Citric Acid and Gypsum Production from Wastes Biomass and Industrial By-products Using Microbial Fermentation



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DECLARATION CERTIFICATE

This is to certify that Examination Roll No. 01, Reg. No. 013, M.Phil. Session-2022-23, Department of Applied Chemistry and Chemical Engineering, University of Dhaka, Dhaka, Bangladesh, has carried out his M.Phil. thesis on **"Citric Acid and Gypsum Production from Wastes Biomass and Industrial By-products Using Microbial Fermentation"** under our supervision.

He has successfully completed his research work and is ready to present his dissertation, which is required to fulfill the M.Phil. degree. The candidate did the work submitted here and no part of this thesis has been submitted elsewhere for any degree or diploma. The thesis does not include any previously published or written works by other authors than those to which it makes reference.

We have reviewed the final draft of thesis and urge you to submit it for the Master of philosophy (M.Phil.) in Applied Chemistry and Chemical Engineering, University of Dhaka

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Abbreviations

%	Percentage
A.niger	Aspergillus niger
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
CA	Citric Acid
FAD	Flavin Adenine Dinucleotide
Mg	Magnesium
mg	Milligram
mg/ml	milligram/Milliliter
mL	Milliliter
Mn	Manganese
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
O.D	Optical Density
^{O}C	Degree Celsius
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PFK-1	Phosphofructokinase 1
PFK-2	Phosphofructokinase 2
RT	Room Temperature
SEM	Standard Error Mean
SEM	Solvent Extraction Method
SF	Surface Fermentation
SmF	Submerged Fermentation
SSF	Solid-state Fermentation
TCA	Tricarboxylic Acid
UV	Ultraviolet
µg	Microgram

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Abstract

The economic potential of microbial citric acid (CA) production is substantial due to its wide-ranging applications in the beverage, food, detergent, cosmetic, and pharmaceutical industries. In Bangladesh, the majority of CA demand is met through imports, while annual consumption is increasing steadily at a rate of 3.5–4%, underscoring the need for sustainable domestic manufacturing alternatives. Large-scale production must therefore adopt eco-friendly processes that utilize readily available and inexpensive agro-industrial byproducts, while ensuring high production yields.

CA is predominantly produced via microbial fermentation using *Aspergillus niger*. The present study aimed to optimize citric acid production from sugarcane molasses employing *A. niger* (F-81) strain under submerged fermentation, with gypsum as a byproduct. The concentration of CA in the fermentation broth was determined using the Marier–Boulet method. Maximum production was observed in the pretreated molasses sample (12.49 mg/ml), compared to the non-pretreated counterpart (7.3 mg/ml), under identical fermentation conditions (10 days, 2% inoculum size, and an initial pH of 5.6). These findings demonstrate the significance of substrate pretreatment in enhancing yield. The production rate of CA is influenced by multiple factors, including regulatory genes of *A. niger*, pH, temperature, inoculum size, substrate concentration, and the presence of heavy metals, which warrant further investigation.

In addition, citric acid biosynthesis was evaluated using six indigenous fungal strains (FS1–FS6) isolated from soil, cultured under standardized fermentation conditions with 5% molasses as the carbon source, at an initial pH of 6.0 and ambient temperature (30^{o}C). The produced citric acid was isolated through precipitation method using calcium hydroxide and sulfuric acid, in which gypsum was found as a by-product. Citric acid and gypsum production was confirmed via FTIR analysis. Among the isolates, FS2 exhibited the highest and most consistent productivity, yielding 0.93 mg/ml 1.09 mg/ml and 1.11 mg/ml after 5 days, 7 days and 10 days of incubation, respectively. Moderate production was observed in FS5 (0.55–0.61 mg/ml) and FS3 (0.51–0.61 mg/ml), whereas FS1 (0.41–0.60 mg/ml) and FS4 (0.32–0.40 mg/ml) displayed comparatively lower yields. FS6 was the least productive, reaching only 0.14 mg/ml by day 10. Overall, the results identify *A. niger* (F-81) strain as the most promising candidate for industrial-scale citric acid production under the tested conditions in Bangladesh.

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CHAPTER 1.

INTRODUCTION

1.1 Background

Citric acid is a frequent metabolite, which found both plants and animal. It is commonly utilized as a preservative, a chelating agent, as well as a sequestering in different industries. In addition, it is used in food and pharmaceutical along with in textile and metallic industries as well. It has high demand due to its antimicrobial properties in food products, is anticipated to be a major factor in the industry's expansion. Bangladesh is a least developed country, therefore, a huge amount of citric acid imports per year to meet the demand of industrial goal [1]. According to the World Integrated Trade Solution report in 2023, Bangladesh imported more than 10,000 metric tons of citric acid from different countries; among them, China was the highest exporter (Figure 1.1). Globally, the demand of citric acid increases significantly and the market value of it was anticipated at USD 3.78 billion by 2022, and is expected to increase at a compound annual growth rate (CAGR) of 4.0% from 2023 to 2030, reaching USD 5.17 billion (Figure 1.2) [2]. Due to its long shelf life and suitability for dry-blend formulations, anhydrous citric acid is expected to hold the biggest product category share in 2025, at 56.2%. Because of its versatility as an acidulated, preservative, and taste enhancer, the food and beverage sector will hold a 65.4% market share in the end-use category. It is the most multipurpose used organic acid in food (60%) and pharmaceuticals (10%) industries. Citric acid has numerous other applications [3], with fermentation being the primary method of production. Almost all of Bangladesh, citric acid demand is met through imports. Every year, its consumption is steadily increasing (3.5-4%), which indicating the need for alternatives production.

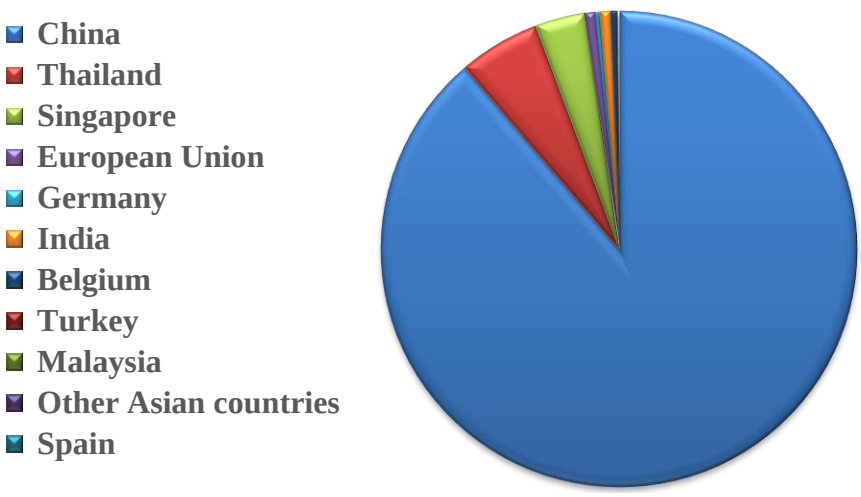


Figure 1.1 *Citric Acid Imports Country of Bangladesh* [4].

On the other hand, Gypsum, mainly composed of calcium sulfate dihydrate, is widely used in construction, agriculture, and industry for its beneficial properties. While naturally occurring gypsum is common in products like drywall and plaster, industrial processes also generate byproduct gypsum such as desulfurization gypsum and phosphogypsum. These byproducts, often high in impurities and produced in large volumes, are underutilized and pose environmental concerns. Although some are used in cement-related applications, the broader potential for industrial gypsum reuse remains limited[5].

According to the business company report 2025 (Figure 1.3), the gypsum market is rapidly growing, projected to rise from $41.25 billion in 2024 to $77.44 billion by 2029, with a strong CAGR of 13–15%.

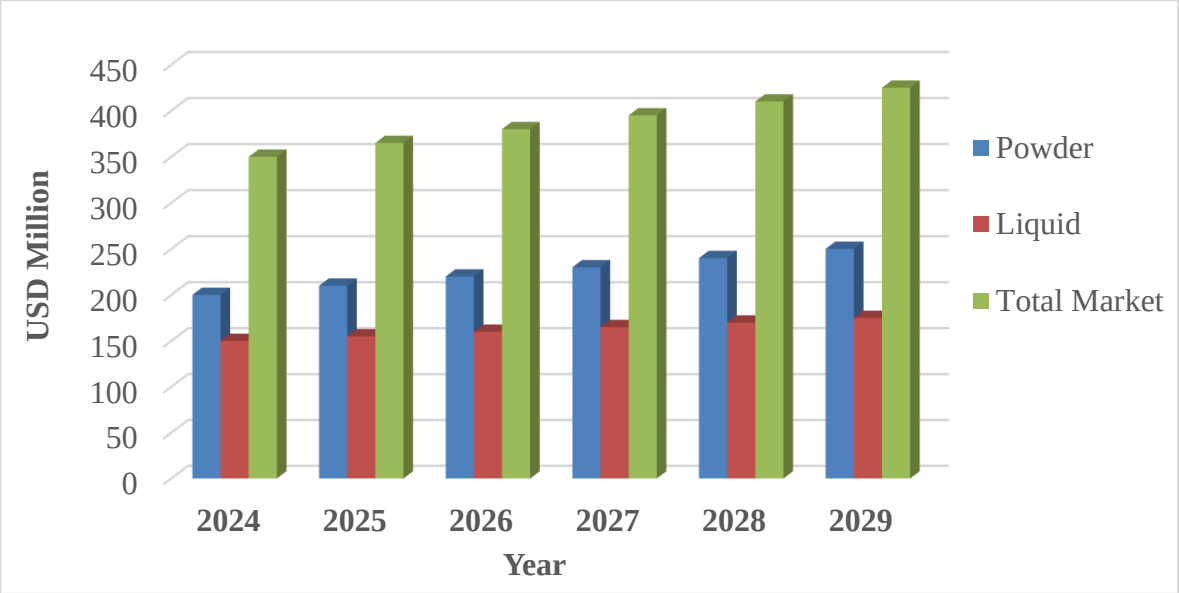


Figure 1.2 *The Global Market Size and Forecast of Citric Acid [2].*

This growth is driven by increased mining activities, government support, rising demand for Plaster of Paris, and higher cement production. Emerging trends include the use of lithium battery-powered haulers, advanced mining technologies, 3D mine visualization, and improved safety measures.

Citric acid can be produced through different method including submerged fermentation, Liquid Surface Fermentation, and Solid-state fermentation. The submerged fermentation (SmF) method is a predominant industrial technique for the production of citric acid where microorganisms grow in a liquid nutrient medium under controlled conditions to produce products like enzymes and organic acids[6]. It offers advantages such as easier monitoring

and control of parameters (pH, oxygen, and temperature), scalability, higher yields, simpler sterilization, automation, and easier product extraction. However, it requires sophisticated equipment, high energy and water input, is sensitive to trace metals, prone to foam formation, less suitable for filamentous fungi, and may produce unwanted secondary metabolites[7].

Liquid Surface Fermentation (LSF) is a technique where microorganisms grow as a thin biofilm on the surface of a static liquid nutrient medium, benefiting from higher oxygen availability at the air-liquid interface. It is useful for producing enzymes, antibiotics, and other metabolites, combining advantages of both solid-state and submerged fermentations[8]. However, LSF has limitations such as low productivity due to restricted microbial growth to the surface, difficulty in scaling up because of reduced surface area-to-volume ratio, higher contamination risk, slower nutrient and oxygen diffusion, and challenges in maintaining uniform environmental conditions, which can lead to inconsistent product quality. In addition, this process are labor-intensive, large floor space needed, contamination-prone, long processing time, and relatively modest yield[9].

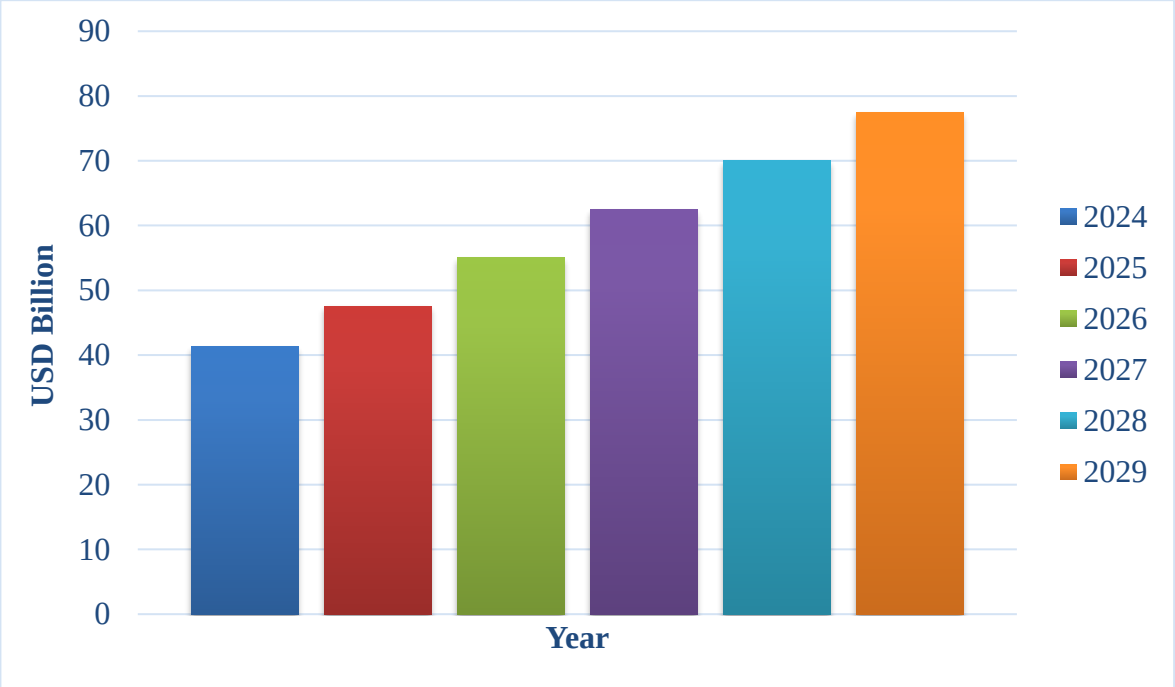


Figure 1.3 *Gypsum Global Market Report 2025*[10].

Solid-state fermentation (SSF) is a microbial fermentation process carried out on solid materials in the absence of free water, making it particularly suitable for producing citric acid using filamentous fungi such as *Aspergillus niger*[11]. SSF offers advantages like

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lower energy consumption, simpler fermentation equipment, and the ability to utilize agro-industrial residues as substrates. However, SSF also presents several disadvantages. The process is often difficult to control due to challenges in maintaining uniform temperature, moisture, and aeration throughout the solid substrate, which can lead to inconsistent fermentation results. Scale-up of SSF is complex because of poor heat and mass transfer, causing hotspots and uneven microbial growth. Additionally, product recovery can be more complicated and costly compared to submerged fermentation, as extracting citric acid from the solid matrix requires more intensive downstream processing[12]. These limitations often restrict the widespread industrial application of SSF for citric acid production despite its potential benefits.

