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Sustainable production of citric acid using *Aspergillus Niger*: Optimization of fermentation parameters and utilization of orange peel

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Abstract

This research paper focuses on the production of citric acid using *Aspergillus niger*, a well-established microorganism in industrial biotechnology. The study outlines the isolation of *Aspergillus niger* from soil samples, its inoculum preparation, and the fermentation process utilizing orange peel powder as a carbon source. Detailed insights into the composition of the fermentation media, including the necessary carbon, nitrogen, minerals, and pH control, are provided to optimize citric acid yield. The downstream process for citric acid recovery, purification, and estimation using titration with sodium hydroxide is discussed. Additionally, the paper examines various fermentation conditions and strategies, contributing to an in-depth understanding of citric acid production and its potential industrial applications.

Keywords: *Aspergillus niger;* Citric acid; Inoculum preparation; Fermentation; Orange peel powder; Titration

1. Introduction

Citric acid, or 2-hydroxy-propane-1,2,3-tricarboxylic acid ($C_6H_8O_7.H_2O$), is a naturally occurring weak organic acid found in all citrus fruits. The name of this organic acid is derived from Latin word *citrus*, which refers to trees of the genus *Citrus*, including lemon trees. Citric acid in its pure form is readily soluble in water and colourless [1]. The global citric acid market in 2023 is projected increase to $3.2 billion [2]. It is estimated that over a million tons of citric acid are produced globally every year[3]. Citric acid has been designated as Safe by the World Health Organization [4]. It is solid at room temperature. Citric acid has a melting point of 153 ℃and it decomposes at higher temperatures [5].

Citric acid has a molecular weight of 210.14 g/mol and possesses three different pK_a values, at pH 3.1, 4.7 and 6.4, owing to the presence of three functional groups of carboxylic acid in its structure [6]. Chemically, citric acid shares the properties of other carboxylic acid. When heated above 175 ℃, it decomposes through the loss of carbon dioxide and water. At the present day most citric acid is produced by using fungi *Aspergillus niger* [7]. Citric acid can be derived from natural sources (e.g. lemon, lime and orange) or synthetic in 1784. The method maintained its monopoly as the only commercial source for citric acid production until the late nineteenth century, when a German botanist, Wehmer, in 1893, first observed the feasibility of obtaining citric acid through the fermentation of a sugar medium containing inorganic salts with *Penicillium glaucum* [8].

Citric acid can be isolated from citrus fruit [9,10]. Citric acid is commonly used in food and beverages, detergents, pharmaceuticals, cosmetics, toiletries and other industries [11]. The beverage and food industries account for about 75% of the world's citric acid consumption, mainly as an ingredient in carbonated drinks and an acidulant. Industrially, metal finishing and cleaning accounts for the largest use of citric acid, followed by lubricants, chelating agents, animal

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feeds and plasticizers [12,13]. ADM and Tate & Lyle have had to shut down their citric acid plants in Ireland and the UK owing to fierce competition from China [12].

Based on market trends, it is apparent that there will be a surge in the global citric acid demand. In light of this, this review discusses the biochemistry of citric acid production, raw material choices, selection of citric-acid producing microorganisms, production methods and strategies, the effects of various fermentation conditions and recovery options. The aim is to provide a thorough and comprehensive review, compared to other reviews that focus on specific areas [13,14,15]. We have used the *Aspergillus niger* strain obtained in the previous isolation done from the soi sample collected from Hindu college for the production of citric acid [16].

