RESEARCH ARTICLE

HARNESSING CITRUS RHIZOSPHERE ISOLATES FOR CITRIC ACID PRODUCTION BY *ASPERGILLUS NIGER*

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Abstract:

Aspergillus niger, which can produce yields of as much as 150 g/L through strain engineering, executes more than 90% of the microbial fermentation in the industrial production of citric acid. This paper reviews key operational parameters, recovery methods, fermentation technologies (submerged fermentation), and metabolic processes that regulate the biosynthesis of citric acid. Recent improvements in genetic engineering and optimal fermentation conditions have substantially boosted production efficiency.

Keywords: Microbial Fermentation, Acid Production, Rhizosphere Isolation, Submerged fermentation, Sources, Industrial Technology, Down Stream Processing, Recovery, Quantification.

Introduction:

Citric Acid (tricarboxylic acid) is a naturally occurring organic acid commonly found in citrus fruits, or in citrus rich soil regions. As a key intermediate in the tricarboxylic acid (TCA) cycle, citric acid plays an important role in cellular metabolism across all living organisms.[1] Global production of citric acid has reached approximately 2 million tons in 2020, with projections indicating growth to approximately 3 million tons by 2026, representing an annual growth rate of 3.5-4.0% in demand and consumption.[2]

The widespread industrial applications of citric acid span multiple sectors: from being utilized in the food and beverage industries as an acidulant, flavour enhancer, preservative, and antioxidant to detergents and cleaning products as a chelating agent and phosphate replacement; as well as in pharmaceuticals, cosmetics, agriculture, and textile industries.[3]

Historical Development:

The pivotal breakthrough in citric acid production occurred in 1893 when German botanist Carl Wehmer first observed that *Penicillium glaucum* could produce citric acid through fermentation of sugar media containing inorganic salts but encountered significant challenges, that included contamination problems and longer fermentation times.[4]

The foundation for modern industrial citric acid production was established in 1917 by James Currie, who discovered that *Aspergillus niger* could produce citric acid under controlled conditions. Currie's groundbreaking findings demonstrated that *A. niger* could thrive at low pH values and that high sugar concentrations favourably influenced citric acid production, leading Pfizer Inc. to establish the first commercial citric acid production facility in year 1923.[5]

***Aspergillus niger*: Industrial Backbone**

Among the diverse microorganisms capable of producing citric acid—*Aspergillus niger* has remained the organism of choice for commercial production. The selectivity of *A. niger* arises from several key advantages:

- Ease of handling and cultivation
- Ability to ferment a wide variety of inexpensive raw materials
- High production yields
- Capability to grow at low pH, which minimizes contamination risks and inhibits the formation of unwanted by products such as oxalic and gluconic acids
- Well-developed homologous and heterologous extracellular enzymatic systems[6]

Aspergillus. niger has been officially recognized as safe for human use. Industrial strains of *A. niger* are capable to produce high citric acid levels which have undergone genetic modifications through mutagenesis techniques, including physical methods (gamma and UV radiation, X-rays) and chemical mutagens. These strain improvement programs have resulted in hyperproducer strains with significantly enhanced citric acid secretion capabilities.[7]

Production Methods:

Chemical synthesis of citric acid was first achieved in 1880 by Grimaux and Adam, who synthesized it using glycerol as the starting material. Other chemical processes have been discovered since then. However, despite these achievements, chemical synthesis methods have proven economically less efficient compared to fermentation processes. The high production costs, complex reaction pathways, and lower yields made chemical synthesis impractical for industrial-scale citric acid production.[4]

The Microbial Fermentation method offers numerous advantages over extraction and chemical synthesis including simple and stable operations, lower energy consumption, less complex control systems and lower technical skill requirements. Additionally, fermentation processes can utilize a broad range of inexpensive raw material sources such as molasses, glucose syrups, starch hydrolysates, and agro-industrial wastes.[1]

Modern fermentation technology primarily employs two main approaches: submerged fermentation (80% of global production) and surface fermentation (20% of global production). *Aspergillus niger* remains the dominant microorganism due to its high citric acid production capacity, easy handling, ability to ferment various substrates, and high yields.[2]

