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Comparative estimation of citric acid production due to the activity of indigenous *Aspergillus niger* and *S. cerevisiae* on kitchen waste

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Abstract
Citric acid is the most versatile and widely used organic acid in the field of food and pharmaceutical industries which is non-toxic GRAS compound with pleasant sour taste. Its a natural constituent and common primary metabolite of plants and animals formed during tricarboxylic acid cycle or Krebs cycle. The production of citric acid have been evaluated upon variety of bacteria, fungi & yeast. The conventional methods have proved to be complex, expensive, and not eco-friendly but microbial production is supposed to be feasible & cost effective. Thus, in present investigation citric acid production by *Aspergillus niger* and *Saccharomyces cerevisiae* is comparatively estimated by using kitchen waste as a source of nutrition. Indigenously isolated *A. niger* and *S. cerevisiae* were inoculated in the homogenized and partially sterilized organic mixture prepare from kitchen waste. The standard peak generated in HPLC chromatogram for pure citric acid marker showed ad retention time of 9.732±0.3min. When compared to the standard, the percentages estimation of citric acid produced due to fermentation activity of *A. niger* on kitchen waste was observed to be 0.569%, while the activity of *S. cerevisiae* results into the yield of 0.325% citric acid in sample extract within 15 days at ambient temperature. Citric acid production by *A. niger*by using homogenized mixture of kitchen waste observed to be higher compared to the yield due to activity of *S. cerevicea* on same substrate. The improvement in yield of organic acid production using such substrate could be further worked out by modifying the nutritional quality of organic substrate by addition of mineral supplements which is matter of further investigation.

Keywords: *Aspergillus niger*, *Saccharomyces cerevisiae*, fermentation, kitchen waste, citric acid, HPLC

Introduction
Citric acid ($C_6H_8O_7$, 2 – hydroxy – 1,2,3 – propane tricarboxylic acid), is the most versatile and widely used organic acid about 75% in the field of food and 12% pharmaceutical industries which is a natural constituent and common primary metabolite of plants and animals formed during tricarboxylic acid cycle or Krebs cycle (Haq *et al*., 2001; Max, *et al*., 2010) [8, 15]. Citric acid is a non-toxic GRAS (Generally Recognized as Safe) compound with pleasant sour taste (Sawant, *et al*., 2018) [17].

The production of citric acid have been evaluated upon variety of bacteria, fungi & yeast such as *Bacillus licheniformis*, *B. subtilis*, *Corynebacterium* spp.; *Aspergillus niger*, *A. awamori*, *A. foetidus*, *Penicillium restrictum*; *Candida lipolytica*, *C. intermedia* and *Saccharomyces cerevisiae* respectively (Kubicek, 1998; Kamzolova *et al*., 2003; Kareem, *et al*., 2010) [14, 11, 12].

Several physical and chemical methods have been carried out for production of citric acid where conventional methods have proved to be complex, expensive, and not eco-friendly. But the production is supposed to be feasible with use of microorganisms compared to plant and animal sources (Gupta *et al.*, 2015; Yin *et al.*, 2017; Yu *et al.*, 2018; Sawant, *et al*., 2018) [22, 17, 23].

Commercial production of citric acid is done either by fermentation of molasses using *A. niger* in submerged state or it is synthetically produced from glycerol or acetone (Torres *et al*., 1998; Haq *et al*., 2004; Kareem, *et al*., 2010) [9, 12, 18]. But in case of microbial production of citric acid which is supposed to be cost effective, itsaccumulation is stronglyinfluenced by the media composition, in fermentation processes where its production could be improved by using genetic modified microorganisms or by adjusting environmental parameters (Max *et al*., 2010; Ali *et al.*, 2016) [15, 1]. Thus, present investigation is intended to evaluate the production of citric acid by indigenously isolated *Aspergillus niger* and *Saccharomyces cerevisiae* using kitchen waste as source of nutrition by solid state fermentation method.