The produced CA can be recovered in several way including Precipitation, Solvent Extraction, and Adsorption Method. Precipitation is the most commonly used technique for recovering citric acid as it can be applied across various processes. However, it necessitates filtration to eliminate micelles, fungal biomass, fermentation broth, and suspended solids. This method provides several benefits—it selectively isolates citric acid from the fermentation broth with high efficiency, minimizing unwanted byproducts. Additionally, the precipitation step streamlines downstream purification since calcium citrate is stable and easy to manage. Furthermore, it allows citric acid to be recovered in a concentrated form after it is converted back using dilute sulfuric acid[13]. A more ecologically friendly technique for crystallizing and purifying citric acid than precipitation is solvent extraction[14]. While it is cost-effective for clear fermentation broths, it is about 50% more expensive than traditional methods like precipitation. In laboratories, citric acid is commonly identified and measured using techniques such as spectrophotometry, gas chromatography, high-performance liquid chromatography (HPLC), high-resolution NMR spectroscopy, and classical titration[15]. Adsorption is a process where citric acid molecules are captured on the surface of solid adsorbents, which selectively extract citric acid from complex fermentation broths containing sugars, proteins, pigments, and other organic acids. Poly(4-vinylpyridine) (PVP) resin is an effective adsorbent for recovering citric acid, achieving adsorption capacities over 30% by weight at low pH and showing high selectivity even in the presence of sulfate ions. This method is valuable for purifying organic acids, pigments, and other fermentation products due to its simplicity, selectivity, and the ability to reuse the adsorbent after regeneration. However, the efficiency of

adsorption depends on factors like the adsorbent type, target compound concentration, and process conditions[15].

Aspergillus niger, because of its superior yield, is chosen over other microbes for the commercial manufacture of citric acid. CA production increases in a considerable amount using a variety of *Aspergillus strains*. Its capacity to flourish at a low pH of roughly 2.5 to 3.5, which reduces the production of byproducts like gluconic and oxalic acids, is a significant benefit. Furthermore, as the concentration of sugar grows, the generation of citric acid increases as well.[16]. Once thought to be a waste product of the sugar industry, molasses is now valued as a byproduct due to its low cost in comparison to other sugar sources and the minerals and other organic and inorganic compounds it contains[17]. In fermentation operations, agro-industrial wastes are frequently used as inexpensive substrates. Fruit peels from kiwis, kumara, orange waste, date syrup, pineapple waste, banana extract, carob pods, carrot waste, coffee husks, maize cobs, grape pomace, and apple pomace are a few examples[18]; Pumpkin and leftover potato chips have been explored as citric acid synthesis substrates with success. It is well known that pumpkin, either by itself or in combination with molasses, produces significant levels of citric acid[19]. In Bangladesh, industries and local street vendors generate substantial amounts of waste biomass and industrial byproducts[20]. These wastes and byproducts are often discarded as garbage, contributing to environmental pollution. However, they can be effectively utilized as substrates in submerged fermentation for citric acid production. Although Bangladesh has significant potential for citric acid production using locally available raw materials, no studies have yet been conducted on its laboratory- or commercial-scale production. Therefore, there is an opportunity to explore the use of such biomass as a substrate, enriched with varying amounts of different nutrients and additional carbon source, for submerged fermentation by microorganisms to produce citric acid. Therefore, the aims of the study were to produce cost effective Citric acid and Gypsum production from locally available raw materials to meet the country and global demand and propre utilization of agro-industrial residues. The objectives of the study are described below.

1.2 Aims and Objective of this Study

The aim of the study is to produce cost effective Citric acid and Gypsum production from locally available raw materials to meet the country and global demand.

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The aims are:

- To use *Aspergillus niger* to produce citric acid from agro-industrial waste, such as sugarcane molasses.
- To compare the generation of citric acid in molasses fermentation by lab-scale *Aspergillus niger* (F-81) strains with isolated desirable microorganisms (*Aspergillus niger strains*).
- To maximize the impact of different factors on the synthesis of citric acid, such as pH, incubation time, inoculum size, substrate concentration, and substrate pretreatment and non-pretreatment
- To recover citric acid on a laboratory scale using diluted sulfuric acid and calcium hydroxide.
- To recover the calcium hydroxide and sulfuric acid as gypsum
- To determine whether citric acid has antibacterial properties for its intended use.

The objective are:

- To utilize the agro-industrial waste (sugarcane molasses).
- To compare the chosen strain with the isolated soil organism (*Aspergillus species strain*).
- The production of citric acid, a crucial organic acid.
- To minimize the pollution, that agricultural waste causes.

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CHAPTER 2. LITERATURE REVIEW

The sustainable production of valuable industrial compounds such as citric acid and gypsum from industrial wastes and biomass has garnered increasing attention in recent years due to environmental and economic concerns. Citric acid, widely used in food, pharmaceuticals, and cosmetics, is traditionally produced through microbial fermentation using pure substrates. However, the growing interest in utilizing agro-industrial residues and biomass as alternative, low-cost raw materials offers promising opportunities to reduce production costs and minimize waste disposal issues. Various agricultural wastes, such as fruit pomaces, molasses, and lignocellulosic biomass, have been successfully employed as substrates for *Aspergillus niger* fermentation, enhancing sustainability in citric acid production. Similarly, gypsum, a versatile material extensively used in construction and agriculture, can be recovered and synthesized from industrial by-products such as fermented citrate recover, flue gas desulfurization sludge and phosphogypsum waste. These approaches not only contribute to waste valorization but also help mitigate environmental pollution associated with industrial effluents. Advances in processing technologies and characterization methods are critical to optimizing production efficiency and quality of citric acid and gypsum derived from waste streams, supporting the transition toward circular bio-economy models.

2.1 Physicochemical and Structural Characteristics of Citric Acid and Gypsum

With the molecular formula of citric acid ($C_6H_8O_7$), a weak organic acid is frequently found in a variety of citrus fruits. The Latin term for "citrus trees," like lemon trees, is whence its name originates. Citric acid is a colorless chemical that dissolves readily in water when it is pure [18]. In ambient temperature, it is typically a solid; around 153°C, it melts; and at higher temperatures, it breaks down. It weighs 192.12 g/mol at the molecular level. Three carboxylic acid functional groups in the citric acid molecule give it three different pKa values approximately of 3.1, 4.7, and 6.4, respectively [21][22].

Yuill and Bennett elucidated the crystalline structure of anhydrous citric acid by cooling a heated, concentrated solution of its monohydrate form[3]. In 1960, Nordman and colleagues posited that two citric acid molecules are interconnected via hydrogen bonding between the COOH groups of each monomer((Figure 2.1(a)) [23]. Furthermore, a 1994 study demonstrated that ab initio quantum chemical calculations of electron-rich citric acid (and the citrate trianion) were executed using a Hartree-Fock level approximation[24]. Researchers identified that citric acid and the citrate trianion exhibit distinctions from

other carboxylic acids due to an intramolecular hydrogen bond between the central hydroxyl hydrogen and a terminal carboxyl group, resulting in the central (C=O) stretch appearing at a lower frequency than the terminal stretches. Bichara et al. corroborated this finding ((Figure 2.1(b))[25]. Data from natural bond orbital and atoms-in-molecules calculations support the formation of citric acid dimers through hydrogen bonding between the COOH groups of each monomer." Glusker conducted X-ray studies as part of biochemical research, and Krebs actually discovered the critical role of citric acid in the metabolic cycle back in 1937. These dimers are now identifiable by numerous vibrational bands of varying intensities that were previously unassigned in the spectra[26].

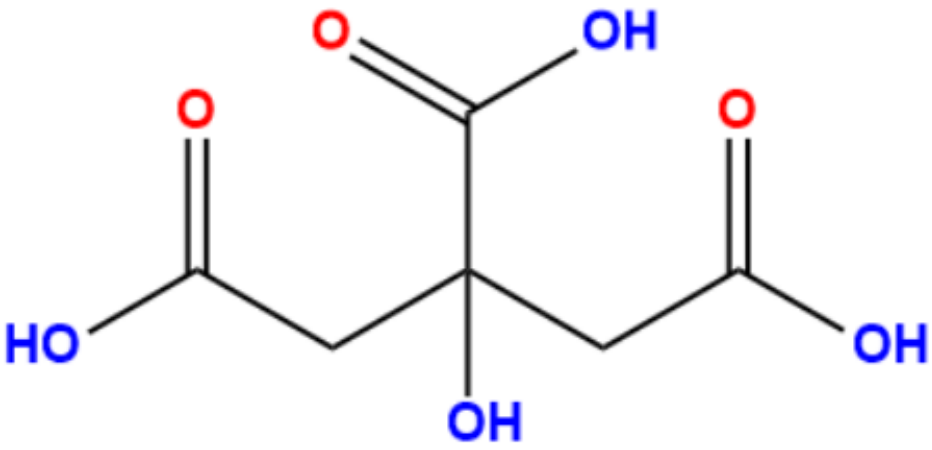


Figure 2.1 *Chemical structural of citric acid*[3].

Estrada recently described the citric acid cycle as a self-amplifying process that splits carbon and recycles both parts. The Krebs cycle uses the four-carbon oxaloacetate carrier to release two CO_2 molecules, regenerating oxaloacetate to accept a new two-carbon acetyl group (see Fig. 2.2, 1972) [27]. Gypsum ($CaSO_4.2H_2O$) is a soft, naturally occurring sulfate mineral known for its unique physical and structural properties. It crystallizes in the monoclinic system with space group C2/c, forming layered structures of calcium and sulfate ions separated by interlayer water molecules. This structural arrangement contributes to its perfect cleavage along the {010} plane, as well as its flexibility and softness (Mohs hardness 1.5–2) [28]. Gypsum has a specific gravity of approximately 2.3 g/cm^3 and typically appears in prismatic, tabular, fibrous (satin spar), or massive

(alabaster) habits. It is moderately soluble in water (~2.0–2.5 g/L at 25 °C) and displays retrograde solubility, meaning its solubility decreases with increasing temperature.

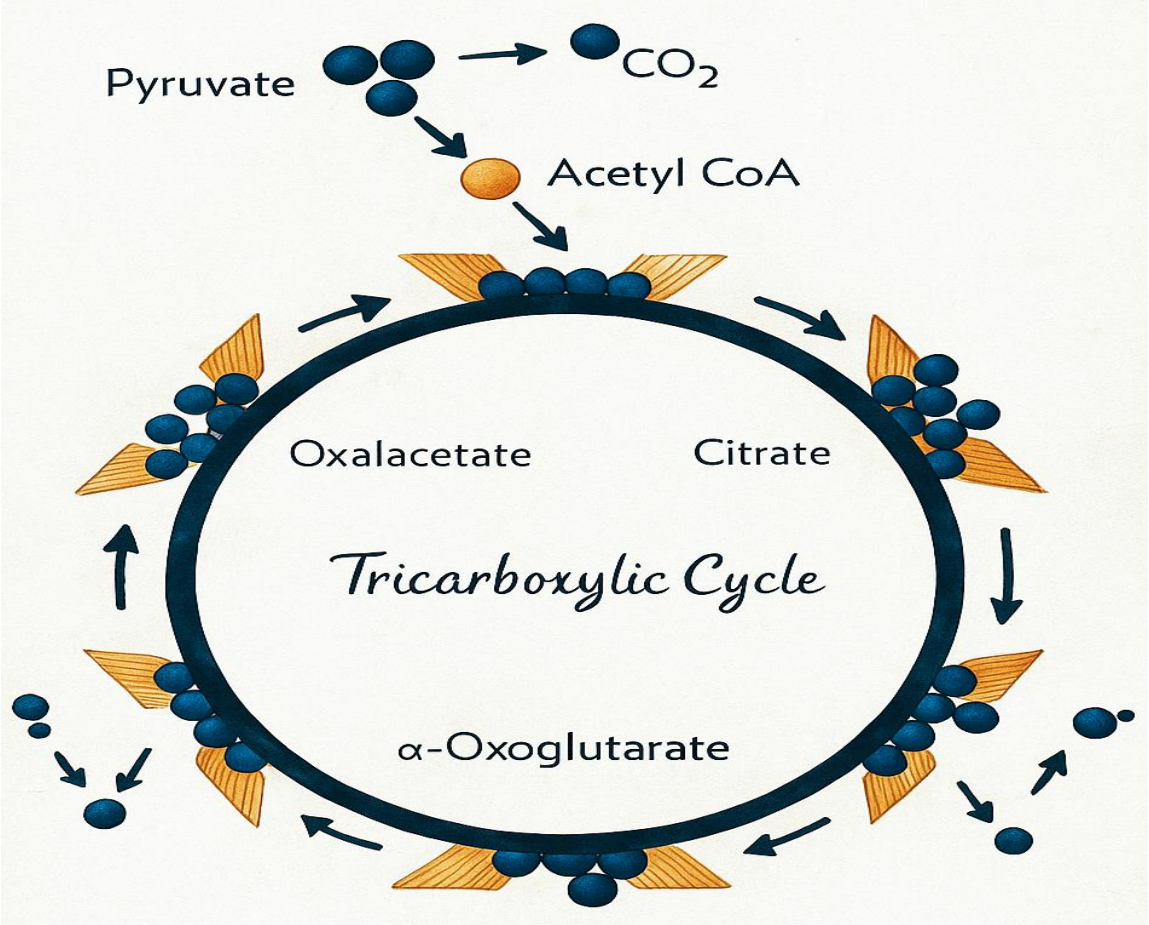


Figure 2.2 *In 1972, Nafissy proposed the citric acid cycle, where the four-carbon oxaloacetate acts as a carrier that binds with a two-carbon acetyl group to form six-carbon citrate*[3].

Thermally, gypsum begins to dehydrate around 100–150 °C, converting to hemihydrate ($CaSO_4 \cdot 0.5H_2O$, or Plaster of Paris), and further heating produces anhydrite ($CaSO_4$), which lacks water in its crystal structure[29]. These phase transitions have been identified using thermal analysis methods like differential thermal analysis (DTA) and Thermogravimetric analysis (TGA).

Additionally, optical studies show that gypsum is biaxial (+) with low birefringence and refractive indices around 1.52–1.53[30]. These combined characteristics—softness, structural hydration, cleavage, and optical properties—make gypsum an essential material in construction, agriculture, and industrial applications.

2.2 Sources of Citric Acid

2.2.1 Natural Sources

Citric acid is a naturally occurring acid found in many fruits and vegetables, especially citrus fruits like oranges, lemons, and limes. It is also found in blackcurrants, cranberries, and pineapples[31]. In addition, citrus fruits and other common fruits vary significantly in their citric acid content, which contributes to their characteristic tartness. Lemons have the highest concentration, ranging from 5-8%, followed by limes with 2-5%. Oranges and tangerines contain lower amounts, typically between 1 and 2%, while grapefruits have slightly more, ranging from 1-3%. Pineapples and tomatoes share similar citric acid levels, both falling between 0.5 and 1%. Strawberries contain the least amount of citric acid, with levels ranging from 0.1-0.5%. This variation in citric acid content influences the flavor profiles and acidity of these fruits[3].

