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Citric Acid Production from Different Organic Wastes by Using *Aspergillus niger*

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Abstract
Citric acid is one of the most important organic acids which have many commercial applications. The present study is based on the production of citric acid using different organic wastes by a mold, *Aspergillus niger*. It was isolated from soil sample using serial dilution and enrichment technique. The isolate was identified on the basis of their microscopic and morphological characteristics and then used for the production of citric acid. Various agricultural wastes used for the production of citric acid were different fruit peels and bagasse. The production was studied by submerged and solid-state fermentation using *Aspergillus niger* as the inoculum. The process has several advantages such as requirement of low energy, less water and fewer chances of bacterial contamination. Citric acid production was monitored by determining the pH of the medium and titration. Production optimization was done on the basis of temperature, pH, incubation period during fermentation, inoculum size and nitrogen source. During present investigation in the process of submerged fermentation (SF) and solid state fermentation (SSF), it was noted that the orange peels and bagasse substrates (after HCl treatment) give the maximum yield of citric acid at 30°C temperature and pH 6 after seven days of incubation period in presence of ammonium sulphate as a nitrogen source. Citric acid was recovered by filtration and then by precipitation by $CaCl_2$. Free citric acid was finally recovered by treatment with H_2SO_4. These experimental designs helped to find out the more efficient and economical way to produce citric acid.

Keywords
Citric acid; *Aspergillus niger*; organic waste; submerged fermentation; solid state fermentation

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INTRODUCTION
Citric acid is a hexa carbon containing tricarboxylic acid with chemical formula $C_6H_8O_7$ which is of one of the most global commercial importance. Citric acid is colourless and in taste, it is strongly acidic. It is highly hygroscopic in moist air. Citric acid is readily soluble in water, soluble in ether and it practically is not soluble in alcohol. It is found in considerable amount in a variety of fruits and vegetables, mainly in citrus fruits. Lemons and lime contain a high concentration of citric acid as high as 8% of the dry weight of the fruit. Citric acid is biodegradable and palatable [1]. It is widely used in food and beverages, but it also has many other uses such as in cosmetics, as cleaning and chelating agent,

in pharmaceuticals and the demand for it is increasing each year [2].

Citric acid is produced by mycological fermentation on an industrial scale using crude sugar solutions such as molasses and peels of various fruits such as orange, banana, sweet lime [3]. Other substrates such as corn starch, wheat and sorghum are also used. Some substrates are used by giving pre-treatment such as bagasse [4]. In these substrates, addition of basal medium (source of nitrogen, phosphorus, potassium and other salts) is needed for better production of citric acid. The presence of trace metals in toxic concentrations can be a significant problem during the fermentation of substrate into product. The treatments include HCL treatment, hot water treatment and distilled water treatment [4].

The commercial production of citric acid is performed primarily with fungus *Aspergillus niger*, but other microorganisms such as other species of the same genus, *Penicillium janthinellum, P. restrictum, P. luteum, Trichoderma viride, Mucor piriformis, Ustulina vulgaris, Candida guilliermondii, C. lipolytica, C. intermedia, Saccharomycopsis lipolytica, Arthrobacter paraffineu*s can also be used [5,6]. The attempts have been made for overproduction of citric acid by using mutant strains of different microorganisms.

The fungus *Aspergillus niger* is grown on sugar-rich by-products to produce citric acid [7]. The starter culture is prepared which is then used as the inoculum for the production. Microbial fermentation is the most commonly applied methodology for large scale production of citric acid. There are different methods for fermentation such as solid-state fermentation (SSF) and submerged fermentation (SF) [8]. The most common method is the submerged fermentation method. The process of citric acid production is significantly influenced by various factors such as microbial strain used, substrate used, fermentation conditions employed like temperature, pH, incubation period etc. Factors such as carbon source and nitrogen source used in the fermentation process also influence the yield of the acid in the medium [1]. Carbon sources such as starch, sucrose, and maltose and the nitrogen sources such as ammonium sulfate, sodium nitrate, ammonium nitrate, yeast extract and peptone can be used. The essential trace elements are required in fermentation media such as ferrous sulphate, potassium hydrogen phosphate, and magnesium sulphate.

A large amount of agro-industrial wastes is generated from the corresponding industries which if persists in environment can lead to serious threats and there is thus the need for effective waste management. This waste in turn can be used for the production of commercially valuable products. In this study, the attempt has been made for the production of citric acid from orange, sweet lime, banana peel and bagasse.