2. Material and methods

2.1. Isolation of microorganism

The production of citric acid by *Aspergillus niger* is a well-established process, commonly utilized in industrial biotechnology. The isolation of *Aspergillus niger* for citric acid production involves several key steps, beginning with the selection of a suitable medium for fungal growth. Sabouraud Dextrose Agar (SDA) is commonly used as it provides the ideal conditions for the growth of fungi, particularly those in the Aspergillus genus, due to its high sugar content and acidic pH. To isolate the *A. niger* strain, a sample containing fungal spores or mycelium is inoculated onto SDA plates. After incubation, individual colonies of *A. niger* are selected based on their distinctive growth patterns and morphology. The identification and confirmation of the strain are performed using the Lactophenol Cotton Blue (LPCB) mounting method, where a small portion of the fungal culture is placed on a microscope slide, stained with LPCB, and observed under a microscope. This staining allows for the clear visualization of *A. niger*'s conidiophores, conidia, and other characteristic features. Once the identity is confirmed, the isolated *A. niger* strain is then cultured for citric acid production, taking advantage of its ability to metabolize sugars and produce citric acid in industrial fermentation processes.

2.2. Inoculum preparation

Inoculum preparation using Sabouraud Dextrose Broth (SDB) involves adding main ingredients such as water, carbon sources, nitrogen sources, minerals, and other supplements in precise quantities to support the growth of *Aspergillus niger* for citric acid production. The composition of SDB typically includes water as a solvent, dextrose (a simple sugar) as the carbon source at around 40 g/L, peptone as the nitrogen source at about 10 g/L, and essential minerals like potassium, magnesium, and calcium to support enzymatic activity and metabolic functions. Other supplements, such as trace elements or vitamins, may be added depending on the specific needs of the organism. These ingredients in pure form, when combined in the correct quantities, are easy to prepare and are generally supportive of *A. niger* growth. However, the same medium may not always guarantee satisfactory growth of the organism, as factors such as an improper carbon-to-nitrogen ratio, imbalances in nutrient concentrations, or pH levels outside the optimal range can hinder growth. Furthermore, certain strains of *Aspergillus niger* may have different nutritional requirements or growth conditions, meaning that slight variations in medium composition or supplementation may be necessary to achieve the desired growth and productivity. Therefore, while the preparation of SDB is straightforward, fine-tuning the composition and conditions for each specific strain is often required to ensure optimal growth and fermentation outcomes.

2.3. Fermentation media

The composition of citric acid fermentation media is designed to support the growth of *Aspergillus niger* and optimize citric acid production. The main components of a typical citric acid fermentation medium include:

- **Carbon Source:** The primary source of carbon is usually a sugar, such as glucose, sucrose, or molasses. Glucose is commonly used in many industrial fermentations and is typically added at a concentration of 100–150 g/L. The carbon source is critical as *Aspergillus niger* metabolizes it to produce citric acid.
- **Nitrogen Source:** Nitrogen is required for the growth and protein synthesis of the microorganism. Common nitrogen sources include ammonium salts (e.g., ammonium sulfate or ammonium nitrate) or organic nitrogen sources like peptone or yeast extract. Nitrogen is usually added in the range of 1–3 g/L.
- **Minerals:** A range of essential minerals is included to support the growth of the microorganism and metabolic processes. Common minerals include:
- **Phosphates (e.g., potassium phosphate):** Serve as a source of phosphorus and help maintain pH balance. Potassium phosphate is usually added in the range of 1-3 g/L

- **Magnesium sulfate:** Acts as a cofactor for enzymes and is essential for cellular metabolism. Magnesium sulfatee is usually added in the range of 0.5–1 g/L
- **Calcium salts (e.g., calcium chloride):** Can aid in the production of citric acid and also help in maintaining proper enzyme activity. Calcium chloride is usually added in the range of 0.1-0.2 g/L
- **Trace Elements:** These may include iron, zinc, manganese, and other micronutrients that are essential for enzyme activation and overall metabolism.
- **pH Adjusters:** The pH of the medium typically needs to be maintained between 2.5 and 5.0 for optimal citric acid production. pH is controlled through the addition of acids (such as sulfuric acid) or bases (such as ammonium hydroxide), and the pH needs to be monitored throughout the fermentation process.
- **Additional Supplements:** Depending on the strain of *Aspergillus niger* and specific fermentation conditions, other growth factors, vitamins, or amino acids may be added to enhance growth and citric acid yield.

