar utilization(Loss of sugar). The same work supported by Khadir (2011).

Citric acid is the most important organic acid produced in large amount preferably by fungal fermentation (Soccol et al., 2006). In this work, A. niger isolated from waste dump site soil and used for citric acid production. Many microorganisms are known to produce citric acid but Aspergillus niger remains the favorite employ for industries. Most of them, however, are not able to reduce commercially acceptable yields. This could be explained by the fact that citric acid is a metabolite of energy metabolism and its accumulation raises in appreciable amount only under conditions of drastic imbalances (Vanderberghe et al., 1999). Only few specific strains of A.niger are capable of overproducing citric acid have been developed for various types of fermentation processes. The

yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source (Papagianni, 2007). Soil isolate Aspergillus niger produced 9.2% of citric acid by using mycelia inoculums but, spore inoculum produced 8.3% of citric acid.

The improvement of citric acid producing strains has been carried out by mutagenesis and selection. The most employed technique is inducing mutation using mutagens (Haq et al.,2001; Vanderberghe et al.,1999). Such mutants of A.niger were used for commercial productionof citric acid (Jianlong et al., 2000). Among the physical mutagens, γ-radiation (Bonatelli and Azevedo,1983; Islam et al.,1986) and UV radiations(Pelechova et al.,1990) have often used. To obtain hyperproducing strains, frequently UV treatment could be combined with some chemical mutagen treatment e.g. aziridine, N-nitroso-N-methylurea or ethyl-methane sulfonate (Musilkova et al.,1983). By using a suitable selection technique on model medium with non-specific carbon sources, a strain yielding high amount of citric acid from unusual substrate can be obtained from the mutants produced (Vanderberghe et al.,1999). In the present study, the Aspergillus niger was subjected to random mutagenesis by UV radiations (physical mutagens). Mutant strains (UV exposure for 15 minute) produced more citric acid (10% of citric acid) with lesser consumption of glucose compared to parental strain (9.2% of citric acid).

In my work the antibacterial effectiveness of citric acid against E.coli (gram-negative) and gram-positive (Staphylococcus aureus) was studied. The highest amount (2.5ml) of citric acid concentration showed no growth on the medium. The same work supported by Vukusic (2009).
Vegetable preservation by using the citric acid soaked cloth wrapped around the tomatoes were observed after 10 days, they showed no signs of spoilage in the experimental tomatoes, but control tomatoes showed spoilage. The citric acid soaked cloth prevented growth of spoilage microorganisms and extended shelf life of tomatoes. Similarly, antifungal activity of a cotton textile material treated with an easy-care finishing agent and citric acid (CA) has been evaluated. The same work supported by Vukusic (2009). Textile materials used in hospitals (e.g., sheets, pillowcases, theater drapes, gowns, masks) are known to be major sources of cross-infection and as such, use of antimicrobials present opportunities to prevent or minimize infection or disease transmission (Lee, 1999).

In the present work, artificial kidney stones formed Calcium hydrogen phosphate dihydrate crystals, they are well known urinary crystals and frequently found in urinary stones. The CHPD crystals were grown by the single diffusion gel growth technique in sodium meta-silicate gel. The grown crystals were having platelet shape. Recently, the effect of herbal extracts such as, Tribulus terrestris and Bergenia ligulata, have been investigated dissolution of urinary stones crystals, by Joshi et al.(2003). Moreover, the growth inhibition study of crystals in presence of tamarind solution and tartaric acid solutions have been reported by Joseph et al. (2005). Citrate plays predominant role in the mechanism of formation of urinary calculi. First of all citrate binds with calcium ions in the urine and reduces calcium ion activity, consequently, the lowering of the urinary super saturation of calcium phosphate and calcium oxalate. Second, citrate has a direct inhibitory effect on the crystallization and precipitation of salts. Citrate inhibits nucleation and growth steps of calcium oxalate monohydrate (COM) as well as CHPD crystallization (Joshi, 2003).

CONCLUSION:

A maximum citric acid was obtained from orange peel using mutated fungal stains. This study indicates that the use of orange peel for fungal production of citric acid might represent an efficient method of minimizing orange peel waste disposal problems and simultaneously producing organic acids of valuable importance for food and pharmaceutical industries. This process contributes to a reduction of environmental pollution also.

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