Methodology:

The worldwide production of citric acid, a significant commercial product, is based on effective microbial fermentation. Isolating high-yielding microbial strains is the first step in the simplified

production process. After fermentation, several treatments are used to remove and purify the citric acid from the culture broth before it is processed into its final form that can be marketed. Various steps involved during the production are enlisted below:

1. Isolation and Screening: For isolation of citric acid-producing organism, natural reservoirs such as soils, decaying fruits or compost are sampled and inoculated onto the solid culture media containing $CaCO_3$ (Calcium Carbonate) along with carbon source like glucose or sucrose. These organisms can be identified by formation of clear halazones around them on GYC Agar (Glucose Yeast Calcium Carbonate Agar). This Clearing is due to acid-mediated solubilization of $CaCO_3$, where secreted citrate reacts with calcium carbonate to form calcium citrate releasing Carbon Dioxide, thereby dissolving opaque precipitate and producing transparent zones around the colonies.[8]

$$CaCO_3 + C_6H_8O_7 \rightarrow Ca_3(C_6H_5O_7)_2 + H_2O + CO_2 \uparrow$$

2. Fermentation: Submerged fermentation (SmF) is the most widely used technique for global citric acid production. This method involves cultivating *A. niger* in liquid medium with controlled aeration, agitation, pH, and temperature. SmF offers several advantages: higher productivity and yields, lower labour costs, reduced contamination risk, better control of process conditions, and wider substrate range. However, it requires rigorous control systems, and management of foam formation through antifoaming agents. Fermentation typically concludes within 5-12 days depending on process conditions.[3]

Table 1: Fermentation Media Composition[9]

Component	Concentration (g/L)	Purpose
Sucrose	100–500	• Primary carbon source • Promotes overflow metabolism for acid buildup
Ammonium Nitrate (NH_4NO_3)	2–3	• Nitrogen source • Limited nitrogen favors citric acid production
Potassium dihydrogen Phosphate (KH_2PO_4)	0.5–1.0	• Supplies phosphate • Essential for ATP production and acts as a buffer
Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$)	0.2–0.5	• Provides Mg^{2+} ions • Vital as an enzyme co-factor in various pathways
Trace Metal Solution	< 0.1	• Supplies micro-nutrients • Iron-limiting condition favors acid yield
Methanol	40 mL/L	• Increases permeability • Enhances citric acid yield

3. Product Recovery: Precipitation is the most used method for industrial citric acid recovery. The classical process involves adding calcium hydroxide (lime) to the fermentation broth to precipitate tricalcium citrate tetrahydrate at pH 6.0-7.5. The precipitate is then filtered and treated with sulfuric acid to form citric acid and gypsum (calcium sulphate). Crystallization produces citric acid monohydrate at temperatures below 36.6°C or anhydrous citric acid above 36.6°C. While effective, this method generates substantial waste, including gypsum and microorganism residues.[10]

4. Quantification: Quantification of citric acid Produced by isolated strains is commonly carried out

by acid-base titration. After fermentation, the culture broth is filtered to remove microbial biomass. And the filtrate is titrated with a standardized sodium hydroxide solution. Using phenolphthalein as an indicator, the appearance of faint pink colour at the end point indicates neutralization of acid. Based on the volume of NaOH consumed, the concentration of citric acid in the sample can be calculated. This straightforward method provides a reliable means for estimating citric acid production by microbial isolates.[11]