2.4. Fermentation process

The fermentation process for citric acid production using orange peel powder as a substrate involves several carefully prepared steps to create the optimal conditions for microbial growth and citric acid synthesis. First, a specific formulation of nutrients and supplements is prepared to support the growth of *Aspergillus niger*. The medium consists of 250 g of orange peel powder, which serves as the primary carbon source. This is supplemented with 10.0 g of ammonium nitrate (NH_4NO_3), a nitrogen source that promotes fungal growth and protein synthesis. Additionally, 4.0 g of potassium dihydrogen phosphate (KH_2PO_4) is added to supply phosphorus and maintain pH stability during fermentation, while 1.0 g of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) provides magnesium ions essential for enzyme activation. Calcium carbonate ($CaCO_3$), added at 2.0 g, serves as a buffer to regulate pH fluctuations, especially as citric acid accumulates in the medium.

The prepared medium is carefully transferred into a 500 mL conical flask using a sterile measuring cylinder, ensuring that the components are evenly distributed. To prevent excessive foaming during fermentation, anti-foaming agents such as vegetable oils, specifically castor oil, are added. This step is crucial to maintain the integrity of the fermentation process and prevent the loss of medium due to over-foaming. The flask is then cotton-plugged and corked with aluminium foil to maintain sterility while allowing for some airflow.

Before inoculation, the flask and medium are sterilized by autoclaving at 121 ℃ for 15 minutes. This process ensures that any contaminants present in the medium or flask are eradicated, leaving only the desired inoculum to grow. After autoclaving, the flask is allowed to cool to room temperature to prevent any thermal shock to the inoculum. Once cooled, 2 mL of inoculum, containing *Aspergillus niger* spores and mycelium, is added to the sterile medium under aseptic conditions. The inoculated flask is then incubated at temperatures ranging from 25 ℃ to 30 ℃, with a pH maintained between 2 and 5 to create an acidic environment conducive to citric acid production.

The incubation period typically lasts from 3 to 7 days, during which *Aspergillus niger* metabolizes the carbon source (orange peel powder) and produces citric acid. The pH of the medium is continuously monitored and may be adjusted if necessary to maintain the desired range, as the production of citric acid tends to acidify the medium. The temperature and pH are critical factors for optimal fungal growth and citric acid production, and they must be carefully controlled throughout the fermentation process. After the designated incubation period, the medium is harvested, and citric acid concentration is measured, with the final product being used for various industrial applications.

2.5. Downstream process

The downstream process in citric acid production involves several critical steps to separate, purify, and concentrate citric acid from the fermentation medium after the fermentation process is complete. When conducted in vitro, such as in a conical flask, the first step is harvesting the fermented broth. Once the fermentation period, typically 3 to 7 days, has elapsed, the culture is terminated, and the fungal biomass must be separated from the liquid medium containing citric acid. This separation is typically achieved through filtration, where the broth is passed through a fine mesh or using vacuum filtration to remove the solid matter, leaving behind a clarified liquid that contains the citric acid. In some cases, centrifugation may also be used to enhance the separation of the biomass from the medium.

After the solid biomass is removed, the liquid phase, which contains dissolved citric acid, is subject to acidification if necessary. This step involves adding a suitable acid, such as sulfuric acid, to adjust the pH of the medium, ensuring that the citric acid is in its protonated form for easier extraction. The next phase of the downstream process is the extraction of citric acid, which can be performed using methods like solvent extraction or liquid-liquid extraction. During this process, an organic solvent is added to the liquid medium to selectively extract citric acid, separating it from other

soluble components. The solvent containing the citric acid is then separated, leaving behind the aqueous portion of the medium.