2.2.2 Citric Acid Production through Synthetic Method

Citric acid is synthesized by fermenting glucose or sucrose with the microorganism *Aspergillus niger*. A series of chemical reactions within the microorganism converts glucose or sucrose into citric acid. The citric acid is then extracted from the fermentation broth and crystallized to purify it[31].

2.3 Microbial Citric Acid Production through Fermentation

2.3.1 Historical Perspective behind the Microbial Citric Acid Production

The production of citric acid by microorganisms is one of the oldest and best-known uses of industrial biotechnology. Citric acid was originally produced from lemon juice in the early 1700s by Swedish chemist Carl Wilhelm Scheele (1784). The commercial extraction of citrus fruits, such as lemons and limes, especially in Italy, happened next[32].

In the early 20th Century, citric acid was produced through Fermentation. In 1917 James Currie who was a British chemist, discovered that the fungus *Aspergillus niger* could efficiently produce citric acid when grown on sugar. During World War-I, when citrus fruits were scarce. At that time, Pfizer (then a chemical company) began fermentative production based on Currie's method[33]. From 1920s to1930s, scientists worked on it with cordially to reduce the cost of CA production with optimization of substrates, pH, aeration, and strain selection. In World War II, the optimization process was further

developed due to the need for preservatives and chelating agents. Between 1940s to1950s, the fermentation process was significantly scaled up, using submerged fermentation instead of surface culture, allowing for higher yields and automation. Due to the Global Expansion & Biotechnological Advances from 1960s to 1980s, CA generation rapidly expanded in worldwide, especially in Eastern Europe, India, and China. Introduction of molasses, corn starch hydrolysates, and waste materials are the main carbon sources of CA generation[34]. From 1990s to 2000s, the strains were continuously improvement through mutagenesis and selection. It is mainly developed by the application of genetic engineering, metabolic flux analysis, and bioreactor design for improved yields. Furthermore, Greater environmental control and downstream processing techniques are another media of CA production improvements[35].

In the modern era, microbial fermentation—especially using *Aspergillus niger* accounts for over 99% of global citric acid production. This acid are used mainly in food and beverages (acidulant and preservative), Pharmaceuticals, Cosmetics, Industrial applications (e.g., cleaning agents). China is currently the largest producer, contributing over 50% of global production[36]**.**

2.3.2 Citric Acid Producing Strains

There are a number of microorganisms including- fungi, bacteria and yeast that have been produced citric acid using different organic substrate via fermentation[37], as shown in (Table 2.1). Citric acid-producing bacteria are microorganisms that can biosynthesize citric acid—a weak organic acid commonly used in food, pharmaceutical, and industrial applications, some bacteria are also known to produce citric acid, among them *Arthrobacter paraffinens, Bacillus licheniformis*, and *Corynebacterium* sp. are a significant organism that can generate large amount of citric acid though they are less commonly used at industrial scales [38]. Furthermore, Citric acid-producing fungi are the primary microbial source of commercial citric acid production. Among fungi, certain Aspergillus species, especially *Aspergillus niger*, dominate the industrial process due to their high yield, low production cost, and ability to grow on a variety of carbon sources. Yeast are the another organism that aggrandize citric acid under the particular environmental condition, such as *Candida, Hansenula, Pichia, Debaromyces, Torula, Torulopsis, Kloekera, Saccharomyces, Zygosaccharomyces,* and *Yarrowia.* are the key organisms. In addition, some of yeasts have shown potential for specialized or alternative

substrate fermentations, such as those involving oils or glycerol. However, there are a number of drawback in CA production that have been reported by André (2007) and Rymowicz (2010), among one of them of is that yeast fermentation they produce substantial quantities of Iso-citric acid an undesired by-product[39][40].

Table 2.1 List of Citric Acid Producing Microbes[34]

Name of Microbe	List of Species
Fungi	*Aspergillus niger, A. niger YW-112, A. wentii, A. niger C192, A. awamori, A. carbonarius, A. aculeatus, A. niger GCB-75, A. fonsecaeus, A. usumii, A. fumaricus, A. saitoi, A. foetidus, A. niger CBX-209, A. luchensis, A. phoenicis, Mutant strain, Mucor piriformis, Penicillium luterum, P. janthinellum, P. citrinum, P. purpurogenum, P. restrictum, Paecilomyces divaricatum,, Trichoderma viride, Yarrowia lipolytica, Sacharomycopsis lipolitica.*
Yeast	*Candida tropicalis, C. guilliermondii, C. fimbriae, C. citroformans, C. intermedia, C. parapsilosis, C. oleophila, C. zeylanoides, C. Subtropicalix, Candida lipolytica, Hansenula anamola*
Bacteria	*Arthrobacter paraffinens, Bacillus licheniformis, Corynebacterium spp.*

The main microbiological source of commercial citric acid production is fungi that produce citric acid. Some *Aspergillus species*, particularly *Aspergillus niger*, are the most common fungus in industrial settings because of their high output, low cost of production, and capacity to grow on a range of carbon sources.

The key microbes that produce a significant amount of CA include *Aspergillus niger, A. awamori, A. wentii, A. luchensis, A. clavatus, A. nidulans, A. fonsecaeus, A. flavus, A. phoenicus, A. saitoi, Absidia sp., Acremonium sp., Botrytis sp., Eupenicillium sp., Mucor piriformis, P. luteum, P. citrinum, P. janthinellu, P. restrictum, Trichoderma viride, Talaromyces sp., and Ustulina vulgaris*[13].

2.4 Microbial Biochemistry of Citric Acid Production in *Aspergillus niger*

It is the well-known compound that is found in widely not only in plants but also in animal tissue, for instance, bone, muscle, and blood. In living beings, it is one of the significant organic acids in the TCA cycle that produces energy through a series of oxidative chemical reactions [30][31]. In *Aspergillus niger*, citric acid production is a regulated metabolic process involving the TCA cycle, glycolysis, and environmental conditions that promote citrate buildup and secretion. Although citric acid is a TCA cycle intermediate, it accumulates in *A. niger* due to inhibition of enzymes aconitase and isocitrate dehydrogenase, which slow downstream reactions. Carbohydrates are first converted to pyruvate outside the mitochondria, then pyruvate dehydrogenase converts pyruvate to acetyl-CoA. Acetyl-CoA combines with oxaloacetate to form citrate, which normally proceeds through the TCA cycle but accumulates here, leading to citric acid overproduction [32]. It is inhibited because of the low manganese and iron (co-factor deficiency), high sugar concentrations, low pH (~pH 2) environment during fermentation[33].

2.4.1 Citric Acid formation in Metabolic Pathways

Citric acid production involves a complex metabolic pathway influenced by multiple nutritional factors. High carbon source levels, dissolved oxygen, acidity, and low phosphate and trace metal concentrations collectively enhance citric acid yield. According to studies, *A. niger's* anabolism is inhibited by limiting nitrogen and eliminating manganese or phosphate from the medium, which results in protein breakdown and elevated ammonium ion levels[41]. Through a vigorous glycolytic process, A. niger generates significant amounts of citrate (Figure 2.4). Citrate is known to inhibit glycolysis, although in some circumstances, this inhibition may be lessened by the buildup of phosphofructokinase-1 (PFK-1) enzyme activators[42]. Oxalosuccinate forms by proton removal, followed by CO_2 release to complete the reaction. Mn^{2+} and Mg^{2+} ions are crucial for enzyme function, binding to active sites to stabilize oxalosuccinate. Both ions can be used without affecting enzyme activity. The $NADP^+$ dependent enzyme is inhibited by citrate and α-ketoglutarate, promoting citric acid accumulation. High glucose and NH_4^+ levels inhibit 2-oxoglutarate dehydrogenase, reducing citric acid breakdown in the TCA cycle. Papagianni et al. (2005) showed that phosphofructokinase inhibition during early citric acid production in *A. niger* is not due to intracellular ammonium. Instead,

ammonium combines with glucose to form glucosamine rather than accumulating inside cells[43]. The study showed that phosphofructokinase is not inhibited by the ammonium pool. Rather, very low intracellular ammonium levels (about 1% of the external concentration), intracellular pH, and the release of glucosamine into the fermentation broth all affect inhibition. In terms of the report of Papagianni et al., (2025), in Tricarboxylic Acid cycle, a considerable amount of glucose as well as ammonium level which direct effect on enzymes synthase including phosphofructokinase, 2-oxoglutarate dehydrogenase, and glucosamine synthase[21]. The production of citric acid is driven by the key enzymes of *A. niger,* where the membrane-bound enzyme invertase breaks down sucrose into glucose and fructose, which are then transported into the cell. In *A. niger*, hexokinase is the dominant enzyme, showing a 1000:1 preference for glucose over fructose. Mutations that inhibit glucokinase can enhance sucrose utilization by creating *A. niger* strains with improved sucrose affinity[17].

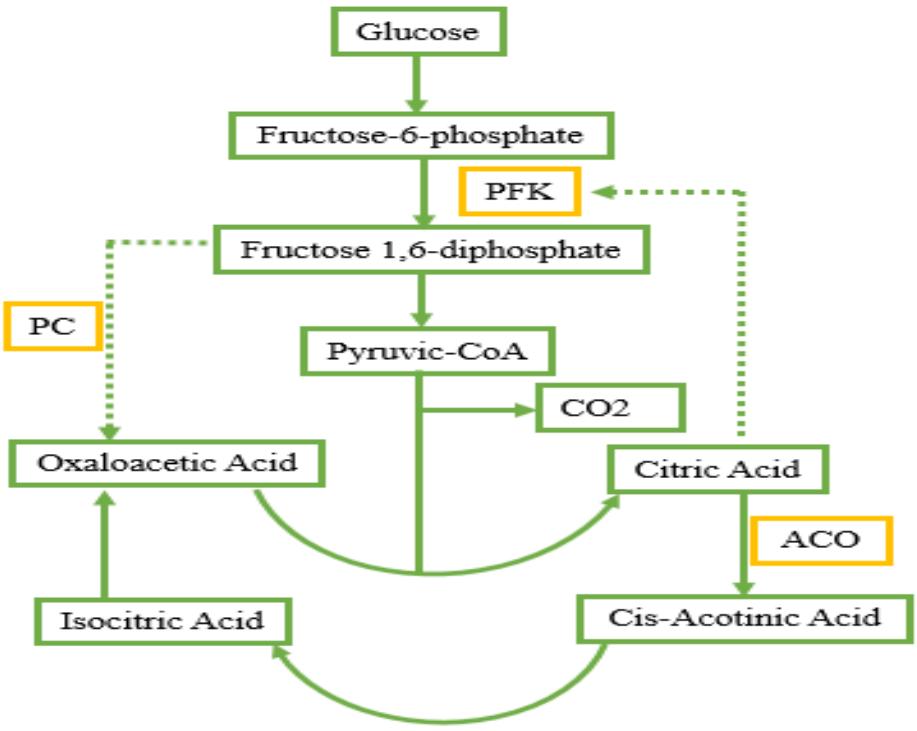


Figure 2.3 *Diagram illustrating the key metabolic reactions involved in citric acid production by Aspergillus niger*[34].

Glucose oxidase in *A. niger* converts glucose to gluconic acid, optimally functioning under strong aeration, low nutrient levels, and high glucose concentration.

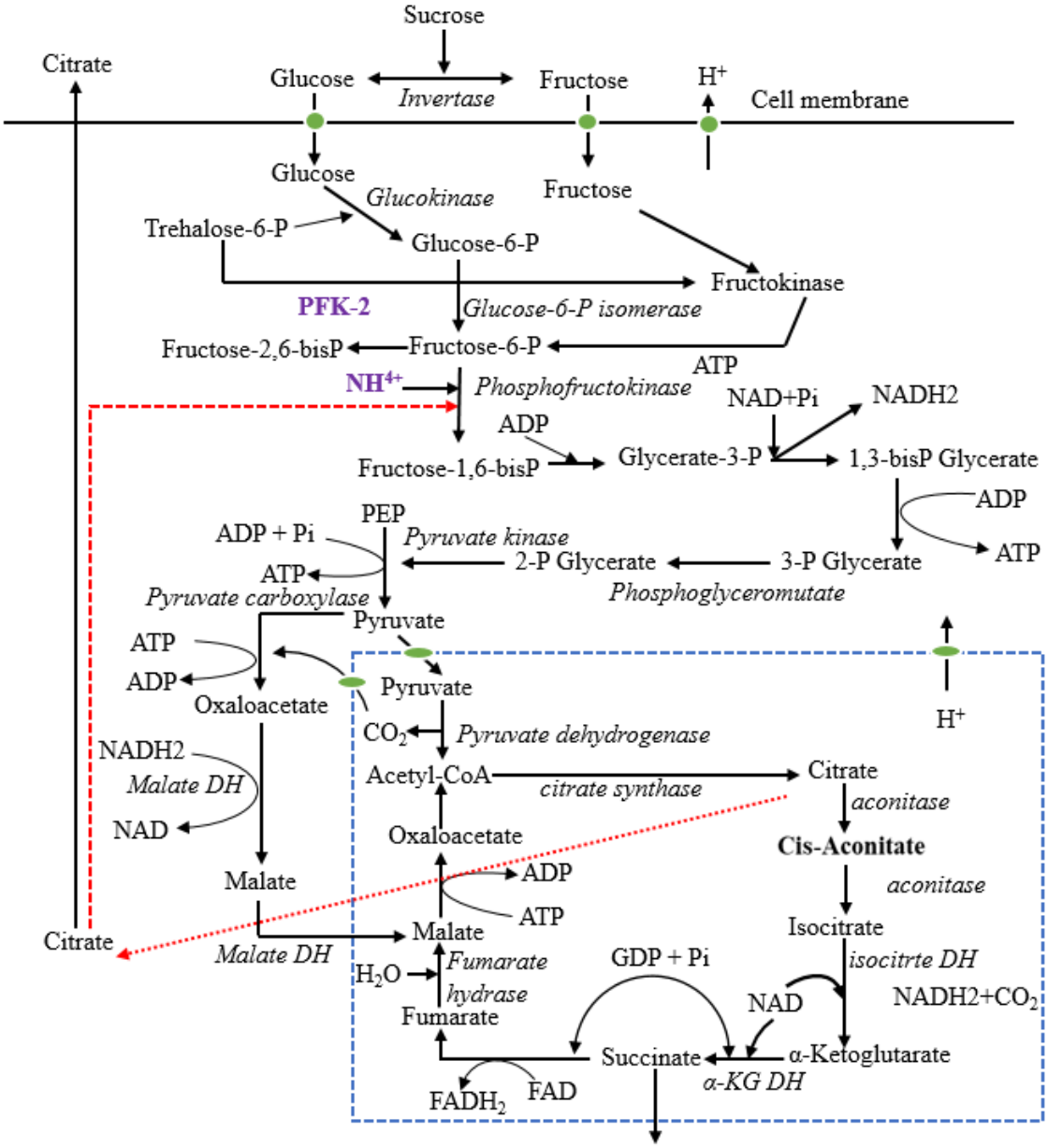


Figure 2.4 *Cellular locations, metabolic reactions and enzymes involved in citric acid production in A. niger*[21].