MATERIALS AND METHODS

Isolation and identification of *Aspergillus niger*

Soil samples were collected from various locations such as onion field and botanical gardens, placed in polythene bags, closed properly and stored appropriately. 1 gram of soil sample was added to 100ml distilled water and shaken properly. Serial dilutions were prepared using distilled water. These serially diluted samples were streaked on different petri plates containing Sabouraud Dextrose Agar (SDA). Streptomycin was also added to the petri plates and was incubated at 30±1°C for 5-7 days.

Identification of the mold was done on the basis of microscopic and morphological characteristics. Wet mounting was performed using lactophenol cotton blue. This enables the fungal elements to be visualized in blue colour. Another technique used in the purpose of identification was slide culture technique. This technique allows in-situ study of the fungi and thus its identification.

Inoculum preparation

The culture of *A. niger* prepared from 5-7 days growth on SDA which was incubated at 30°C was used as inoculum. The final concentration of spores was adjusted to 1.3 x 10^8 cells per ml of inoculum. The isolate was aseptically inoculated to sucrose salt media. The media used was composed as sucrose 15%, $MgSO_4.7H_2O$ 0.025%, NH_4NO_3 0.25%, KH_2PO_4 0.1% [8]. The inoculated formulation is incubated at 30°C for 3 days on the rotary shaker.

Collection and preparation of the substrates for fermentation

Fruit peels: This material was used for submerged fermentation. Various fruits like orange, sweet lime and banana were collected from the local market and washed several times with sterilized water. The peels

were separated and dried in oven at 40-50°C and ground. These preparations were packed in transparent zip lock polythene bags and stored at room temperature until further study [3,8,9].

Bagasse: The substrate was collected from Shri. Chhatrapati Sahakari Sakhar Karkhana Ltd, Bhavaninagar. These materials were given different treatments as stated below [4]. These were referred to as pretreatments applied to the substrates. These substrates were used for solid state fermentation.

HCL treatment: Bagasse was treated with diluted 1N HCl using solid/liquid ratio of 10% (w/v). It was pretreated in water bath at 100°C for one hour. After cooling, the preparation was washed with distilled water and dried in an oven at 100°C.

Urea treatment: Bagasse was soaked with 2% (w/v) urea solution, using a solid/liquid ratio of 5% (w/v). It was pretreated in a water bath at 60°C for one hour. The preparation was cooled and washed with distilled water followed by drying in oven at 100°C.

Hot water treatment: Bagasse was mixed with distilled water using solid/liquid ratio of 10% (w/v). This was followed by boiling at 100°C for an hour. The preparation was cooled and washed with distilled water followed by drying in oven at 100°C.

Distilled water treatment: Bagasse was treated with sterile distilled water followed by drying at 100°C.

Production of citric acid

***Submerged fermentation*:**

The basal medium containing ammonium phosphate, potassium hydrogen phosphate and peptone (0.5%) was prepared and peels of different fruits were mixed into it respectively. Finely ground fruit peel (150 g) was mixed with 500 ml of basal medium [3]. Sterilization of the medium was done at 121°C for 15 minutes. It was cooled to room temperature and inoculated with the suspension of *A. niger* followed by incubation on a rotary shaker at 30°C for 5 days [10]. After the incubation period, the fermentation broth was diluted and filtered for further process.

Solid state fermentation:

The fermentation was carried out in 250 ml conical flask containing 5 grams of pretreated substrates and 10 ml of Prescott salt (NH_4NO_3, 2.23 g/l, K_2HPO_4, 1.00 g/ l and $MgSO_4.7H_2O$, 0.23 g/l) [10]. The contents of flask were mixed thoroughly and autoclaved at 121°C for 20 minutes. After cooling the flasks containing production media were inoculated with inoculum (106 spores/ml) and incubated under static condition at 30°C for 5 days [4].

Estimation of citric acid by titrimetric method

Production of acid was estimated by using simple titration method in which the fermentation broth obtained is titrated against sodium hydroxide (NaOH) [11]. The part A comprises the standardization of NaOH. It is done by titrating 0.1N NaOH from the burette with 10 ml of 0.1N succinic acid using phenolphthalein indicator. The end point of the reaction is colourless to pink. This procedure is repeated for three times and mean of the three readings is taken. The part B is the estimation of citric acid from fermented broth. 20 ml fermented broth was taken in 100 ml conical flask and 2–3 drops of phenolphthalein indicator was added to it and titration was carried out against 0.1N NaOH from burette. The end point of this reaction was colourless to pink. Same procedure was repeated for two more times and the mean of the values from these three readings was calculated.