Once extracted, the citric acid solution typically undergoes concentration to remove excess water and increase the acid's concentration. This is often done by vacuum evaporation, where the solution is heated under reduced pressure, preventing the degradation of citric acid while removing water. The concentrated solution is then subjected to purification, where crystallization techniques are applied. This involves cooling the concentrated citric acid solution or adding a suitable solvent to promote the formation of citric acid crystals. The crystals are then separated from the remaining liquid by filtration or centrifugation, resulting in a purified citric acid product.

Finally, the citric acid crystals are dried to remove any remaining moisture, usually through rotary vacuum evaporation or other drying methods. Once the citric acid is completely dried, it is subjected to quality control tests to ensure it meets the desired purity, pH, and other specifications. Once it passes these tests, the citric acid is ready for packaging and distribution for various industrial applications. This entire downstream process, from fermentation to final product purification, ensures that high-purity citric acid is obtained efficiently and is suitable for use in food, pharmaceuticals, and other industrial sectors.

2.6. Reagents

In the titration method for estimating the acid content in citric acid production, 0.1N sodium hydroxide (NaOH) is typically used as the titrant, and phenolphthalein is employed as the indicator. The NaOH solution is of known concentration (0.1N), which is crucial for accurately determining the amount of citric acid in the sample. During the titration, the NaOH is slowly added from a burette into a flask containing the citric acid solution.

2.7. Estimation of acid content using titration

The estimation of acid content in citric acid production is typically carried out using a titration method, which involves neutralizing the acid with a known concentration of a base, often sodium hydroxide (NaOH), and measuring the volume required to reach the equivalence point. A sample of the citric acid solution is first prepared, and a few drops of a suitable pH indicator, such as phenolphthalein, are added to it. The NaOH solution is then gradually added from a burette to the acid solution while stirring continuously. The endpoint of the titration is indicated by a color change in the indicator, signaling that all the citric acid has reacted with the base.

Phenolphthalein, a pH indicator, is used to signal the endpoint of the titration. It is colorless in acidic solutions and turns pink as the solution becomes slightly alkaline, indicating that all the citric acid has reacted with the NaOH. The transition occurs when the pH of the solution reaches around 8.3, which corresponds to the neutralization of the citric acid by NaOH. The volume of NaOH required to reach this endpoint allows for the calculation of the citric acid concentration in the sample using the titration formula mentioned earlier. This method is highly reliable and commonly used in laboratory analysis for acid content determination in citric acid production.

To estimate the citric acid content in a fermented medium using titration, the first step involves preparing a sodium hydroxide (NaOH) solution, which will act as the titrant. Start by dissolving 4 grams of NaOH in 100 mL of distilled water to create a concentrated NaOH solution. To achieve the desired 0.1N concentration, dilute the solution further by adding distilled water until the final volume reaches 1 liter. This dilution ensures that the NaOH is at the appropriate concentration for the titration, and it is important to ensure the NaOH is fully dissolved and the solution is homogeneous.

Once the NaOH solution is prepared, the next step is to prepare the fermented medium that contains citric acid. The medium should be filtered through cheesecloth to remove any solid particles and impurities. This filtration process ensures that only the liquid portion containing the dissolved citric acid is used for the titration. After filtration, transfer a measured volume of the filtrate (typically 10 mL) into a clean conical flask. To help identify the endpoint of the titration, add 2-3 drops of phenolphthalein indicator to the flask. Phenolphthalein is ideal for this titration as it is colorless in acidic solutions and turns faint pink as the solution becomes slightly alkaline, signaling that the acid is neutralized.

Next, fill a burette with the prepared 0.1N NaOH solution. Before starting the titration, ensure that the burette is clean and free from air bubbles, as this can affect the accuracy of the titration. Record the initial volume of NaOH in the burette. Then, begin the titration by slowly adding NaOH from the burette to the conical flask containing the citric acid solution. As NaOH is added, the solution will start to neutralize the citric acid. The phenolphthalein will remain colorless until the endpoint, where a faint pink color will appear and persist for about 30 seconds, indicating that the titration is complete.