However, its activity is limited below pH 3.5 due to proton accumulation lowering the fermentation broth's pH. It remains unclear if gluconic acid is later converted into citric acid during fermentation. Fructose-6-phosphate is phosphorylated by PFK-1 and Phosphofructokinases-2 (PFK-2); PFK-1 forms fructose-1,6-bisphosphate by adding a phosphate at the C-1 site. PFK-1 is inhibited by high ATP, citrate, and manganese, while PFK-2 is activated by these compounds[44]. Citric acid is produced through glycolysis. Aspergillus species metabolize glucose and other carbohydrates via glycolytic and pentose phosphate pathways for growth and maintenance. Although pyruvate kinase was once

considered a key regulatory enzyme in citric acid production, studies show its activity is minimally affected by metabolic inhibitors. Citrate synthase catalyzes the reversible condensation of oxaloacetate and acetyl-CoA to form citrate. Thioester hydrolysis takes place during the process because it favors the synthesis of citrate. The following chemical equation is presented below[45]:

$$\textbf{Acetyl Co-A + Oxaloacetate} \leftrightarrow \textbf{Citrate}^{3-} + \textbf{H}^{+} + \textbf{CoA–SH}$$

Aspergillus niger contains three isoforms of isocitrate dehydrogenase, distinguished by their proton extraction methods from isocitrate (Figure 2.5).

These enzymes are classified into NAD^+-dependent, located only in mitochondria, and $NADP^+$-dependent, found in both mitochondria and cytoplasm. $NADP^+$-dependent isoenzymes are more abundant than NAD^+-dependent ones[46][47].

2.4.2 Accumulation of Citric Acid

Citric acid is a key intermediate of the **tricarboxylic acid (TCA) cycle**, and its **overproduction** by microbes especially by *Aspergillus niger* is one of the most successful examples of **microbial fermentation** in industrial biotechnology.

Under specific physiological and environmental conditions, microbial metabolism can be manipulated to accumulate citric acid in the culture medium. Citric acid accumulation depends on the number factors in biochemical reaction in the microbial cell. To the beginning, high concentration of sugar substrates that proliferate the glycolytic pathway that feeding as a pyruvate to acetyl-CoA in the TCA cycle. After that, this CoA aggrandizes with oxaloacetate to form citric acid with the help of citrate synthase. This following it converts to isocitrate through acotinase in the metabolic reaction in the living organism. Isocitrate dehydrogenase, which converts isocitrate to α-ketoglutarate, at the times requires Mn^{2+} and Fe^{2+} as cofactors. Under the metal-deficient conditions, the enzyme is inhibited, leading to citrate accumulation in the living life[48][49]. Under manganese-deficient conditions, transcription of the citrate exporter gene cexA is up-regulated, boosting citrate export. This export step is critical for extracellular accumulation; manganese supplementation suppresses both export and accumulation. Citrate builds up in the mitochondria because the cycle is halted beyond this point.

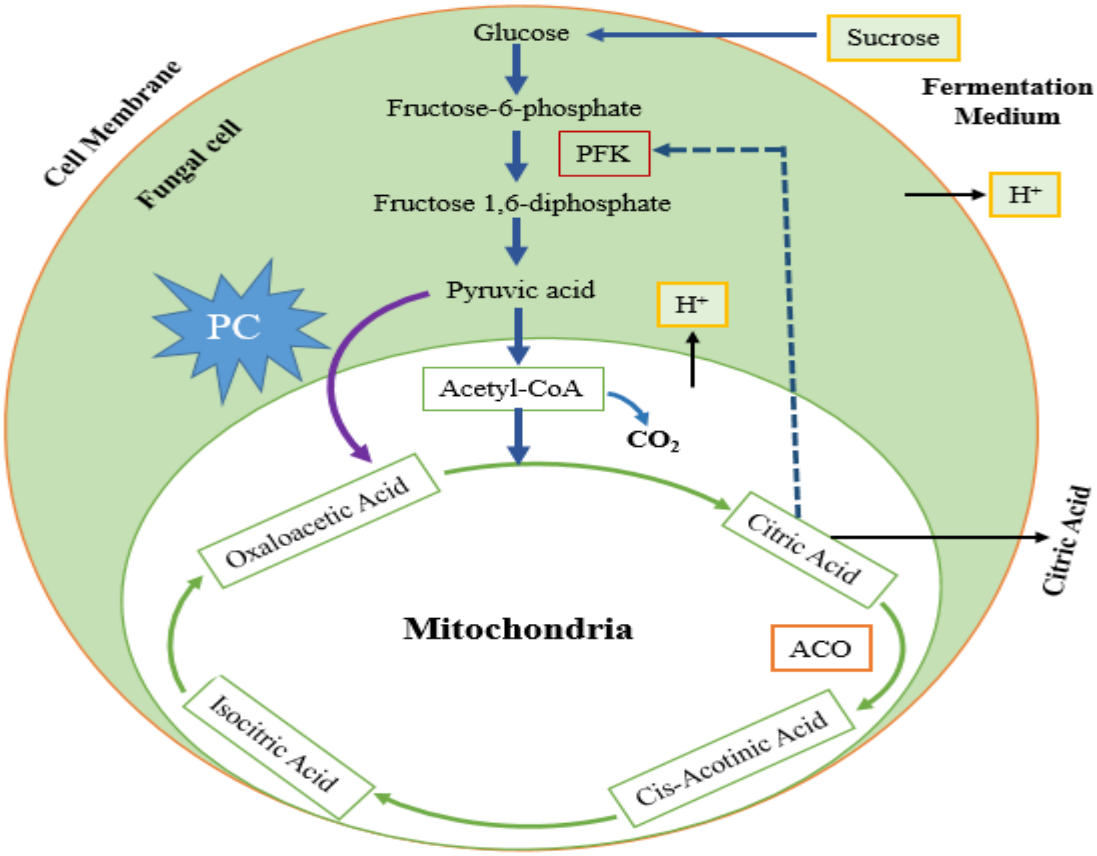


Figure 2.5 *Production of citric acid by A. niger*[50].

This is following, this organic acid transport to cytosol via specific citrate transporters and Secretion into the extracellular medium (figure 2.6)[51].

Citric acid accumulation is typically the result of aconitase and isocitrate dehydrogenase inactivation. The intermediates required in order to formation of biomass during the generation of citric acid are produced by activity in the Krebs cycle [49]. The Krebs cycle consists of eight events that occur in the mitochondrion. These mechanisms completely oxidize the two-carbon compound acetate to carbon dioxide. The cycle is summed up in the following chemical equation [22]:

Acetyl CoA + $3NAD^+$ + FAD + ADP + HPO_4^{-2} → $2CO_2$ + CoA + 3NADH + $FADH_2$ + ATP

As a result, citric acid accumulation is most likely the result of enhanced (deregulated) biosynthesis rather than inhibited degradation.

It does this by connecting it to the tricarboxylate transporter, which competes with aconitase for citric acid. The tricarboxylate transporter removes citric acid from the

mitochondrial matrix without disturbing other cycle enzymes, as its affinity for citric acid surpasses that of aconitase[7]. Citric acid accumulation is influenced by pH, ammonia, and manganese ions through their association with glutamate synthase. During the production stage, maintaining a highly acidic condition. As a result, the biological contamination and the by-products generation such as gluconic and oxalic acids are inhibited, thereby enhancing product recovery[52]. Isocitrate is converted to α-ketoglutarate through a two-step process. First, proton removal leads to the formation of oxalosuccinate, followed by decarboxylation that releases carbon dioxide and completes the reaction. The activity of the enzymes involved requires the presence of Mg^{2+} or Mn^{2+} ions, which bind to the active sites, stabilize the intermediate oxalosuccinate, and support substrate–enzyme interactions. Both ions can function interchangeably without affecting enzyme performance. However, citrate and α-ketoglutarate act as inhibitors of the $NADP^{+}$-dependent form of the enzyme, thereby limiting citric acid accumulation[53].

2.4.3 Improvement of Strains for Citric Acid Production

There are a number of strategies used to improve microbial strains—primarily *Aspergillus niger* and *Yarrowia lipolytica*—for enhanced citric acid production. In Metabolic & Genetic Engineering in *A. niger*, Overexpression of Biosynthetic Enzymes, for instance Citrate synthase and ATP-citrate lyase are upregulated to direct flux toward citrate production. In addition, Hu et al. (2019) reported that relieved feedback inhibition on phosphofructokinase (PFK-1), boosting citric acid by about 70%[54]. In knocking out competing pathways, with the help of CRISPR/Cas9-mediated disruption of pyrG (orotidine-5′-decarboxylase) led to increased yields in *A. niger*. As a result, disabling the isomaltose gene (agda) lowers byproduct formation and raises citric acid output[55]. CA production increase up to 42.6% through Silencing chsC gene that led to compact pellets, better oxygen transfer[54]. Overexpression of the high-affinity glucose transporter HGT-1 in A. niger CGMCC 10142 enhanced sugar uptake and significantly increased citric acid production at industrial scale [56]. In Yarrowia lipolytica, metabolic engineering strategies such as overexpressing pyruvate carboxylase (PYC) and isocitrate lyase (ICL), along with deleting ATP-citrate lyase (ACL), achieved titers of 70–110 g/L with yields of 0.64–0.76 g/g. Further modifications—including AMPD and YHM2 upregulation, combined with DGA-1/DGA-2/ACL deletions—enabled production of 83.6 g/L citric acid directly from undetoxified corn stover[57].

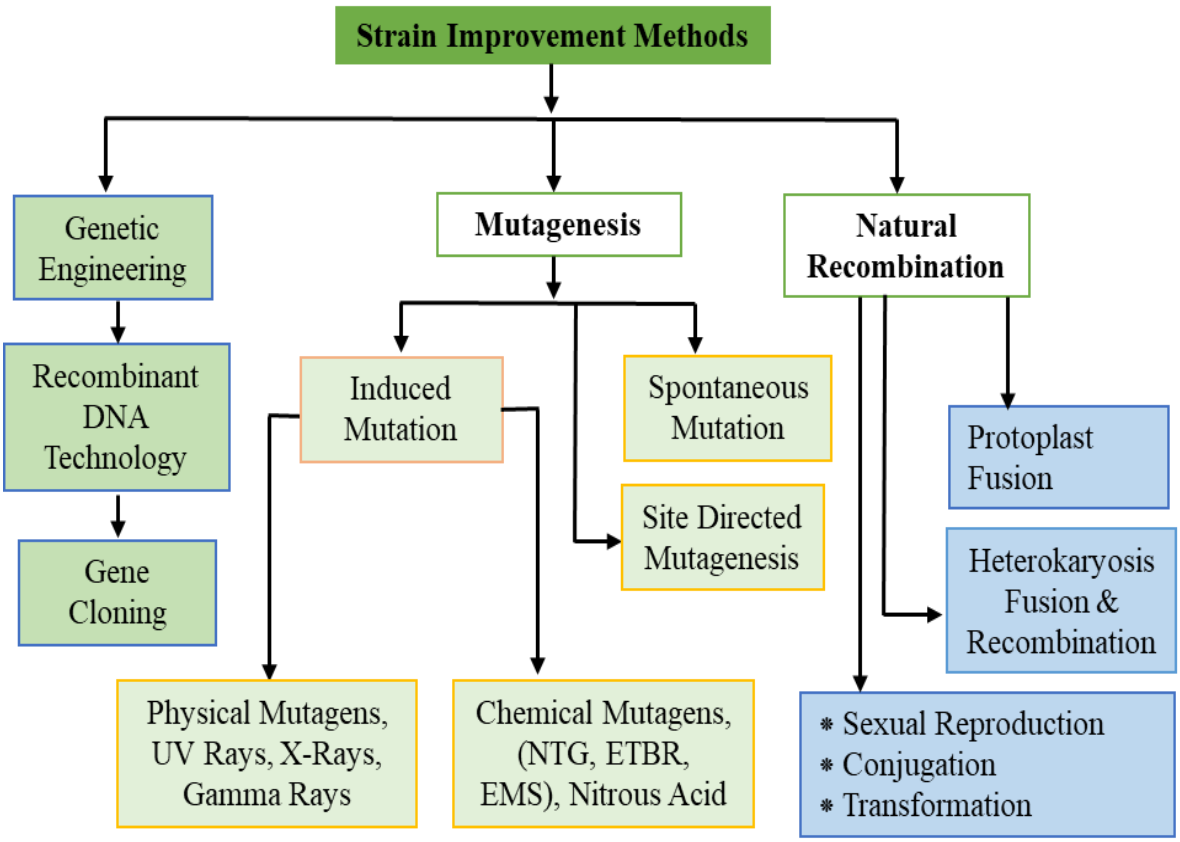


Figure 2.6 *Overview of strain improvement for CA production*[58].

According to the Sayın Börekçi et al. (2022) optimization of the environmental condition such as pH, fermentation time, and adaptive evolution strategies that improved the strain. As a result the productivity of CA increases than non-optimize condition[59]. UV and chemical mutagenesis on *A. niger* LPB strains on citric pulp yielded mutants with 19–37% higher titers in solid-state fermentation (SSF) that help to CA production[60]. Optimal conditions: low pH (<2 during production) to suppress contaminants and non-target acids. Trace metal limitation (Zn, Mn, Fe) and controlled aeration enhance yield[7]. Overexpression of malate dehydrogenase (mdh-2), fumarate reductase (FumR/Frds-1) and enhancing acetyl-CoA/ oxaloacetate pools increased CA levels. It also, Cofactor balancing (e.g., NADH/NADPH) can help redirect metabolic flux to favored the CA production pathways[54]. A variety of techniques, such as gene cloning, recombinant DNA technology, protoplast fusion, and mutations, are employed to enhance industrially significant microorganisms, as seen in (Figure 1.10) Protoplast fusion and random mutagenesis are the most fundamental and frequently applied methods. Site-directed mutagenesis, chemical mutagenesis, and physical mutagenesis are mutagenic methods for strain improvement. Strain enhancement has been taken into consideration in the

commercial fermentation process to address the overproduction of industrial products. Using physical or chemical mutagens to induce mutations in the microorganisms can also change their metabolism and produce improved strains. The most often employed physical mutagens are ultraviolet (UV) and gamma radiation. The most prevalent chemical mutagens include diethyl sulfonate, N-methyl-N-nitroso-guanidine, ethidium bromide, aziridine, N-nitroso-N-methyl urea, and ethyl methane-sulfonate[61][62].