Materials & Methods

Isolation and Identification of Fungi

For isolation of fungal species of interest, 2-3 days old domestic or kitchen waste collectively including food waste, raw vegetable waste, fruit peals was collected and subjected crushing followed by serial dilution. A dilution of 10^{-7} was used to inoculate malt extract agar media plates and yeast extract peptone dextrose agar media plates (Himedia India) by spread plate technique. The culture plates were than incubated at 30ºC for 3-5days in an incubator (Navyug, India) as per the method described in earlier literatures (Clark, *et al*., 1958; Iqbal, *et al*., 2015; Yadav & Tiwari, 2016) [5, 10, 20] with slight modifications. The suspected fungal colonies of interest were subjected to pure culture followed by identification on the basis of colony morphology and lactophenol anilin cotton blue stain according to Aneja (2003) [2] and Arumugam *et al.* (2014) [3].

Fermentation activity

Collection of kitchen waste

Once again, fresh kitchen wastes was collected including including food waste, raw vegetable waste, fruit peals; all present in equal ratio. The food and kitchen waste was cut into small pieces, crushed and homogenized to paste like consistency. The homogenized kitchen waste material was kept in stainless steel container and treated incubated for 2 hours at 60ºC in a water bath for partial sterilization. After the process of pasturilzation, the domestic or kitchen waste mixture was transferred to well hygiene transparent PET container with lid to be inoculated with test fungal species.

Inoculation of Fungal Cultures

The indigenously isolated *Aspergillus niger* and *Saccharomyces cerevisiae* were pre-inoculated separately in 100 ml of potato dextrose broth for 3 days at 30 ºC with intermittent shacking. After the development of heavy fungal and yeast mass in broth, the liquid culture was used to inoculate the homogenized kitchen waste and whole content was allowed to stand for fermentation activity at ambient temperature for 15 days.

Quantitative estimation citric acid

The possible citric acid production by fungal activities on kitchen or domestic waste was confirmed and estimated by HPLC analysis. The liquid discharge of the fermentating substrate was collected from each container and passed through a course nylon type filter screen followed by centrifugation at 5000 rpm. The supernatant was retained was again cellulosic syringe filter dissociable unit (Moxcare) of pore size 0.22 µm and collected in a sterile tube with screw cap.

Preparation of stock standard solution

10 mg citric acid (Merk) was mixed with acetonitrile to give a stock solution of 1000ppm. From stock solutions of 0.05, 0.1, 0.15, 0.20 and 0.25 ml was taken and diluted up to 10 ml with methanol, gives standard concentrations of citric acid solution as 5, 10, 15, 20, and 25µg/ml.

Analysis of samples

10 ml of each collected fermentation extract sample was taken in 10 ml volumetric flask and filtered through Whatmann filter paper and finally samples were mixed with Acetonitril in a ration 1:1. The solution was further passed through 0.22 µmpore size cellulosic syringe filter dissociable unit (Moxcare) followed by sonication for 10 min.

For chromatographic evaluation at ambient temperature on a RP-C_{18} analytical column with a mobile phase composed of 10mM KH_2PO_4: Acetonitrile 20:80 v/v, pH 3.0 with OPA) samples were isocratically eluted at 1 ml/min. flow rate. 20 µl of prepared samples were injected intoHPLC system (Waters India Pvt. Ltd.) fitted with C_{18} (250 X 4.6 mm, 5µm from Thermo) column which was detected at a wavelength of 210nm and analysis & chromatogram done on Data Ace software.

Results & Discussion

Isolation & Characterization of *A. niger* and *S. cerevisiae*

Fungi utilize wastes to perform their metabolism while producingmany useful compounds playing significant role in soil health, plant growth and overall to keep a natural ecosystem well balanced. During biological processes, solid waste absolutely gets degraded and mineralized into simple carbonaceous and hydrogenous compounds and mineral salts due to the activity of aerobic microorganisms (Gautam, *et al*., 2011) [6]. In present study, *A. niger* and *S. cerevisiae* were observed to be present in fermentating kitchen waste which were isolated and identified according to Aneja (2003) [2] and Arumugam *et al.* (2014) [3] on the basis of culture characteristics and microscopic features of mycelium, sporangium & spores in case of *A. niger*; while growth and budding characteristics in case of *S. cerevisiae.*

Citric acid estimation

HPLC Analysis

The estimation of citric acid production by the indigenously isolated fungal strains by utilizing the required nutrition from the homogenised kitchen waste containing vegetable, fruits, and cooked food waste as mixture was done by comparing peaks of citric acid in sample extract of fermentum with the standard peaks of citric acid. The standard plot generated for citric acid as marker compound is described by table 1 and figure 1 here.

Table 1: Standard curve plot in terms of concentration of citric acid vs area under peak generated in HPLC analysis.