Process optimization

The process parameters optimized and evaluated in the present study were temperature, pH, fermentation period, inoculums size and nitrogen source [1,4,10].

Effect of temperature: Different temperature ranges were selected i.e. 25°C, 30°C, 35°C, 40°C and 45°C and the fermentation media were allowed to produce citric acid under these temperature ranges. The effect of temperature was checked on the amount of citric acid produced at different temperatures.

Effect of pH: A range of pH, 4-9 was selected for fermentation process utilizing fruit peels and bagasse as substrate for citric acid production by *A. niger*.

Effect of fermentation period: The effect of fermentation period was determined on citric acid production at different intervals of 5-8 days.

Effect of inoculum level: In fruit peel fermentation, the effect of an inoculum size was determined using various concentrations ranging 1-3%.

Effect of nitrogen sources: Five different nitrogen sources were used to find the better suitedsource for citric acid production. The sources selected were peptone, ammonium sulphate, ammonium nitrate, sodium nitrate and yeast extract.

Recovery of citric acid

The method used for the recovery of citric acid used required 20.9 ml 98% H_2SO_4, 28.5 g $CaCl_2$ and 10% NaOH. Fermented broth was added with 10% NaOH till

the pH becomes 8.5-9. It is then filtered to separate the filtrate and the filtered product is taken to which $CaCl_2$ (28.5 g in 70 ml distilled water) is added. Boil this to provide the heating treatment during which trisodium and calcium chloride reacts to give tricalcium dicitrate and sodium chloride. Stir it well and then filter it, followed by washing for three times. The fluid is discarded, and solid part is taken for further process. Add H_2SO_4 (20.9 ml 18% dissolved in 200 ml water) and mix well. Tricalciumdicitrate and sulphuric acid gives citric acid and calcium sulfate. It was filtered and the filtrate was taken for evaporation to remove the water by heating. The above process was repeated again, and it was allowed for the evaporation for 2 to 6 days. After evaporation, crystals of citric acid were formed which were allowed to dry on a paper and weighed [7].

RESULTS AND DISCUSSION

Since the last few decades, citric acid production has been greatly studied and the alternatives for the process of its production are in heavy demand. The use of alternative raw materials for the production of citric acid by SF and SSF seems to be a suitable possibility. But it is important to make the choice of right type of substrate to the technique to be used e.g. fruit peels used in SF, or cane bagasse employed as substrate in SSF. It has been found that some pretreatment of substrate can significantly increase the efficiency of fermentation.

Isolation and identification of *A. niger*

Isolates were obtained from the soil samples on SDA media which were later successfully passaged on SDA media plates. As mentioned above, the organism was identified on the basis of microscopic and morphological properties. The observed characteristics of the selected isolate were those of the typical *Aspergillus niger* which revealed it to be of 1-2 mm in size, circular, black, regular margin and smooth and globular 3-4 µm conidia with septate hyphae.

Estimation of citric acid

Fermentation broth of the fruit peel batch was filtered through Whatman filter paper and estimation of citric acid was carried out titrimetrically using 0.1N NaOH and phenolphthalein indicator. In case of bagasse used as a substrate for citric acid production, 50 ml distilled water was added to fermented substrate and it was kept for shaking for an hour at 150 rpm at room temperature. It was then filtered through Whatman filter paper and estimation of citric acid was done titrimetrically [4]. The tables below show the results of the titration process using fruit peels and bagasse and the comparative analysis between them.

Table 1: Estimation of citric acid production from fruit peels and bagasse

Sr. No.	Type of fermentation process used	Substrate	Titration reading (ml)	Citric acid production by *A. niger* (mg)
1.	Submerged fermentation	Orange	14.5	0.63
2.		Sweet lime	11.5	0.50
3.		Banana	0.7	0.03
4.		Untreated	11.5	0.50
5.		HCl	18.1	0.79
6.	Solid state fermentation	Urea	13.0	0.56
7.		Boiling water	12.1	0.53

Table 2: Comparative view of estimated citric acid production using submerged fermentation and solid state fermentation

Sr. No.	Type of fermentation process used	Substrate	Titration reading (ml)	Citric acid production by *A. niger* (mg)
1.	Submerged fermentation	Orange	14.5	0.63
2.	Solid state fermentation	Bagasse (HCL treated)	18.1	0.79