2.5 Substrates of CA Production

In fermentation for citric acid production, substrate plays a crucial role in lowering costs and achieving optimal yield. As a result, it is more important in terms of for fermentation productivity. There are several types of raw material used in fermentation process. In the fermentation process through microorganisms, a wide variety of substrates are used, shown in table 1.4[31]. Molasses, starchy materials, and hydrocarbons are commonly utilized. Carob pod extract, n-paraffin, glycerol, maize starch, hydrolysate starch, yam bean starch, wood hemicellulose, olive oil, rapeseed oil, palm oil, and soy bean oil were among the examples mentioned by Soccol et al. (2006)[38].

To lower citric acid production costs and promote environmental sustainability, inexpensive substrates such as non-crystallizable sugar refinery byproducts (molasses) are often utilized. Molasses, which contains 40–55% sugars (mainly fructose, glucose, and sucrose), is a cost-effective option, though its quality varies depending on the source. To make it appropriate for fermentation, pretreatment is necessary, therefore it blends with $K_4Fe(CN)_6$ at pH 4.5 and 90 °C for 15 minutes, then filtering to remove precipitates. Although, Differences in sugarcane and sugarbeet cultivars, farming methods, and storage conditions are the primary causes of molasses composition variability[63]. According to Chen et al. (2014), beet molasses supports higher yields than cane molasses, as the latter contains elevated levels of trace metals (e.g., magnesium, zinc, manganese, calcium, and iron) that negatively affect citric acid synthesis [64]. To enhance citric acid productivity, particularly with Aspergillus niger, researchers have tested various supplements during fermentation. These additives improve yield by optimizing microbial metabolism, regulating environmental conditions, or enhancing substrate utilization. For example, Lu et al. (1998) reported that supplementing molasses with phytate during the incubation of tripling CA assortment[65][66]. Similarly, Anita et al. (2010) demonstrated that adding natural oils rich in unsaturated fatty acids to beet molasses increased production[67]. With

rising citric acid demand and growing the concern environment, attention has recently shifted toward the exploring novel substrates derived from agricultural residues and byproducts. However, some of these alternative elements insufficient essential nutrients like nitrogen and phosphate, which are crucial for efficient citrate formation. In such cases, supplementation with compounds like KNO_3 or NH_4NO_3 (as nitrogen sources) and K_2HPO_4 or KH_2PO_4 (as phosphate sources) is necessary for CA formation[18].

2.5.1 Utilization of Agricultural and Food Processing Wastes for Citric Acid Production

Agricultural and food processing wastes present a valuable resource for citric acid fermentation, with varying yields depending on the raw material used. Among these, palm oil and olive oil stand out with exceptionally high yields of 155% and 119%, respectively, followed by rapeseed oil at 115%. Cane molasses also demonstrates a perfect yield of 100%, while coconut oil yields 99.6%. Other notable raw materials include apple pomace and grape pomace, both capable of producing yields up to 88%, and brewery waste, which varies between 42% and 78.5%. Several other agricultural residues such as beet molasses (68.7%), cassava bagasse (67%), corn starch (62%), and pineapple waste (74%) provide moderate yields [41. Conversely, some materials like de-oiled rice bran (22%), whey permeate (19-33%), and turnip whey enriched with molasses (27-46%) yield significantly less. This data highlights the potential for optimizing citric acid production by selecting suitable agricultural by-products, with oils and molasses showing the greatest promise for industrial applications [34]**.**

2.6 Fermentation Process for Citric Acid Production

The most popular and economical way to obtain citric acid is by fermentation. More than 90% of the CA used today worldwide is produced by fermentation. Benefits of this approach include easier, dependable, and less complex operations; less technical expertise and complex control systems are needed; less energy is used; and frequent plant power outages have no negative effects[35].

There are three stages generally needed for every fermentation techniques: inoculation and preparation, fermentation, and citric acid recovery. The raw material and microorganism utilized determine the fermentation process used to produce CA. The raw material and microorganism utilized in the fermentation process used to produce CA. The raw materials

utilization in the manufacturing process of CA production were separated into two groups by Ramesh et al. (2011): (i) Cane or beet sugar, dextrose syrups, and crystallized dextrose are examples of low-ash raw materials that can be eliminated using standard methods; (ii) raw materials that have a high ash content and a high concentration of other non-sugar substances, such as cane and beet molasses and crude unfiltered starch hydro-lysates. A large variety of substrates may be employed for the practical and effective generation of CA, depending on the type of fermentation. Three techniques for commercial CA production in fermentation including—surface, submerged, and solid-state by using various substrates—each have pros and cons. However, media preparation, inoculation, fermentation, and CA recovery are required for each of these fermentation techniques[68].

2.6.1 Liquid Surface Fermentation

Liquid Surface Fermentation (LSF) is a type of fermentation process where microorganisms grow and metabolize on the surface of a liquid nutrient medium, typically under aerobic (oxygen-rich) conditions. It contrasts with submerged fermentation (SmF), where microbes grow within the liquid, and solid-state fermentation (SSF), where growth occurs on solid substrates with minimal free water[69].

The roots of LSF go back centuries and were traditionally used before modern bioreactor technologies were developed. It has a historical milestone, during the ancient, there was many traditional fermentation methods (e.g., Koji fermentation in Japan) in Asian and European used. Late 19th to early 20th Century, a few industries began to use and expand with the discovery and industrial production of citric acid and antibiotics. In the early of 20^{th} century, Pfizer's started production of penicillin involved surface fermentation before submerged processes took over. In the mild of 20^{th} century, SmF increased while LSF used decline in industry but remains important in certain niche applications, traditional methods, and for specific fungal fermentations[70][8].

In Liquid Surface Fermentation use especially filamentous microbes like *Aspergillus and Penicillium* grow as a mat on the liquid surface. The process is done at aerobic condition, particularly oxygen need to diffuse into the surface layer. *A. niger* grows as a thick mycelial mat on the surface of nutrient medium held in shallow trays or pans. At that time the production rate accounts for 10-20%. In this process, the medium needed to place in the shallow trays that stacked in the ventilation chambers. The cultivation time, temperature and pH of LSF were 8–12 days , 28–30 °C and 1.5–2.0 respectively [11]. In

this process usually produce different types of primary and secondary metabolites such as antibiotics, organic acid. In addition, numerous types of enzymes for example amylase, lipase and so on are also generated in LSF[71]. The merit of the system are lower energy consumption, foam-free, simpler equipment. In addition, More tolerant to trace metal variations and dissolved oxygen fluctuations[58]. However, this process are labor-intensive, large floor space needed, contamination-prone, long processing time, and relatively modest yield (70–75%)[9].

2.6.2 Submerged Fermentation

Submerged fermentation (SmF) is a microbial fermentation process where microorganisms grow in a liquid nutrient medium, usually under aerobic or anaerobic conditions, to produce valuable products such as enzymes, organic acids, antibiotics, and biofuels. The liquid medium typically contains dissolved sugars, nitrogen sources, minerals, and other growth-promoting substances. The process occurs in bioreactors or fermenters with controlled parameters such as pH, temperature, aeration, and agitation. Submerged fermentation developed as a response to the limitations of solid-state fermentation (SSF), especially for large-scale, industrial needs[72].

In the early 20th century, submerged fermentation began replacing SSF due to its ease of process control and scalability. In 1940s, it came breakthrough with the large-scale production of penicillin during World War II. Penicillin was initially produced using surface fermentation, but submerged fermentation allowed for vastly higher yields, marking a major turning point in industrial biotechnology. This is following of Post-World War II, the technique expanded rapidly into the production of other antibiotics, vitamins, and enzymes[73].

In the modern era, SmF is a standard in the biotechnology industry, used in producing a wide range of pharmaceuticals, food additives, and industrial enzymes. The key product of this process are enzymes (e.g., amylase, cellulase), antibiotics (e.g., penicillin), alcohols (e.g., ethanol), amino acids, and organic acid (e.g., citric acid). In addition, it is done in liquid (broth based medium) and using through bacteria, yeast, and fungi as well. The process is highly suitable for industrial-scale production[6]. In SmF fermentation, Growth and production phases are often separated by adjusting pH (e.g., dropping from pH 5–6 to 2–3), with pellet formation of 0.5 mm facilitating mass transfer. The process is done in 5–10 days, and up to 70–90% citric acid can be yielding. In terms of the substrate

concentration, the production rate up to (110–140 g/L) and although in low quality biomass, it was (10–15 g/L)[7].

There are a number of advantages of this process. It is Easier to monitor and control environmental parameters namely- pH, oxygen, temperature. Moreover, it is Scalable in industrial fermenters. Furthermore, in this fermentation system, higher product yields due to optimized conditions. The main precedence of the process is that easier sterilization and automation together with easier downstream processing system (e.g., product extraction)[58]. The disadvantage of this process requires sophisticated equipment and high energy input. In addition, it is more sensitive to trace metal inhibition and prone to foam formation (which must be mitigated with anti-foam agents or headspace allowance). Moreover, the process need high amount water and energy for maintenance. Furthermore, filamentous fungi are not suitable for SmF which perform better in SSF. Another disadvantage is that some secondary metabolites are produced in this fermentation[7].

2.6.3 Solid-state Fermentation

Solid-State Fermentation (SSF) refers to microbial fermentation processes carried out on solid materials with low moisture content and without free-flowing water. The SSF is known as Koji-style fermentation. Microbial growth on moist solid substrates (e.g. agro-industrial residues) with minimal free water—historically called the Koji process[11]. The Koji process has been used in Japan for over 1,000 years and is also integral to fermentation practices in China, Korea, and Southeast Asia. The "Koji-style" is a traditional Japanese method of SSF where cooked grains (typically rice, barley, or soybean) are inoculated with specific molds such as *Aspergillus oryzae* to promote enzymatic fermentation. Cultural significance of the Koji became the cornerstone of Japanese fermentation, forming the basis of foods like, Sake (rice wine), Soy sauce (shoyu), Miso (fermented soybean paste), Mirin (sweet rice wine[74]. The process used a number of microbes among them *Aspergillus oryzae* (Koji mold) is most popular as a primary microbe. In addition, in this process, there are a number of substrates are used for fermentation such as stream grains (rice, soybean). Furthermore, dried wastes, for instance, pineapple core, banana peel, coffee husk, corn cob, apple pomace, etc. are also used[75]. The environmental condition of this process need to control to get optimum products. The moisture condition of substrates have to up to be 30 to 60%. It also, the temperature, pH, and time have to be 28–30 °C, 4.5–6.0 and 4–5 days respectively[58].

There are a lot of merits of this process. The SSF is a greater output, the capacity to use widely accessible and reasonably priced agricultural and industrial leftovers as bio-production ingredients, reduced effluent, and environmentally acceptable substrate reuse. Additionally, compared to submerged fermentation, it is more beneficial to the environment[76]. In addition, it is a low cost and low energy usage. Furthermore, the process is higher specific yield besides it is a low bacterial contamination [12]. The disadvantage of this Process is that it need to control pH, humidity, heat that is difficult. On the contrary, the scale-up is challenging as well as it is often also lower consistency, and product recovery is costlier in most of the times. Furthermore, strains with high nitrogen and phosphorus requirements are ineligible, and this method's restricted supply of viable microorganisms and inadequate heat and oxygen transmission in the substrate mean that it does not effectively utilize available nutrients[77].

2.6.4 Types of Raw Materials in CA Production in Different Fermentation Process

Three main approaches have been used to manufacture citric acid. Historical and small/medium operations have employed inexpensive, sugar-rich wastes for surface (liquid-surface) fermentation, including turnip whey fortified with molasses, carob-derived sugar, cotton processing waste, brewery byproducts, and tubers of Asphodelus aestivus. These substrates are listed in compilations of raw materials for citric-acid fermentations and are echoed in contemporary reviews of A. niger processes[31][9][78].

In submerged fermentation (SmF)—the current industrial workhorse—feedstocks span sugar and starch streams (e.g., beet/cane/blackstrap molasses, corn starch and starch hydrolysates, whey permeate), lignocellulosic hydrolysates (e.g., bagasse, xylan/hemicellulose liquors), and hydrophobic carbon sources (e.g., n-paraffins, vegetable/edible oils such as coconut, palm, olive, soybean, rapeseed) as well as glycerol; these are well documented in classic and recent reviews and patents of SmF practice[7]. Finally, solid-state fermentation (SSF) leverages agro-industrial residues as both support and carbon source—e.g., apple or grape pomace, wheat/rice bran, coffee husks, corncobs, cassava bagasse, okara, citrus (orange/pineapple) peels, kiwi peels, molasses/press-mud, mussel-processing waste, and other fruit/vegetable wastes frequently with A. niger and documented across SSF surveys and substrate-specific studies (e.g., orange-peel SSF)[79].

Table 2.2. Comparative Evaluation of Various Fermentation Methods for the CA Production.

Method	Advantage	Disadvantage
Solid-state	Reduced energy and cost demands, higher product yield, inexpensive natural media, improved oxygen circulation, lower susceptibility to trace element inhibition, minimal risk of bacterial contamination, and reduced post-recovery waste generation.	Challenges in scale-up, difficulty in controlling process parameters, and higher product recovery costs.
Surface	Reduced effort in operation and installation, along with lower energy costs.	Generates a large amount of heat, is time-consuming and requires extensive space, and is highly susceptible to contamination by *Penicillia*, other *Aspergilli*, yeasts, and lactic acid bacteria.
Submerged	Advanced control mechanisms, reduced labor costs, and increased productivity and yield.	High media costs, susceptibility to trace metal inhibition, and substantial wastewater generation.

2.7 Factors Affecting the CA Production in Fermentation

2.7.1 Carbon source

Citric acid output is directly influenced by the carbon source, according to decades of research. The fungus prefers monosaccharides and disaccharides as carbon sources because they are digested more quickly and produce a larger yield than polysaccharides[32]. Since polysaccharides take too long to break down to achieve the rate of sugar catabolism needed to produce citric acid, they are not acceptable as raw materials.

Reduced enzymatic activity drives the sluggish pace of polysaccharide hydrolysis and affects the fermentation medium's pH[43]. The citric acid output of sucrose is superior to that of lactose, fructose, and glucose. It has been demonstrated that sucrose is superior[18]. *A. niger's* potent extracellular mycelium-bound invertase, which hydrolyzes sucrose quickly at low pH, has been credited with sucrose's supremacy[80]. Pure glucose or sucrose could not be commercially viable on an industrial scale. Consequently, low-quality carbon sources are used, like cane and beet molasses, which are waste products from sugar refineries. Pretreatment can be necessary because these raw components come from a variety of sources. Since cations are the most prevalent pollutants, cation-exchange resins or precipitation with potassium ferrocyanide are the most often used pretreatment techniques[18]. For citric acid manufacturing to be successful, the carbon source's concentration is just as crucial as its kind. Since the amount of citric acid produced is proportional to the concentration of sugar, it increases as the concentration of sugar does. However, the findings indicated that 14–22% sugar was the optimal concentration. Xu et al. (1989) discovered that, with the exception of glucose, which had a maximum yield of 7.5% (w/v), the maximum yield for sucrose, glucose, fructose, mannose, and maltose was 10% (w/v)[81].