S.N.	Concentration (µg/ml)	Area
1.	0	0
2.	5	205.658
3.	10	412.256
4.	15	621.458
5.	20	825.56
6.	25	1012.25

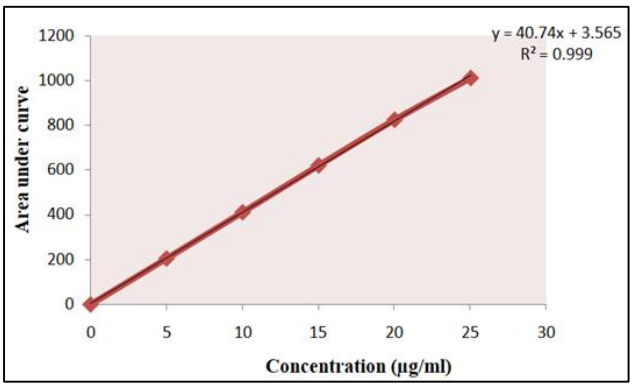

Fig 1: Graph of calibration curve for standard concentration of citric acid and standard peaks generated at 210 nm

The fermentation extracts used to detection and quantification of citric acid produced by activities of indigenous fungal

species *A. niger* and *S. service* over kitchens waste were designated as S-1 and S-2 respectively. The detection of citric acid in fermentation extracts was confirmed by matching the standard citric acid solution whose retention time in HPLC run with reference to the method validated and suggested by Kim *et al*., (2017) with some modifications in present study was estimated to be 9.732±0.3min with reference to the peak generated in chromatogram as depicted in figure 2. When compared to the standard, the percentages estimation of citric acid produced due to fermentation activity of *A. niger* on kitchen waste was observed to be 0.569%, while the activity of *S. cerevisiae* results into the yield of 0.325% citric acid in sample extract within 15 days at ambient temperature (see table 2). The chromatographic peaks can be seen in figure 3 and 4 for the citric acid production due to activity of *A. niger* and *S. cerevisiae* respectively.

Table 2: Percentage estimation of standard lactic acid and citric acid in fermentation extracts

Extract	% Citric acid Concentration
S-1	0.569
S-2	0.325

Thus in present study also it is evident that the indigenously isolated *A. niger* and *S. cerevisiae* both can utilize the organic waste of domestic origin specifically from the kitchen, as a source of nutrition and thereby acting upon it have the ability to produce several types of organic substances in addition to the citric acid with reference to the chromatogram as depicted in figure 2 and 3 indicating the presence of many other peaks of unknown compounds besides the peak matches to standard citric acid. In numerical terms, *A. niger*is ahead in citric acid production compared to the *S. cerevisiae* using waste organic matter from the kitchen.

Kareem *et al* (2010) [12] reported 6.061% yield of citric acid production by *A. niger* KS-7 utilizing pineapple peels as substrate but externally supplemented with sucrose, ammonium nitrate and methanol. Chirova, *et al*., (2016) [4] notified the substantial amount of citric acid production by *A. niger* rice extract media supplemented with 5% sucrose and 0.25% ammonium nitrate (w/v) while estimating it through titration. *S. cerevisiae* is a type of yeast species that also generally a common inhabit the fruit juices or liquid rich in carbohydrate specifically sucrose or glucose (Yadav and Tiwari, 2016) [20]. Due to the presence of the waste fruit peals in the substrate in present study also, *S. cerevisiae* cells were isolated. By utilizing variety of carbon sources, thermal aerobic & anaerobic conditions *S. cerevisiae* may produce succinic acid, citric acid, malic acid, acetic acid, lactic acid etc., (Walker and Stewart, 2016) [19]. Estimation citric acid production by using organic kitchen waste was the case of present study. Yalcin, *et al*., (2010) [21] as well as Morgunov, *et al*., (2018) [16] worked out with yeast called *Yarrowia lipolytica* for citric acid production while also most all investigators used titration as method for citric acid estimation. But in present study, the use of HPLC like sophisticated techniques yield precise results in terms of quantification.