It is recommended to perform the titration at least two more times to ensure accuracy and consistency of the results. After completing the titrations, record the volume of NaOH used in each trial by subtracting the initial volume from the final volume. The average volume of NaOH used in the titrations can then be used to calculate the concentration of citric acid in the sample. The formula for this calculation is:

$$\text{PERCENTAGE OF CITRIC ACID} = \left[\frac{N \times V \times 6.4}{W \times 100}\right] \times 100$$

Where, N = Normality of NaOH
V = Volume of burette reading
W = Volume of the sample

3. Results and Discussion

The process involves growing *Aspergillus niger* on Sabouraud Dextrose Agar (SDA) plates to isolate the microorganism, followed by the preparation of an inoculum in Sabouraud Dextrose Broth (SDB). The medium for fermentation was composed with orange peel powder, ammonium nitrate (NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), calcium carbonate ($CaCO_3$), and anti-foaming agents. The fermentation was incubated at temperatures between 25 °C to 30 °Cto facilitate optimal growth for 3-7 days and citric acid production by *Aspergillus niger*.

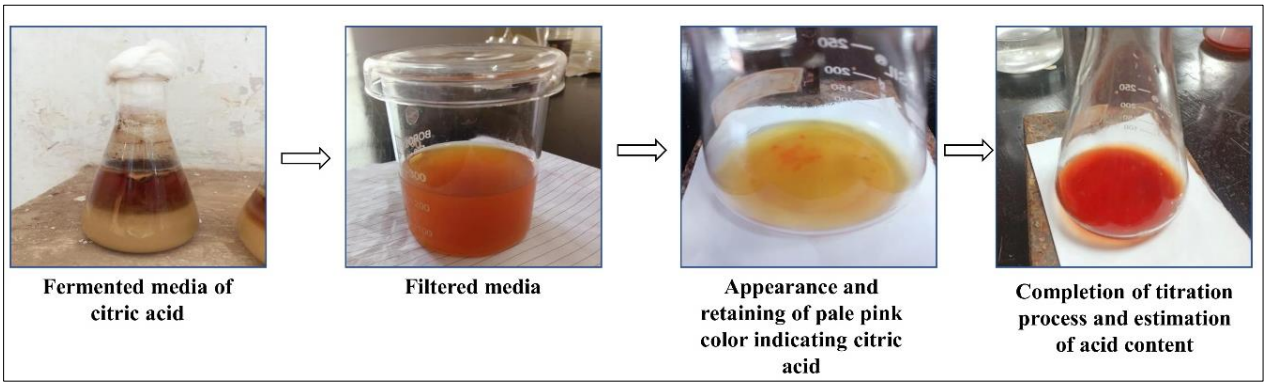

Figure Estimation of alcoholic percentage in citric acid

This titration method allows for accurate determination of the citric acid content in the fermented medium, ensuring reliable and consistent results.

Table Titration values

S.no	Initial burette reading	Final burette reading	Volume of naoh (sodium hydroxide)
1.	0	2.5	10
2.	0	2.6	10
3.	0	2.7	10

3.1. Calculation

$$\text{PERCENTAGE OF CITRIC ACID} = \left[\frac{N \times V \times 6.4}{W \times 100} \right] \times 100$$

$$= \left[\frac{0.1 \times 2.5 \times 6.4}{100} \right] \times 100$$

$$= 1.6$$

After fermentation in vitro conditions in conical flasks, the citric acid content was estimated through titration using 0.1N sodium hydroxide (NaOH) and phenolphthalein as an indicator. The titration results indicated a citric acid concentration of 1.6%, confirming successful citric acid production. This data provides insight into the fermentation efficiency and allows for further optimization of the process to potentially increase citric acid yield.