2.7.2 Nitrogen Source

The concentration of nitrogen has been proven to have a significant impact on the generation of citric acid since it is a component of a cell's proteins and is necessary for cellular metabolism. Although ammonium salts must be added to laboratory medium, sugarcane as well as industrial media are typically high in nitrogen. Both fungal growth and citric acid synthesis are influenced by the kind of nitrogen source. While ammonium sulfate encourages more vegetative growth, ammonium nitrate encourages less. Because oxalic acid builds up at concentrations higher than 0.25% and lowers citric acid output, nitrogen limitation is necessary[82]. Researchers found optimal nitrogen levels of ~0.1–0.4 g N/L (i.e., ~0.01–0.04%) for maximizing citric acid production while minimizing unwanted by-products[83]. In one study using a sugar-analogue–resistant *A. niger* strain, the highest specific citric acid production rate was observed when intracellular ammonium ion concentrations were ~2–3 $mmol \cdot g^{-1}$ biomass. At higher external nitrogen concentrations, growth was favored and citric acid onset was delayed. When internal ammonium dropped below ~1 $mmol \cdot g^{-1}$, citrate production ceased [84].

In a study on *Yarrowia lipolytica*, citric acid production was mapped across a range of ammonium sulfate concentrations (2–10 g/L). The highest yields (~99.9 g/L CA) occurred at 4 g/L ammonium sulfate—indicating moderately limiting nitrogen supports strong citric acid excretion. Excess nitrogen increased biomass but reduced CA yield[85]. Another paper showed that excess nitrogen suppresses citric acid yield via nitrogen catabolite repression. Both batch and chemostat studies under phosphate limitation showed an inverse relationship between citric acid yield and nitrogen consumption rate—confirming that nitrogen limitation is essential for high CA production[86]. Research exploring ammonium ion dynamics indicates that during early fermentation, ammonium is attached to glucose inside cells forming compounds like glucosamine. High ammonium levels later promote fungal growth but briefly suppress citric acid production, after which CA biosynthesis picks up later during the production phase[43].

2.7.3 Phosphorus Source

Phosphate has also been demonstrated to be an important component, in addition to nitrogen. A phosphorus content of 0.5–5 g/l is necessary for fungal development in order to produce citric acid. Even in cases when nitrogen is not limited, CA accumulates with restricted phosphate, and the addition of phosphorus has only a minor impact on this process[82]. According to early foundational studies, fungal metabolism and citric acid production required a potassium dihydrogen phosphate (KH_2PO_4) concentration of 0.5–5 g/L in chemically defined media; however, citric acid yield improved under phosphate-limited conditions, even when there was enough nitrogen present[87]. Furthermore, under phosphate-limited conditions, citric acid accumulates, even when nitrogen is not limiting. In contrast, some other reports found that phosphate need not be limiting, but excess phosphate promotes biomass and side-product formation still aligning with the conclusion that limiting phosphate favors citric acid accumulation[7].

In studied, phosphate salts (NaH_2PO_4, Na_2HPO_4, Na_3PO_4, KH_2PO_4) in *A. niger* fermentation showed that 0.5–5 g/L phosphate was necessary for growth and production, with certain sources like Na_3PO_4 enabling higher citric acid yields but phosphate limitation overall beneficial for acid accumulation[87]. What is more, Phosphate limitation can stimulate citric acid production, even if nitrogen is present at non-limiting levels; high phosphate encourages biomass and secondary reactions, which reduce citric acid yields[9].

2.7.4 pH in Culture Medium

The pH of the medium is constantly changing due to microbial metabolic processes, namely the release of natural acids like CA and the unwanted gluconic and oxalic acids. Microbes, for instance, *Aspergillus*, *Rhizopus*, and *Penicillium* species can rapidly lower the pH to less than 3. Although *Sporotrichum* and *Pleurotus* species provide a more constant pH range of 4 to 5. During the germination phase, developing spores release protons and absorb ammonia, making the medium more acidic and encouraging the production of citric acid[88]. The fermentation medium's pH is crucial during the sporulation and production stages. The spores that are germinating absorb ammonia and release protons during the germination stage, which makes the medium more acidic and encourages the formation of citric acid. A pH of less than 2 facilitates citric acid recovery by preventing the production of undesirable byproducts like oxalic and gluconic acid as well as the potential for contamination by other microbes[7]. It is necessary to set and optimize the starting pH value of medium to suit the microorganism in manufacturing process. According to Dashen and his colleagues demonstrated that the substrate and manufacturing method had an impact on pH kinetics in different compounds[1].

2.7.5 Concentration of Alcohols

In the presence of methanol, the rate of citric acid production increases. The type of microbe and media composition have an impact on how much alcohol is needed to produce citric acid. It also explains how alcohols promote the excretion of citric acid by altering the phospholipid content of membranes and so enhancing permeability. Optimal alcohol concentrations are generally 1–5%, helping to offset negative effects of metal ions like Mn^{2+} and Fe^{2+}[83]. An orange peel/molasses-based fermentation showed that adding 3.5% v/w methanol increased citric acid yield by 25% over the control; ethanol showed no significant effect, and isopropanol inhibited production. Yield improvements depended on strain, alcohol type, and medium composition[89]. A study using galactose as a carbon source revealed that 1% v/v methanol significantly increased citric acid yields. Under these conditions, enzyme activities shifted: aconitase decreased, pyruvate carboxylase increased, and methanol increased membrane permeability leading to higher citrate excretion[90]. In a controlled fermenter, 1% v/v methanol doubled citric acid yield (90 g/L vs control) and improved pellet morphology and membrane permeability. Meanwhile, ethanol did not significantly enhance production[91]. Other studies across different A.

niger strains showed methanol consistently enhances production, while ethanol's effect varies by strain; the optimal alcohol addiction typically lies between 1–4% v/v, depending on strain and medium[7]. This finding suggests that in order to produce citric acid as efficiently as possible, the amount of methanol utilized must be limited.

2.7.6 Trace Elements

Researcher shown that the concentration of trace metal play the crucial role in CA production. Karaffa et al. (2024) showed that external Mn^{2+} concentration regulates hyphal morphology and citric acid production, primarily via the NRAMP-family transporter DmtA. Excess manganese (> 5 ppb) drastically reduces CA yield, while deficiency enhances citrate export[92]. Furthermore, if the concentration of manganese less than (< 3 µg/L) in the fermenter media that assist the high yield. Moreover, the optimum enzyme activity remained when the magnesium sulfate concentration was 0.02–0.025% (w/v). In Iron, it required of 1mg/L to produce optimum CA production but exceed iron induced oxalic acid. Therefore, the result of CA production was reversed. Copper is the significant compound in CA generation in fermentation. Equivalent of 78/L of it that mitigated the iron toxicity in media and influence the pellet morphology of the CA. In zinc, in fermentation should be carefully maintained[9].

2.7.7 Aeration

The bio-production of citric acid is very aerobic during fermentation and the amount of oxygen provided is crucial. Therefore, the performance and yield of fermentation may be adversely affected by different aeration rates[93]. The partial pressure of dissolved carbon dioxide in the medium falls at high aeration rates. Since carbon dioxide substitutes for the oxaloacetate source for citrate synthase, it acts as a substrate for pyruvate carboxylase. Although carbon dioxide is produced by the pyruvate decarboxylase cycle, excessive aeration may cause some losses. The final biomass and citrate concentrations are negatively impacted by excessive carbon dioxide levels[18].

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Although carbon dioxide is produced by the pyruvate decarboxylase cycle, excessive aeration may cause some losses. The final biomass and citrate concentrations are negatively impacted by excessive carbon dioxide levels[66]. This is in line with the research of Kubicek et al. (1989), who found that the organism's capacity to produce and accumulate citric acid was irreversibly destroyed while preserving its viability during an idiophase disruption process (i.e., aeration interruptions) that lasted up to 20 minutes[94]. Low aeration is meant to decrease A. niger's respiratory activity, which will cause the metabolism to change from producing biomass to producing citric acid. Additionally, it was found that greater aeration causes sporulation to rise and citric acid accumulation to decrease[60].

2.7.8 Other factors

Lipids play a significant role in CA production in fermentation process.

Table 2.3. Factors Influencing the Production of Citric Acid [31].

Factor (source)	Positive effect	Negative effect
Carbon	Glucose, fructose, galactose and sucrose.	Arabinose, pyruvic acid, starch, sorbitol, xylose.
Phosphorus	Concentrations of potassium dihydrogen phosphate range from 0.5 to 5.0 g/L.	
Nitrogen	Urea, peptone, malt extract, ammonium nitrate, and ammonium sulfate (0.1–0.4 g N/L).	More biomass is produced at higher concentrations.
Lower alcohols	Methyl acetate, methanol, ethanol, n-propanol, and isopropanol at 1–4% (v/w).	
Trace elements	Low levels of magnesium sulfate, copper, and zinc	
pH	Depending on the species, less than 3.	

Groundnut oil (also known as peanut oil) as a carbon source for CA production has been explored as an alternative to carbohydrates like glucose or molasses. These lipids can serve as rich energy sources for microorganisms, particularly Aspergillus niger, which is the most common industrial producer of citric acid[95], and sodium mono-fluoroacetate are other factors that influence citric acid production. Lipid improves citric acid yield while having no effect on mycelium dry weight. Kareem et al. (2010) investigated the effects of calcium fluoride, sodium fluoride, and potassium fluoride on citric acid industrial production[96]. Table 1.5 shows the main factors that influence citric acid production.

2.8 Procedure for Citric Acid Recovery

Citric acid can be recovered via three fundamental unit processes: precipitation, extraction, and adsorption (usually using ion-exchange resin). Although crystallization is an option, there is a risk of contamination because of microbial cell autolysis and undesirable elements in the raw material. Different methods can be used to retrieve CA.

2.8.1 Precipitation Method

The most common method for extracting citric acid is precipitation since it works for all procedures; however, it must be filtered to remove the micelles from the fungus, fermentation broth, and other detritus[13]. The precipitate that results from adding calcium oxide hydrate, commonly referred to as milk lime, to the medium is tricalcium citrate tetrahydrate. The precipitate is then filtered and washed with water. It was then treated with sulfur, which produced calcium sulfate, commonly known as gypsum. Cation and anion exchangers are used to process the mother liquor following filtering and active carbon treatment. At 40°C, it is further concentrated by vacuum-assisted evaporation. Last but not least, a vacuum crystallizer produces anhydrous citric acid at temperatures greater than 36.5°C and citric acid monohydrate crystals at temperatures between 20 and 25°C. Microorganism residues that contain inorganic matter, sugar, colloid, color, and amino acids, as well as calcium sulfate, are waste products from this process. These resources could be dried and utilized in feed plants or supplied to cement mills[32]. This method offers several advantages—it allows for the selective removal of citric acid from the fermentation broth with high efficiency, reducing the load of unwanted byproducts. The precipitation step also simplifies the downstream purification process because calcium

citrate is relatively stable and easy to handle. Moreover, it enables the recovery of citric acid in a concentrated form after reconversion with dilute sulfuric acid, yielding high-purity product while producing gypsum as a manageable byproduct.

2.8.2 Solvent Extraction Method

Solvent extraction serves as an alternative approach to purifying and crystallizing citric acid, offering reduced environmental impact compared to precipitation methods. It successfully inhibits the production of gypsum by extracting citric acid using n-octyl alcohol and tridodecylamine as its solvent. The U.S. Food and Drug Administration has suggested this method for use in the food and pharmaceutical sectors[14]. This technology is still around 50% more expensive than traditional recovery techniques like precipitation, even though it has been shown to be cost-effective for processing pristine fermentation broths. Numerous analytical methods, including as spectrophotometry, gas chromatography, high-performance liquid chromatography, high-resolution nuclear magnetic resonance (NMR) spectroscopy, and the traditional titration method, are used in laboratory settings to identify and quantify citric acid[97].

2.8.3 Adsorption Method

Adsorption is a surface phenomenon where citric acid molecules are retained on the surface of solid adsorbents. These adsorbents selectively bind citric acid from the complex fermentation broth (containing sugars, proteins, pigments, and other organic acids). Adsorption and Selective Recovery of Citric Acid with Poly (4-vinylpyridine). In adsorption method, poly (4-vinylpyridine) (PVP) resin is the effective reagent that recover citric acid from fermentation broth. Its adsorption capacity achieve more than 30% of weight at low pH, with high selectivity in the presence of sulfate ions. Along with, resin can be regenerated easily with methanol or ethanol, outperforming common strong and weak ionic resins in selectivity and reusability[98]. Researcher exploring on 18 ion-exchange resins for citric and lactic acid adsorption. Among them, they were identified weakly basic resins like Amberlite IRA 67, as most effective for citric acid recovery[99]. CA can recover through a Synthetic Fermentation Broth by Ion-Exchange Resins. Weak base resin to recover citric acid from synthetic broths is the most effective way to achieve up to 85% recovery and 92% purity at optimized conditions (30–70 °C, pH 1.5–6.5), with an adsorption capacity of 0.46 g CA/g resin [100]. Production of citric acid from molasses integrated with in-situ product separation by ion-exchange resin

adsorption. Researcher demonstrates that an integrated fermenter-resin column approach for in-situ adsorption separation of citric acid boosted the productivity from 0.338 to 0.543 g/L·h and sugar conversion up to 94.8%, outperforming traditional batch fermentation[101]. This method is particularly useful for purifying organic acids, pigments, or other fermentation products due to its simplicity, selectivity, and the ability to reuse adsorbents after regeneration. However, adsorption efficiency depends on factors such as the nature of the adsorbent, concentration of the target compound, and process conditions.

2.9 Application and Uses of Citric Acid

Citric acid is a multifunctional organic acid extensively used in food, beverage, pharmaceutical, cosmetic, and industrial applications due to its acidifying, chelating, and antioxidant properties. In the food sector, it serves as an acidulant, pH regulator, emulsifier, and preservative, enhancing flavor, texture, and microbial stability in products such as candies, dairy, jams, frozen fruits, and fats/oils. In beverages, it imparts tartness, stabilizes juices, prevents browning in wines, and inhibits oxidation. Pharmaceutical applications include facilitating rapid dissolution of active ingredients, serving as an effervescent agent in combination with bi-carbonates, enhancing solubility of cathartics, and acting as an antioxidant in vitamin preparations. In cosmetics, it functions as a metal-ion chelator, pH adjuster, and antioxidant, while in industrial processes it is employed as a buffering agent, metal cleaner, and oxide remover in electroplating and related operations. These diverse functionalities highlight citric acid's significance as a critical additive in multiple sectors[34].