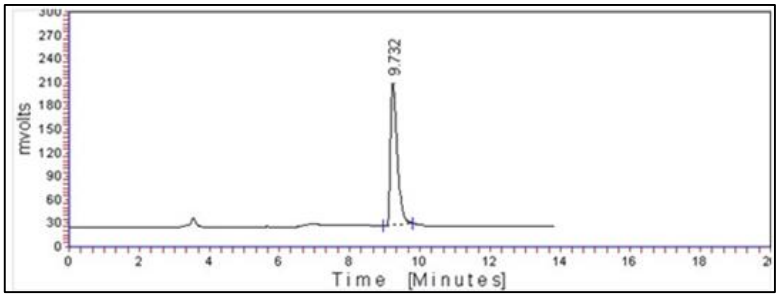

Fig 2: Chromatogram of standard peaks of citric acid generated at 210 nm

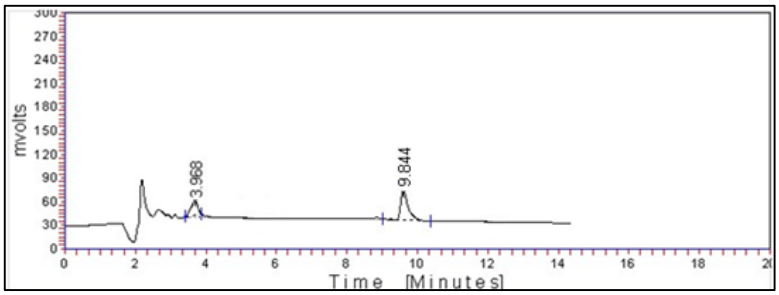

Fig 3: Chromatogram of sample extract S-1 for quantitative estimation of citric acid generated at 210 nm

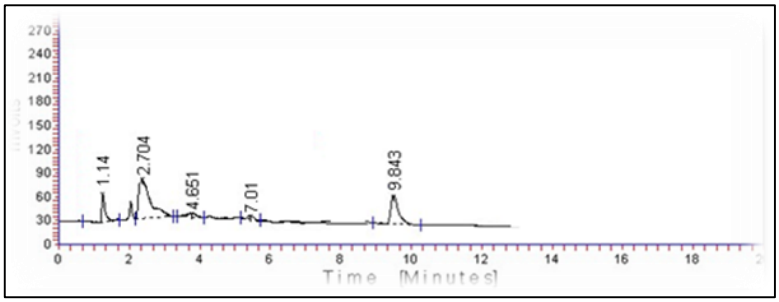

Fig 4: Chromatogram of sample extract S-2 for quantitative estimation of citric acid generated at 210 nm

Conclusions

Referring to the outcomes of experiments for the production of citric acid by the two microbial species, *A. niger* and *S. cerevicea*, the waste coming out from kitchen and domestic garbage of organic nature could be utilized as nutritional substrate for fermentation activity of microbial strains specifically fungi and yeasts. Citric acid production by *A. niger*by using homogenized mixture of kitchen waste observed to be higher compared to the yield due to activity of *S. cerevicea* on same substrate. The improvement in yield of organic acid production using such substrate could be further worked out by modifying the nutritional quality of organic substrate by addition of mineral supplements which is matter of further investigation.