$$C_6H_8O_7 + 3\ NaOH \rightarrow (C_6H_5O_7)\ Na_3 + 3\ H_2O$$

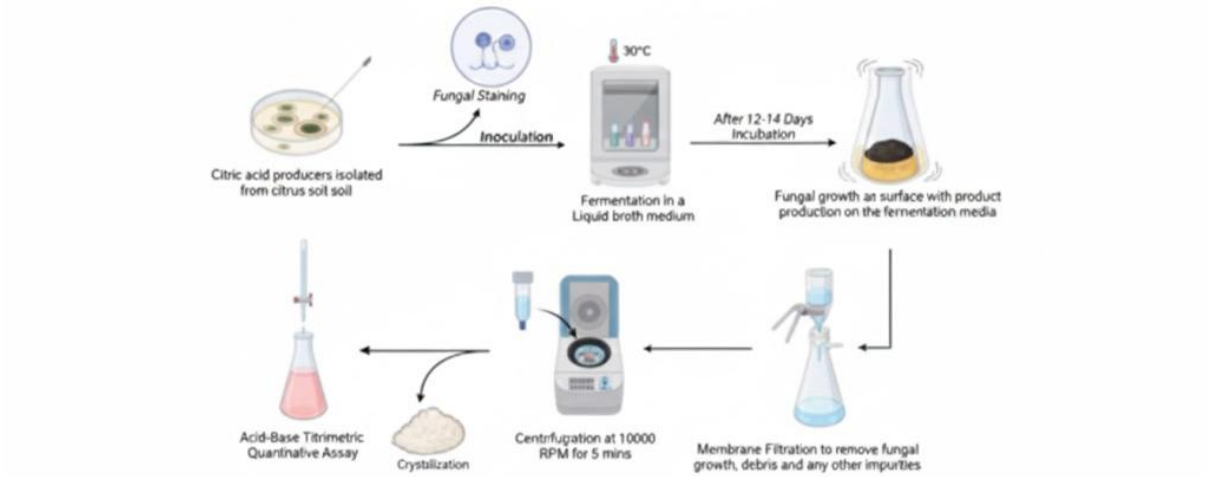

Figure 1: Steps of Citric Acid Production[9][12]

Protocol:

This serial dilution table represents a stepwise dilution process used to reduce the concentration of microorganisms present in the original soil sample. Each of the five tubes (Tube 1 to Tube 5) contains 0.5 g of sample mixed with 4.5 mL of sterile saline, producing dilution factors ranging from 10^{-1} to 10^{-5}. By diluting the sample progressively (from Tube 1 at 10^{-1} to Tube 5 at 10^{-5}), microbial load is systematically lowered, making it easier to obtain distinct and countable colonies when plated on agar media. The final tube marked "Discard" is not used further, ensuring that only the appropriate dilution range is selected for isolation and enumeration of microorganisms. This method improves accuracy and reliability during microbial screening and isolation.

Table 2: Serial Dilution

Tube No	**1**	**2**	**3**	**4**	**5**	
Dilution Factors	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Discard 0.5ml
Sample (g)	0.5	0.5	0.5	0.5	0.5	
Saline Solution (ml)	4.5	4.5	4.5	4.5	4.5	

Serial Dilution Observations

This table summarizes the microbial growth observed after plating different dilution factors of the soil sample. At a dilution factor of 10^{-3}, approximately 9 well-separated colonies were recorded, indicating moderate microbial density. The 10^{-4} dilution yielded around 5 colonies, showing further reduction in microbial load and improved isolation clarity. At the highest dilution of 10^{-5}, only about 3 colonies were visible, representing a significantly diluted sample useful for obtaining pure, countable

isolates. These progressive reductions in colony numbers confirm the effectiveness of the serial dilution technique in producing distinct and manageable colony growth suitable for screening citric acid–producing fungi.

Table 3: Observations for Serial Dilutions

Sr. No.	Dilution Factor	Observation	Number of Countable Colonies
1	10^{-3}		**~ 9**
2	10^{-4}		**~5**
3	10^{-5}		**~ 3**

Sub-Culturing – Description

Sub-culturing confirmed the successful isolation and maintenance of *Aspergillus niger*. The Sabouraud's agar plate showed characteristic black, powdery, radial fungal colonies typical of *A. niger*. Transfer to a Sabouraud's agar slant resulted in a stable, well-defined growth suitable for short-term storage and further experimental use. A pure culture inoculum was then prepared by suspending isolated *A. niger* mycelium in sterile saline, creating a uniform fungal suspension. This ensured that a contamination-free, morphologically confirmed culture was available for use in subsequent fermentation experiments.