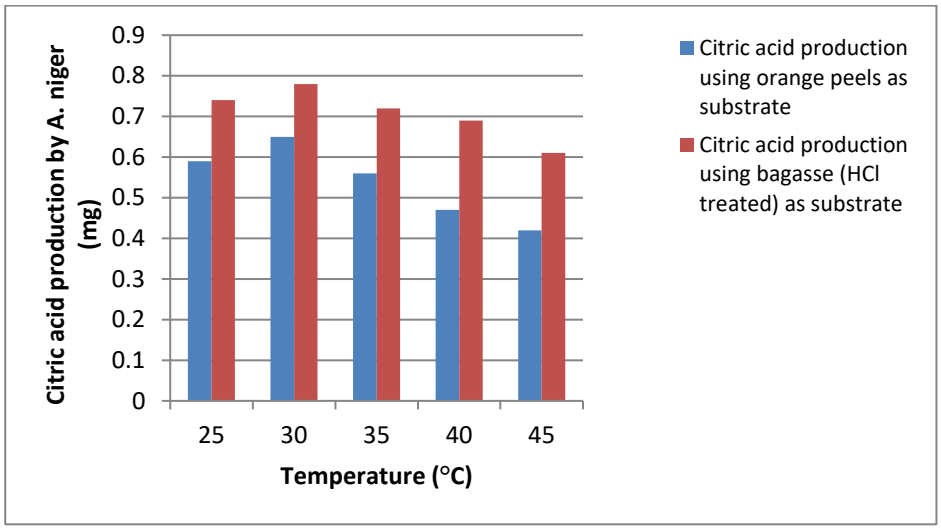
Optimization of process parameters

The process of fermentation was optimized by following the process at different parameters so as to

obtain maximum citric acid production. The data for corresponding parameter has been presented in the graph.

Effect of temperature

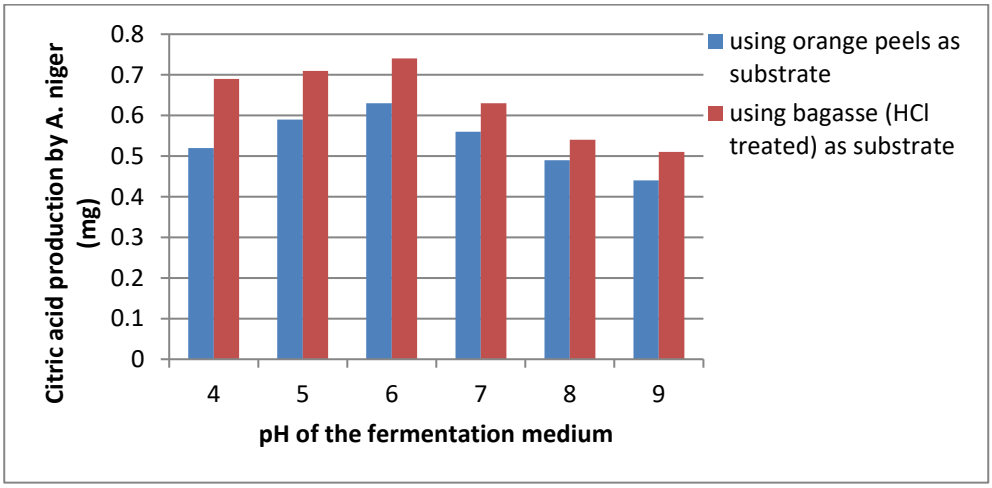
It is one of the most influencing parameters in citric acid production. Microbial growth and metabolism has significant impact of the temperature [4]. The influence of temperature on citric acid production was studied by carrying fermentation at temperature ranging from 25-45°C. As per result, maximum citric acid production was obtained at 30±1°C (0.65 mg) and (0.78 mg) for orange peels and bagasse respectively. At a temperature of 45°C, lower concentration of citric acid (0.42 mg and 0.61 mg) was produced. The decrease in citric acid production with further increase in temperature was probably due to low enzyme activity [1,10].