4. Conclusion

The study successfully demonstrated the production of citric acid using *Aspergillus niger* through a well-established fermentation process, with orange peel powder as the primary carbon source. The titration method, employing 0.1N sodium hydroxide and phenolphthalein indicator, confirmed a citric acid concentration of 1.6%, validating the efficiency of the fermentation process under the described conditions. The results highlight the potential for utilizing agricultural waste, such as orange peel, as a cost-effective and sustainable substrate for industrial citric acid production. This process can be further optimized by fine-tuning fermentation parameters, medium composition, and recovery techniques to enhance yield and scalability, contributing to the growing global demand for citric acid in various industries.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest in this work.

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References

[1] Angumeenal A, Kamalakannan P, Prabhu HJ, Venkappayya D. 2003. Effect of transition metal cations on the production of citric acid using mixed cultures of *Aspergillus niger* and Candida gulliermondii. J Indian Chem Soc. 80:903–906.

[2] Behera, B.C.; Mishra, R.; Mohapatra, S. Microbial citric acid: Production, properties, application, and future perspectives. Food Front. 2021, 2, 62–76.

[3] Hu, W.; Li, W.; Yang, H.; Chen, J. Current strategies and future prospects for enhancing microbial production of citric acid. Appl. Microbiol. Biotechnol. 2019, 103, 201–209.

[4] West TP. Citric Acid Production by *Aspergillus niger* Using Solid-State Fermentation of Agricultural Processing Coproducts. Applied Biosciences. 2023; 2(1):1-13. https://doi.org/10.3390/applbiosci2010001.

[5] Show, P. L., Oladele, K. O., Siew, Q. Y., Aziz Zakry, F. A., Lan, J. C. W., & Ling, T. C. (2015). Overview of citric acid production from *Aspergillus niger*. Frontiers in Life Science, 8(3), 271–283. https://doi.org/10.1080/21553769.2015.1033653

[6] Papagianni M. 2007. Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol Adv. 25:244–263.

[7] Pratik Bezalwar, Ashok V. Gomashe, Harshal M.Sanap and Pranita A. Gulhane. Production and Optimization of Citric Acid by *Aspergillus niger* using Fruit Pulp Waste. ISSN: 2319-7706 Volume 2 Number 10 (2013) pp. 347-352.

[8] Soccol CR, Vandenberghe LPS, Rodrigues C, Pandey A. 2006. New perspectives for citric acid production and application. Food Technology & Biotechnology. 44:141–149.

[9] Angumeenal, A.R.; Venkappayya, D. An overview of citric acid production. LWT—Food Sci. Technol. 2013, 50, 367–370.

[10] Lende, A.V.; Karemore, H.; Umekar, M.J. Review on production of citric acid by fermentation technology. GSC Biol. Pharm. Sci. 2021, 17, 85–93.

[11] Majumder L, Khalil I, Munshi MK, Alam K, Rashid H, Begum R, Alam N. 2010. Citric acid production by *Aspergillus niger* using molasses and pumpkin as substrates. European Journal of Biological Sciences. 2:01–08.

[12] Bauweleers HMK, Groeseneken DR, Van Peij NNME. 2014. Genes useful for the industrial production of citric acid. In: Google Patents.

[13] Pazouki M, Panda T. 1998. Recovery of citric acid–a review. Bioprocess Eng. 19:435–439.

[14] Anastassiadis S, Morgunov IG, Kamzolova SV, Finogenova TV. 2008. Citric acid production patent review. Recent Patents on Biotechnology. 2:107–123.

[15] Dhillon GS, Brar SK, Verma M, Tyagi RD. 2011a. Recent advances in citric acid bio-production and recovery. Food Bioprocess Technol. 4:505–529.

[16] Tallapalli Mohana, Mandugula Hemachandra Chari, Yekula Anupriya & Devandla Anitha, Isolation and Identification of Aspergillus Species from Soil Sample, Hindu College, Guntur, ISSN: 2456-883X, DOI: https://doi.org/10.38177/ajast.2023.7201