Sabouraud's agar plate containing *Aspergillus niger*

Sabouraud's agar Slant containing *Aspergillus niger*

Pure Culture Inoculum for Fermentation (*Aspergillus niger* in Sterile Saline)

Quantitative Assay:

The titration data represent the quantitative estimation of citric acid in the fermented broth. Three successive titrations were performed using 0.1 N NaOH, and the initial and final burette readings were recorded for each trial. The titration endpoints were indicated by the appearance of a faint, persistent pink color using phenolphthalein as the indicator. The average burette reading, calculated from all trials, resulted in a Constant Burette Reading (CBR) of 7.3 mL. This consistent value confirms the reliability of the titration procedure and provides a basis for calculating the final citric acid concentration in the sample.

Table 4: Observations for Titrations

Sr. No.	Initial Burette Reading (ml)	Final Burette Reading (ml)	Average Reading (ml)
1	0.0	7.0	**7.3**
2	7.0	14.1	
3	14.1	22.0	

Results and Discussion:

The present study successfully demonstrated the isolation, identification, fermentation, and quantification of citric acid produced by *Aspergillus niger*. After 48 hours of incubation, black pigmented hyphal colonies were observed on both Sabouraud's agar and GYC agar plates. The presence of clear halo zones around colonies on GYC agar confirmed $CaCO_3$ solubilization, indicating organic acid production. This visual evidence is a well-established screening criterion for citric acid–producing microorganisms, particularly strains belonging to the genus *Aspergillus*[14].

Morphological identification using Lactophenol Cotton Blue staining at 40× and 100× magnification revealed septate hyphae and a swollen vesicle bearing chains of conidia, confirming the organism as *Aspergillus niger*. Similar morphological characteristics have been documented in studies where *A. niger* was used for industrial-scale citric acid production[15].

During submerged fermentation, dense black growth with extensive hyphal formation was visible after 14 days. The turbid appearance of the broth indicated the accumulation of metabolic products. *A. niger* is known to efficiently convert carbohydrate substrates into citric acid under appropriate growth conditions, as previously reported by Kareem (2017) [16].

Upon vacuum membrane filtration and centrifugation, the supernatant containing the crude citric acid was separated and purified using calcium carbonate followed by dilute sulfuric acid treatment. Product recovery through precipitation and acid re-liberation remains one of the simplest and most economical methods of citric acid extraction[17].

Quantitative titration established that 100 mL of fermented broth contained 5.13% citric acid. This concentration aligns with earlier findings where optimized strains of *A. niger* produced between 3% and 8% citric acid under laboratory fermentation conditions[18]. The result confirms that *A. niger* is a highly promising and efficient microbial candidate that can be exploited for commercial and industrial-scale production of citric acid due to its high yield, easy cultivation, and ability to utilize inexpensive substrates.

Conclusion:

This work successfully demonstrates the potential of harnessing rhizosphere isolates from citrus-rich soils for citric acid production. The isolation protocol using GYC agar proved effective for screening acid-producing fungi, with the formation of clear halo zones acting as a primary indicator of citrate production. The isolated organism was morphologically identified as *Aspergillus niger*, confirming it as a potential candidate for microbial fermentation.

The submerged fermentation process, carried out under optimized conditions of low pH and specific nutrient concentrations, resulted in the successful production of citric acid. The recovery method involving precipitation with calcium hydroxide and treatment with sulfuric acid effectively yielded purified citric acid.

The Quantitative analysis through acid-base titration estimated the citric acid concentration in the fermented broth to be 5.13 gm %, proving the efficiency of the isolated *Aspergillus niger* strain.

In conclusion, the integration of isolation, fermentation, recovery, and quantification steps outlined in this work provides a reliable and replicable process for citric acid production. The significant yield achieved highlights *Aspergillus niger's* potential to act as a powerful agent for industrial citrate production. Future efforts should focus on further strain improvement and the optimization of parameters using low-cost, agro-industrial wastes as substrates to enhance yield for large-scale industrial applications.

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