Graph 1: Effect of temperature on citric acid production using orange peels and bagasse (HCl treated) respectively

Effect of pH

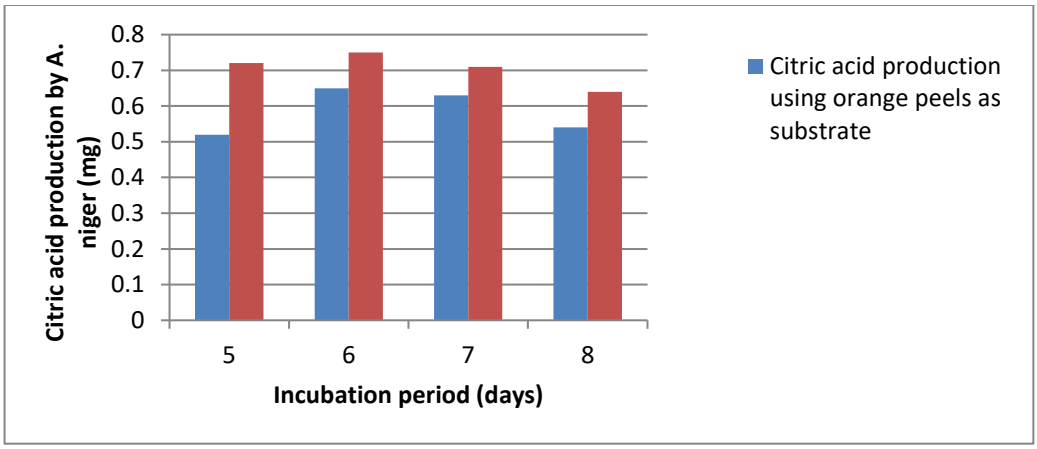
The pH of the medium is another important factor in the fermentation process. In this study, a range of initial pH 4-9 range were used for citric acid production from orange peel and bagasse respectively. After fermentation, maximum citric production (0.63 mg and 0.74 mg) was seen from orange peels and bagasse at pH 6. There was a significant increase in citric acid production as pH was increased to 6 but further increase to pH 9 showed decreased citric acid yield. It is already known that the nature of substrate influences the pH of the medium. The pH was maintained with the help of HCl and NaOH solutions [1,4].



Graph 2: Effect of temperature on citric acid production using orange peels and bagasse (HCl treated) respectively

Effect of incubation period

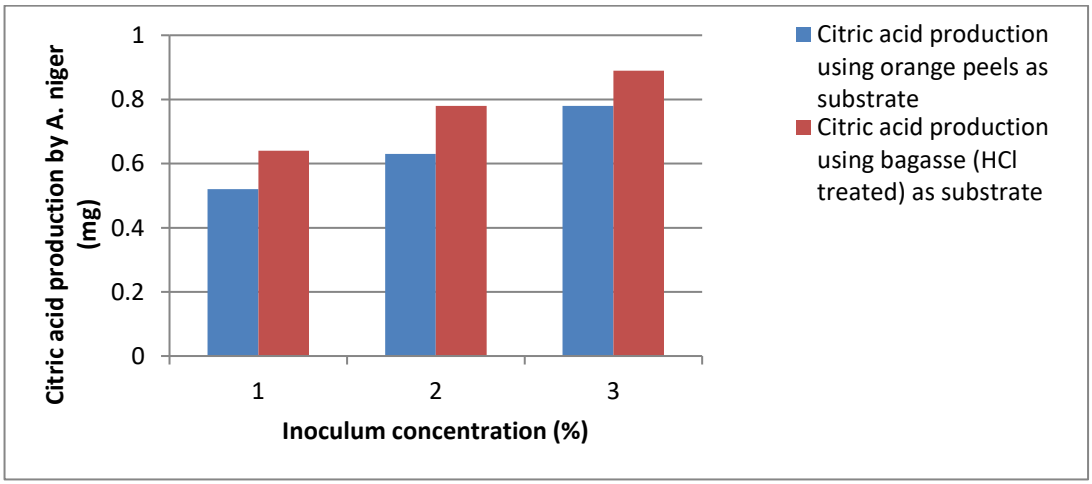
To determine the effect of incubation period, fermentation was carried out for various time periods ranging 5-8 days at 30°C having neutral pH [4]. Citric acid production increased gradually during the fermentation period and attained its maximum values at 6 days after inoculation (0.65 mg) for orange peel and maximum value 6 days after inoculation (0.75 mg). Further, increasing incubation period did not enhance production of additional quantity of citric acid. This decrease in productivity might be due to inhibitory effect of high concentration of citric acid, decreased availability of nitrogen in the fermentation medium, the age of fungi, depletion of sugar contents and decay in enzyme system responsible for citric acid biosynthesis [12,13,14].