2.10 Global Market and Bio-economy of Citric Acid

Citric acid plays a significant role in both the global market and the bio-economy due to its wide applications, natural origin, and increasing demand in various sectors. It has a number of applications in our daily life, more than 55% CA used in the bio-based sector revenue. In addition, growing 25–30%, in the Pharmaceutical manufacturing due to stringent purity. Moreover, 20%, used in cleaning, metal processing, and surfactant industries[83]. In 2022, global production reached approximately 2.8 million tons, with the industry valued at USD 3.87 billion. This market saw notable growth in 2023, rising to USD 4.2 billion, and is projected to climb further to USD 5.52 billion by 2027, reflecting strong demand and expanding applications. China stands as the clear leader in production,

contributing over half of the global output, while Europe and the United States follow as key players in the sector[9]. This analysis explores various substrates, microbial strains, and fermentation strategies—including surface, submerged, and solid-state methods—used in citric acid production. It also provides comprehensive market insights, highlighting a global production volume of approximately 2.8 million tons. Looking ahead, the market is expected to grow steadily at a compound annual growth rate (CAGR) of around 5.9%, reaching an estimated value of USD 9 billion by 2030, driven by rising demand across food, pharmaceutical, and industrial sectors[102]. In 2023, the global citric acid market was valued at approximately USD 3.484 billion. With consistent demand across food, beverage, pharmaceutical, and industrial applications, the market is projected to grow at a compound annual growth rate (CAGR) of around 4.0%, reaching an estimated USD 5.17 billion by 2030. This steady expansion reflects the compound's versatility and its increasing role in sustainable and bio-based product development [2]. Microbial production of citric acid continues to gain momentum due to its efficiency and sustainability, particularly when integrated into bio-refinery systems that utilize renewable biomass. This integration not only enhances resource utilization but also supports the development of a circular bio-economy. Additionally, citric acid is playing a growing role in the formulation of emerging biodegradable polymers and eco-friendly surfactants, further expanding its applications in green chemistry and sustainable industrial practices[3]. Recent literature highlights the promising use of lignocellulosic biomass and agro-waste as alternative feedstock's for citric acid production, aligning with the principles of a sustainable bio-economy. These renewable and low-cost resources not only reduce reliance on conventional carbon sources but also contribute to waste valorization and environmental conservation. By converting agricultural residues into valuable biochemical, such approaches support circular economy models and promote eco-friendly industrial biotechnology[38]. The market value of citric acid has seen a notable increase in recent years, driven by rising demand across multiple regions. Asia leads the global market, accounting for approximately 35% of the total share, largely due to its rapidly expanding economy and industrial growth. Europe follows closely with a 30% share, reflecting strong consumption in food, pharmaceutical, and cleaning product sectors. The Americas also represent a significant portion, contributing around 30%, with North America alone responsible for about 20%. Other countries collectively make up the remaining 8%, underscoring the widespread and growing global reliance on citric acid[9].

The global gypsum market is growing steadily and is expected to reach USD 68 billion by 2035, driven by construction demand. Key sources include natural, synthetic (especially FGD), and recycled gypsum. The bio-economy aspect focuses on reusing industrial by-products like citrogypsum and phosphogypsum, supporting circular economy efforts. Developed regions lead in gypsum recycling, achieving rates up to 80%. Innovations in gypsum bio-composites using agricultural waste enhance building sustainability. Despite challenges such as transport costs and reliance on coal-based gypsum, gypsum's role in eco-friendly construction and soil improvement is expanding alongside global sustainability goals[103].

2.11 Production of Gypsum

Gypsum, a widely used and eco-friendly mineral binder mainly composed of calcium sulfate, offers neutral pH, attractive white color, and excellent decorative qualities. Its production requires lower calcination temperatures (135–180°C), leading to reduced fuel use and CO_2 emissions. Gypsum products set quickly, are lightweight, moldable, fire- and sound-resistant, but have low water resistance, which can be improved with additives. Industrially, gypsum is often produced by reacting calcium-containing materials with sulfur dioxide from emissions through processes like flue gas desulfurization (FGD), which simultaneously reduces pollution and generates valuable synthetic gypsum[104].

Additionally, gypsum can be obtained as a byproduct from phosphate fertilizer manufacturing, known as phosphogypsum, which results from the treatment of phosphate rock with sulfuric acid. Both synthetic and natural gypsum undergo purification steps involving precipitation of calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$). This hydrated calcium sulfate is then separated from the slurry through filtration or centrifugation, followed by thorough washing to remove impurities and residual acids[105].

2.12 Application and Uses of Gypsum

Gypsum ($CaSO_4 \cdot 2H_2O$) is a widely utilized mineral known for its multifaceted applications across several industries due to its unique physicochemical properties. In the construction sector, gypsum is primarily used in the form of plaster of Paris and gypsum boards (drywall) owing to its fire resistance, light weight, and ease of application, making it essential for wall partitioning, ceiling systems, and decorative molding. In agriculture, gypsum acts as a soil conditioner, improving soil structure, drainage, and aeration, while

also supplying vital nutrients such as calcium and sulfur; it is especially beneficial for reclaiming sodic and saline soils. The medical field employs gypsum for orthopedic casts and dental molds, given its biocompatibility and ability to form detailed impressions. Additionally, gypsum finds application in the food industry as a coagulant in products like tofu, and in environmental engineering for flue gas desulfurization and wastewater treatment. These diverse applications are enabled by gypsum's solubility, non-toxicity, and rehydration characteristics, which allow it to set into a hard mass upon mixing with water[106].

2.13 Microbes Used in this Study

To conduct this study Citric Acid producing *Aspergillus niger* (strain: F-81) was grow with appropriate nutrient media. It was collected from "Molecular Biology Lab" of department of Biochemistry and Molecular Biology, University of Dhaka.

For further experiment, we were collected, over 14 soil samples from various regions in Bangladesh. Particularly, samples were obtained from Curzon Hall and Mokarram Bhaban at the University of Dhaka, as well as from Suhrawardy Udyan and the Padma River bank near Shimulia Ghat. In addition, soil was collected from Madai Khamar in Badarganj, Rangpur. Among these samples, more than six fungal isolates were identified and selected for use in fermentation for citric acid production.

2.13.1 Habitat of *Aspergillus niger*

Aspergillus niger is a soil-borne fungus found in a variety of environments such as soil, compost, and decaying plant material. It's also found in foods like bread, cereal, and fermented foods, as well as in industrial settings like paper mills, sugar factories, and textile mills. It is also found in air conditioners and humidifiers.

2.13.2 Mophology of *Aspergillus niger*

Aspergillus niger is a filamentous fungus that forms hyphae (filamentous colonies) on nutrient-rich surfaces. Septate hyphae are those that are divided into segments by cross-walls called septa. These septa have perforations that allow organelles and nutrients to move between hyphal cells. *A.niger* produces aerial hyphae, which rise from the substrate to form a fluffy, cottony growth known as a mycelium.

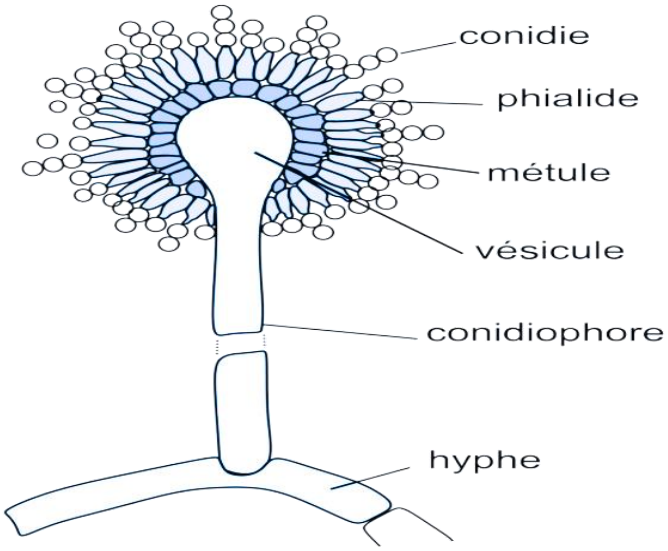


Figure 2.7 *Morphology of A.niger*[107].

This mycelium is typically white or cream-colored, and its thickness varies depending on the growth conditions. *A.niger* also produces distinctive black spores known as conidia on specialized structures known as conidiophores.



Figure 2.8 *Culture of A. niger in the potato dextrose agar (PDA) media. The figure shows the spore of A. niger after 10 days culture in PDA media at 28^{o}C.*

These conidiophores are formed by aerial hyphae and can be found on the mycelium's surface. Conidia form clusters at the tips of conidiophores and are dispersed by wind or water.

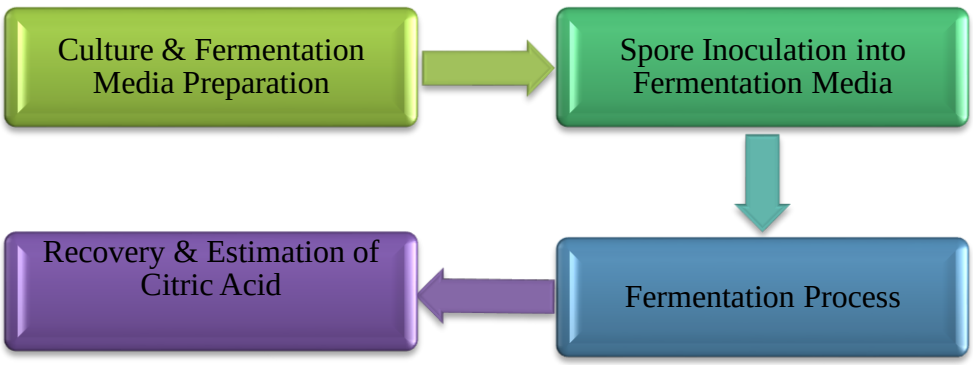
2.13.3 Life Cycle of *Aspergillus niger*

- Asexual reproduction in Aspergillus niger takes place through the generation of conidial spores.
- The life cycle starts when conidia are dispersed onto a surface with favorable temperatures ranging from 25 to 40°C.
- Following dispersal, the conidia germinate, giving rise to a vegetative cell.
- These cells develop into hyphal mycelium that branches dichotomously, leading to the formation of aerial hyphae.
- The aerial hyphae extend and differentiate into conidiophores, which swell at their tips to create a vesicle structure.
- Phialides serve as the primary sterigmata and give rise to secondary sterigmata, which initiate the production of conidial spores.
- The spores are organized in multiple columns stacked on top of the phialides.

2.13.4 Industrial Application of *Aspergillus niger*

Aspergillus niger is commonly recognized for synthesizing citric acid, widely applied as a preservative in the food industry. It also produces enzymes called glycoside hydrolases that facilitate the conversion of plant materials into biofuels. In addition, this microorganism generates various bioactive substances and pharmaceutical compounds. Due to the high transfructosylation activity of its enzymes, it can produce significant amounts of fructooligosaccharides. Its strong capacity for bioadsorption makes it effective in eliminating contaminants like Congo red and Blue 9 dyes.

2.14 Study Design



2.15 Significance of this Study

Microbial production of citric acid and gypsum recovery from molasses, an agro-industrial waste in Bangladesh, holds significant potential for economic and environmental sustainability. Utilizing microbes like *Aspergillus niger*, citric acid can be produced locally, reducing import dependence and meeting demand from various industries. Simultaneously, gypsum can be recovered as a valuable by-product, addressing Bangladesh's lack of natural gypsum reserves. This integrated biotechnological approach supports waste valorization, circular economy goals, rural industrialization, and sustainable development, while fostering backward linkages and contributing to SDG targets.

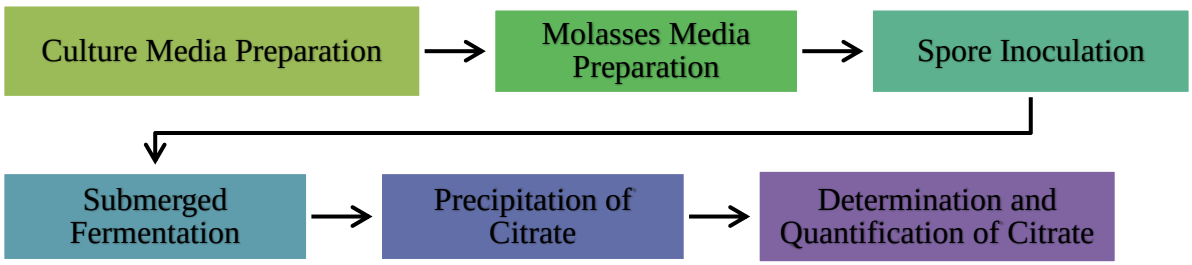
CHAPTER 3. MATERIAL & METHOD

Part-I:

3.1. Background of Research Method

The current research titled "**Eco-friendly Production of CA and Gypsum from Locally Available Biomass and Agro-industrial Wastes using Fermenter Microbes**" was conducted at the Genomics and Proteomics Core Laboratory under the Department of Biochemistry and Molecular Biology, as well as Analytical & Environmental Laboratory of the Department of Applied Chemistry and Chemical Engineering, University of Dhaka, Bangladesh.

The following steps to conduct this study:



3.2 Materials and Chemicals Used in this Experiment

- Measuring cylinder
- Conical flask
- Falcon tube
- Forceps
- Aluminum foil
- Beaker
- Tips
- Had globe
- Inoculation loop
- Masking tape
- Micro piptte
- Parafilm
- PTFE filter
- Syringe
- Petridis
- Tissue paper
- Mask
- Glass spreader
- Burner
- Forceps
- Magnet
- $Ca(OH)_2$
- NaCl
- H_2SO_4
- KH_2PO_4
- NH_4NO_3
- $K_4Fe(CN)_6$
- Ethanol
- Pyridine
- Acetic anhydride
- standard sodium citrate
- PDA media
- Czapek dox agar media
- Chloramphenicol
- Whattman filter paper

3.3 Sterilization

In microbiological experiments, all glassware and equipment were sterilized prior to use using an autoclave (Hirayama HL-41, Japan) at 121 °C and 15 psi for 20 minutes. The same sterilization protocol was applied to all culture media and solutions. Fresh agar plates were subsequently prepared and allowed to dry at ambient temperature. All inoculation procedures were carried out under aseptic conditions within a biosafety cabinet (ESCO, Singapore) to prevent contamination.

3.4 Collection of Fungal Strain for the Production of Citric acid

To conduct the experiment, the fungus strain *Aspergillus niger F-81(91)* was obtained from the Molecular Biology Laboratory in the Department of Biochemistry and Molecular Biology at the University of Dhaka in order to do the experiment. Because of its potential for producing CA via the submerged fermentation approach, this strain was chosen

.