References

1. Ali SR, Anwar Z, Irshad M, Mukhtar S, Warraich NT. Bio-Synthesis of Citric acid from Single and Co-Culture Based Fermentation Technology using Agro-Wastes. Journal of Radiation Research and Applied Science. 2016. 9:57-62. https://dx.doi.org/10.1016/j.jrras.2015.09.003
2. Aneja KR, Experiments in Microbiology. Plant pathology and Biotechnology. New Age International Publishers 2003. 4-978-81-224-1494-3
3. Arumugam G, Sadiq AM, Nagalingam M, Panneerselvam A. Production of invertase enzymes from *Saccharomyces cerevisiae* strain isolated from sugarcane and grape juices. European Journal of Experimental Biology. 2014; 4(5):29-32.
4. Chirova TK, Kumar A, Panwar A, Citric acid Production by *Aspergillus niger* using Different Substrates. Malaysian Journal of Microbiology. 2016; 12(3):199-204.
5. Clark DS, Bordner P, Galdrich EH, Kabler PW, Huff CB. Applied Microbiology. International Book Company, New York. 1958, 27-53.
6. Gautam SP, Bundela PS, Pandey AK, Awasthi MK, Sarsaiya S. Isolation, Identification and Cultural Optimization of Indigenous Fungal Isolates as a Potential Bioconversion Agent of Municipal Solid Waste. Annals of Environmental Science; 2011; 5:23-34.
7. Gupta GK, De S, Franco A, Balu AM, Luque R. Sustainable Biomaterials: Current Trends, Challenges and Applications. Molecules, 2015; 21(48):1-11.
8. Haq I, Ali S, Qadeer MA. Fed-batch Culture Studies during Citric Acid Fermentation by *Aspergillus niger* GCMC-7. Biologia, 2001; 45:32-37.
9. Haq I, Ali S, Qadeer MA, Iqbal J. Citric acid Production by Mutants of *Aspergillus niger* from Cane Molasses. Bioresource Technology. 2004; 93:125-130.
10. Iqbal J, Haq IU, Javed MM, Hameed U, Khan AM, Parveen N *et al.* Isolation of *Aspergillus niger* Strains from Soil and their Screening and Optimization for Enhanced Citric Acid Production Using Cane Molasses as Carbon Source. Journal of Applied Environmental and Biological Sciences. 2015; 5(4):128-137.
11. Kamzolova SV, Shishkanova NV, Morgunov IG, Finogenova TV. Oxygen Requirementsfor Growth and Citric Acid Production of *Yarrowialipolytica*. Federation of European MicrobiologicalSocieties *FEMS Yeast Research* 2003; 3:217-222.
12. Kareem SO, Akpan I, Alebiowu OO. Production of citric acid by *Aspergillus niger* using pineapple waste. Malaysian Journal of Microbiology. 2010; 6(2):161-165.
13. Kim HJ, Lee MJ, Kim HJ, Cho SK. Development of HPLC-UV Method for Detection and Quantification of Eight Organic Acids in Animal Feed. Journal of Chromatography Separation Techniques; 2017; 8:385. doi: 10.4172/2157-7064.1000385
14. Kubicek CP. The role of sugar uptake and channeling for citric acid accumulation by *Aspergillusniger*. Food Technology and Biotechnology 1998; 36:173-175.
15. Max B, Salgado JM, Rodríguez N, Cortés S, Converti A, Domínguez JM, Biotechnological Production of Citric Acid. Brazilian Journal of Microbiology; 2010; 41:862-875.
16. Morgunov IG, Kamzolova SV, Lunina JN. Citric Acid Production by *Yarrowia lipolytica* Yeast on Different Renewable Raw Materials. Fermentation; 2018, 4:36; doi:10.3390/fermentation4020036
17. Sawant O, Mahale S, Ramchandran V, Nagaraj G, Bankar A. Fungal Citric Acid Production Using Waste Materials: A Mini-Review. Journal of Microbiology Biotechnology and Food Sciences; 2018; 8(2):821-828.
18. Torres NV, Lopez JC, Rivero MG, Rojas MG. Kinetics of Growth of *Aspergillus niger* During Submerged, Agar Surface and Solid-State Fermentations. Process Biochemistry. 1998; 33:103-107.
19. Walker GM, Stewart GG. *Saccharomyces cerevisiae* in the Production of Fermented Beverages. *Beverages*; 2016; 2: 30. doi:10.3390/beverages2040030
20. Yadav SS, Tiwari A. Isolation, Identification and Characterization of *Saccharomyces cerevisiae* strains, Obtained from Sugarcane Juice and H_2S Screening from Different Areas of India. International Journal of Advanced Technology in Engineering and Science. 2016; 4(5):303-309.
21. Yalcin SK, Bozdemir MT, Ozbas ZY, Citric acid Production by Yeasts: Fermentation Conditions, Process Optimization and Strain Improvement. Current Research Technology and Education Topics in Applied Microbiology and Microbial Bio technolog, A. Mendez-Vilas (Ed.). 2010, 1374-1382. https://www.researchgate.net/publication/292475444
22. Yin X, Hyun-dong Shin H, Li J, Du G, Liu L, Chem J. Comparative Genomics and Transcriptome Analysis of *Aspergillus niger* and Metabolic Engineering for Citrate Production. Science Reporter, 2017; 7:41040. http://dx.doi.org/10.1038/srep41040
23. Yu B, Zhang X, Sun W, Xi X, Zhao N, Huang Z, Ying H. Continuous Citric acid Production in Repeated-Fed Batch Fermentation by *Aspergillus niger* Immobilized on a New Porous Foam. Journal of Biotechnology. 2018; 276-277:1-9. https://doi.org/10.1016/j.jbiotec.2018.03.015