Graph 3: Effect of incubation period on citric acid production using orange peels and bagasse (HCl treated) respectively

Effect of inoculum concentration

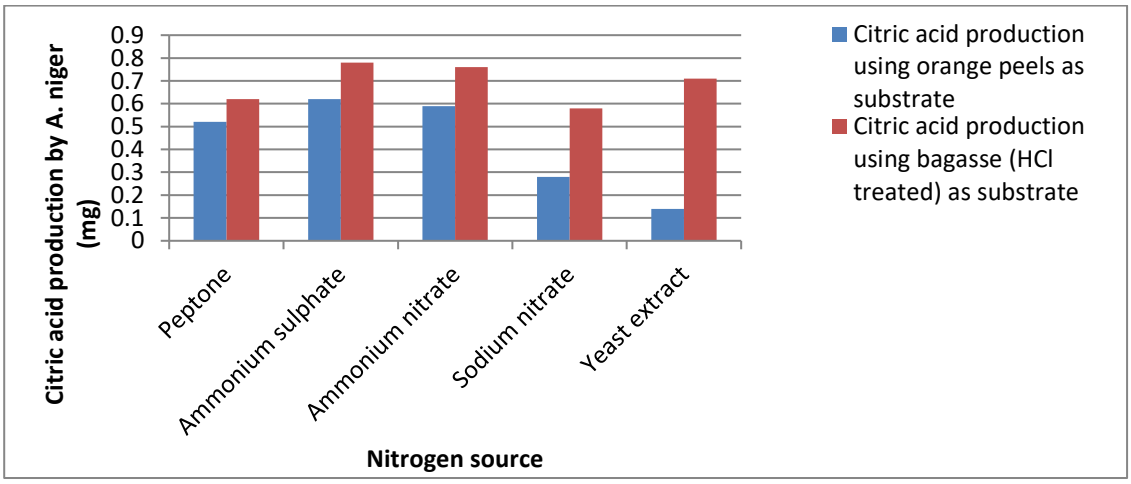
It is a major factor that affects production of citric acid in case of orange peel as well as bagasse. Production of citric acid gradually increases with increase in the concentration of inoculum 1%, 2%, 3%, as 0.52, 0.63 & 0.78 (mg) and 0.64, 0.78, & 0.89 (mg) for orange peels and bagasse respectively.



Graph 4: Effect of inoculum concentration on citric acid production using orange peels and bagasse (HCl treated) respectively

Effect of nitrogen source

The effect of various nitrogen sources was analyzed for citric acid production. In this study, five different nitrogen sources i.e. peptone, $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3 and Yeast Extract were used to check the suitability of each as a source of nitrogen in the fermentation medium for citric acid production. The maximum production of citric acid was seen in the medium containing $(NH4)_2SO_4$, while the other nitrogen sources did not yield results as good as using $(NH4)_2SO_4$. The maximum amount of citric acid production (0.62 mg and 0.78 mg) for orange peel and bagasse respectively is clearly influenced by the concentration and nature of nitrogen sources. The pH of the fermentation medium is impacted by the nitrogen source used. High concentration of nitrogen in the medium promotes fungal growth and concentration of sugar but it has serious effects on citric acid production [1,4,10].



Graph 5: Effect of nitrogen source on citric acid production using orange peels and bagasse (HCl treated) respectively

Recovery of citric acid

After following the Filtration, citric acid was precipitated using CaCl2 and the final product was obtained in the form of crystal. The crystal of citric acid formed from the fermented broth of orange and bagasse (HCl treated) is more as compared to other substrates.

CONCLUSION

Citric acid is a globally required organic acid with various industrial applications. It can be synthesized and produced at large scale by utilizing locally available agro-residue and organic wastes such as sugarcane bagasse, peels of different fruits like orange, sweet lime, banana, etc. Solid state fermentation is very effective technique to be applied for such types of industrial experiments. The problem of agro-wastes disposal and its impacts on environment can be reduced by using such wastes for the production of value-added products. By recycling and reusing waste materials for production of citric acid can be easily done by using microorganisms such as *Aspergillus niger*. The result of this study indicates that the use of sugarcane bagasse as a substrate gives higher yield of citric acid as compared to fermentation using other substrates which were used in this study. The factors such as temperature, pH, different nitrogen sources, incubation period and inoculum concentration affect the citric acid production.

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