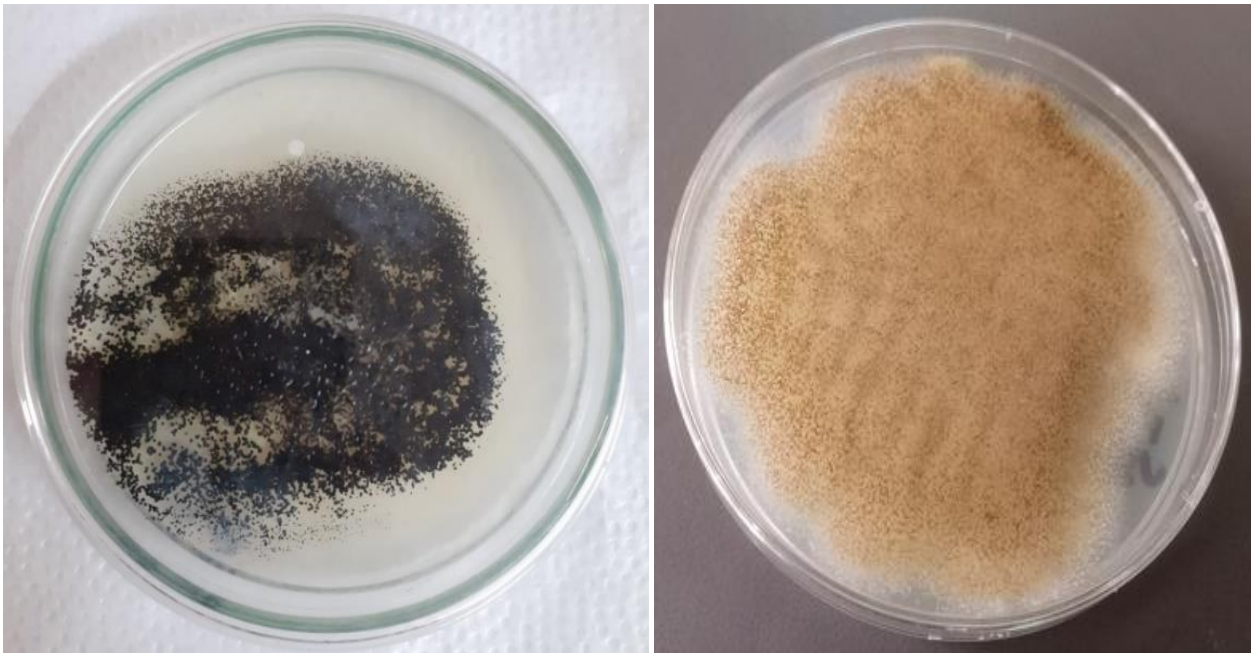


Figure 3.1. *Culture of A. niger in the Potato Dextrose Agar (PDA) media*.

3.5 Preparation of PDA Media for Culturing Fungus (*A. niger)*

To support vigorous *Aspergillus niger* cultivation, a classical PDA medium was prepared using 200 g/L potato infusion, harvested by boiling sliced potatoes to extract starches, vitamins, and growth-promoting phytochemicals. Dextrose (20 g/L) supplied an easily

metabolizable carbon and energy source, while 15 g/L agar provided the solid matrix necessary for surface colony development. The medium was adjusted to a final pH of 5.6 ± 0.2 at 25 °C, creating a slightly acidic environment that favors *A. niger* growth while inhibiting many bacterial contaminants. This formulation is widely recognized and routinely employed for morphological studies, sporulation assessments, and fungal isolation in both research and industrial mycology settings. The specification of PDA composition was shown in table 2.1.

Table 3.1. Composition of PDA Media[108].

Name of ingredient	Amount of ingredients(g/L) in media preparation
Potatoes infusion from	200.00
Dextrose(glucose)	20.00
Agar	15.00
Final pH 5.6 ± 0.2	

3.6 Preparation of PDB Media for Fungal Growth Curve Observation (*A. niger)*

To monitor the fungal growth, Potato Dextrose Broth (PDB) was employed as the growing medium in order to track fungal growth in liquid culture. A Shimadzu ELB200 balance (Japan) was used to weigh a precise amount of PDB powder, which was then put into a 250 ml conical flask. After adding the necessary amount of distilled water, a magnetic stirrer was used to homogenize the liquid. After that, the prepared medium was autoclaved for 20 minutes at 121°C and 15 pressure to sanitize it. Following sterilization, a 2% A. niger spore suspension was added to the broth for inoculation. A shaking incubator with settings of 28 °C and 160 rpm was used to incubate the culture flask. To track fungal growth, optical density was recorded at 240 nm every 24 hours.

3.7 Sample (Sugarcane Molasses) Specification

A Sugarcane Molasses sample source was collected from old Dhaka. Its quality was assessed by analyzing key parameters, including moisture content, pH, ash content, and total sugar concentration.



***Figure** 3.2. Sugarcane Molasses*

3.7.1 Determination of Moisture Content of Sugarcane Molasses

A common gravimetric technique was used to ascertain the sample's moisture content. The sample was first put in a crucible, then an analytical balance was used to precisely record its wet weight (w). To guarantee total moisture removal, the crucible containing the sample was thereafter placed in a hot air oven and dried for 30 minutes at 106°C. Following drying, the sample's dry weight (d) was determined. The formula was then used to determine the moisture content (MC)[109]:

$$\boldsymbol{MC}\ (\%) = \frac{(w - d)}{w} \times 100$$

Where MC is the moisture content (%), “w” represents the wet weight, and “d” the dry weight of the sample.

3.7.2 Determination of Ash Content of Sugarcane Molasses

A high-temperature combustion procedure was used to ascertain the sample's ash content. The sample was first put into a crucible that had been previously weighed, then an

analytical balance was used to determine its moist weight. To guarantee full combustion of the organic debris, the crucible containing the sample was subsequently moved to a muffle furnace and heated to 800°C for two hours. The ash residue was cooled in a desiccator and then weighed. The following formula was used to determine the amount of ash[110]:

$$\boldsymbol{Ash}\ (\%) = \frac{(weight\ of\ Crucible + \ weight\ of\ ash)\ - crucible\ weight}{Sample\ weight} \times 100$$

3.7.3 Determination of Sugar Content of Sugarcane Molasses

3.7.3.1 Preparation of Standard Curve of Maltose

In order to create a maltose working solution, 180 mg of maltose were dissolved in 100 ml of distilled water. Five different test tubes were filled with aliquots of 0.2, 0.4, 0.6, 0.8, and 1 ml of this solution.

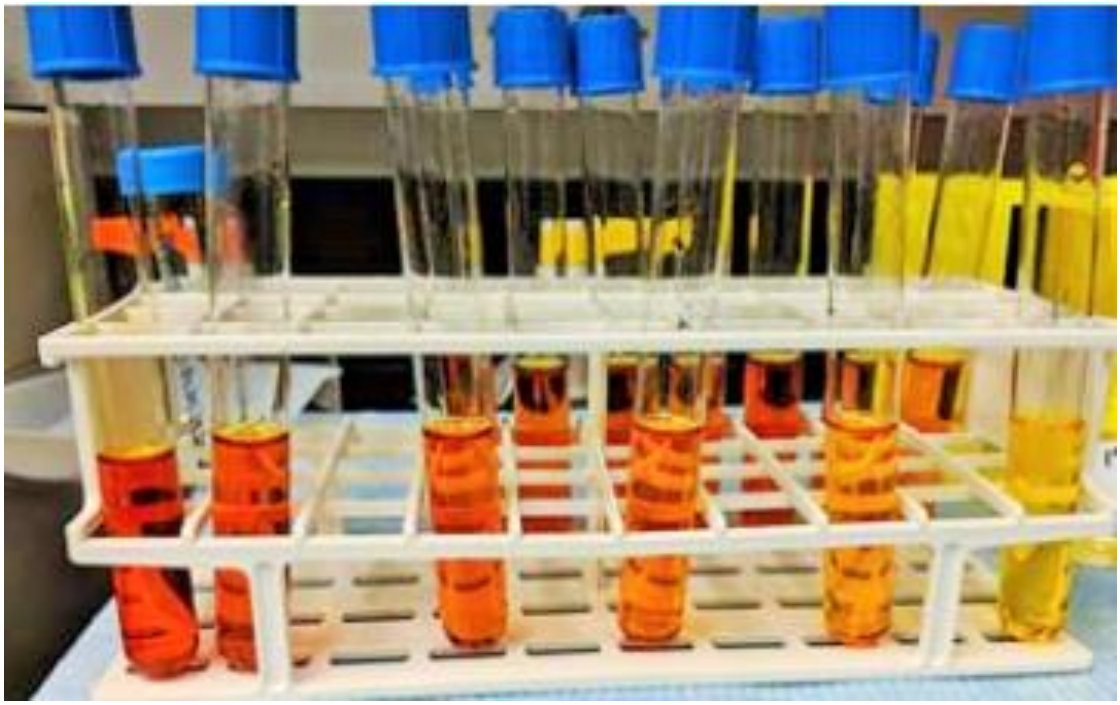


Figure 3.3. *Standards with Color Produced by Maltose Color Reagent and Heat.*

Additionally, a blank sample made entirely of distilled water was made. After that, distilled water was added to each test tube until the volume, including the blank, reached 2 ml. After that, each tube received 1 ml of DNS reagent before being wrapped in aluminum foil. After five minutes of heating in a boiling water bath, the tubes were allowed to cool at room temperature. Following cooling, each tube received 9 ml of distilled water, which

was thoroughly mixed. The resultant dark orange-red (Figure 3.3) solution's absorbance was measured at 540 nm. Ultimately, a calibration curve was created by graphing the absorbance values on the Y-axis against the maltose concentration on the X-axis[111].

3.7.3.2 Preparation of Sample and Estimation of the Total Sugars

To encourage hydrolysis, 1 ml of the diluted molasses solution was combined with 20 µl of strong hydrochloric acid, and the combination was then incubated at 90ºC for five minutes. As required for the DNS assay, 0.05 ml of 5 N potassium hydroxide was then added to neutralize the acid and bring the solution's pH down to an alkaline level. The DNS procedure's next phases were carried out using the previously described methodology. Lastly, the established standard curve was used to calculate the sugar concentration[1][112].

3.7.4 Determination of pH of Sugarcane Molasses

The sample was first diluted with distilled water, after which its pH was determined using a digital pH meter (HANNA, Germany).

3.8 Preparation of Sugarcane Molasses Media

3.8.1 Direct (without Pretreatment) Media Preparation

First, distilled water was added to a conical flask containing raw sugarcane molasses to dilute it to the necessary volume. After that, the prepared medium was autoclaved for 20 minutes at 121°c and 15 pressure to sanitize it.

3.8.2 Pretreated Media Preparation

Initially, distilled water was added to a conical flask containing raw sugarcane molasses until the desired volume was reached. After adding potassium ferrocyanide ($K_4Fe(CN)_6$) to the molasses, it was heated to 90°C for 15 minutes to precipitate heavy metals, which were then filtered out. A 500 ml conical flask was quantitatively filled with the clarified molasses. As sources of nitrogen and phosphate, respectively, ammonium nitrate (NH_4NO_3) and potassium dihydrogen phosphate (KH_2PO_4) were introduced. To get the required final volume, the medium was further diluted with distilled water. After measurement, the pH was brought within the range of 5.0 to 5.6. The medium was then

prepared for fungal spore inoculation by autoclaving it for 20 minutes at 121°C and 15 pressure to sterilize it.

3.9 Inoculum Preparation

Under aseptic conditions, spores were removed from a culture plate that had been incubated for seven to nine days by gently scraping the spore clusters with a sterile wire loop after the plate had been rinsed with sterile distilled water. A hemocytometer (Neubauer chamber, Dolphin Pharmacy Instruments, India) was used to measure the spore concentration in the resultant spore suspension, which was then transferred to a test tube and examined under a light microscope. After that, the suspension's spore count was changed from 1 to 5 million per ml. Finally, the prepared molasses medium was aseptically mixed with the calibrated spore suspension.

3.10 Submerged Fermentation

The fermentation process was carried out by incubating the molasses medium seeded with spore suspension in a shaking incubator (Witeg, Germany). To assess the effects of various physical parameters on CA generation, slight modifications to the fermentation conditions were implemented.

3.10.1 Fermentation Conditions Maintained for CA Production

Three 500 ml Erlenmeyer flasks were filled with 10 ml of untreated sugarcane molasses each, and the mixture was diluted with 190 ml of distilled water to reach a substrate concentration of 5%. Similarly, to achieve a 7.5% substrate concentration, 15 ml of untreated sugarcane molasses were put into three more 500 ml flasks and diluted with 185 ml of distilled water. Prior to sterilization, all medium had their initial pH adjusted to 5.5. After that, the flasks were autoclaved for 20 minutes at 121°C and 15 pressure. Following chilling, 2% spore suspension was added to each flask, and they were then cultured for eight days at 28 °C and 160 rpm in a shaking incubator (Figure 3.4).[113].

3.10.2 Fermentation Conditions Maintained for CA Estimation with Fermentation Time

To reach a 5% substrate concentration, 15 ml of untreated sugarcane molasses were put into a 500 ml Erlenmeyer flask and diluted with 285 ml of distilled water. The medium's

initial pH was brought down to 5.5. After that, the prepared medium was autoclaved for 20 minutes at 121°C and 15 pressure to sanitize it. Following chilling, a 2% spore suspension was added to the molasses medium, which was then incubated for 20 days at 28 °C and 160 rpm in a shaking incubator (Figure 3.4).



Figure 3.4. *Incubator Shaker (New Brunswick Scientific, USA).*

3.11 Optimization of Fermentation Conditions

3.11.1 Effect of Pretreated Molasses in CA Production

To achieve a 5% substrate concentration, 10 ml of untreated sugarcane molasses were introduced to a 500 ml Erlenmeyer flask and diluted with 190 ml of distilled water. Another 10 ml of sugarcane molasses was processed with potassium ferrocyanide ($K_4Fe(CN)_6$) at 90 °C for 15 minutes in a different 500 ml Erlenmeyer flask to get rid of heavy metals, which were then filtered out. After processing, nitrogen and phosphorus sources, potassium dihydrogen phosphate (KH_2PO_4) and ammonium nitrate (NH_4NO_3), were introduced, respectively. After that, 190 ml of distilled water were added to the medium to keep the substrate content at 5%. A pH of 5.5 was set as the starting point. Both medium were autoclaved for 20 minutes at 121°C and 15 pressure to disinfect them.
3.11.2 Fermentation Conditions Maintained for CA Estimation with Fermentation Time

To reach a 5% substrate concentration, 15 ml of untreated sugarcane molasses were put into a 500 ml Erlenmeyer flask and diluted with 285 ml of distilled water. The medium's initial pH was brought down to 5.5. After that, the prepared medium was autoclaved for 20 minutes at 121°C and 15 pressure to sanitize it. Following chilling, a 2% spore suspension was added to the molasses medium, which was then incubated for 20 days at 28 °C and 160 rpm in a shaking incubator (Figure 3.4).

3.11.2 Effect of Substrate Concentration in CA Production

To get a 5% substrate concentration, 10 ml of untreated sugarcane molasses was added to a 500 ml Erlenmeyer flask and diluted with 190 ml of distilled water. Similarly, three distinct 500 ml Erlenmeyer flasks were filled with 15 ml, 20 ml, and 25 ml of molasses, which were then diluted with 185 ml, 180 ml, and 175 ml of distilled water, respectively, to reach substrate concentrations of 7.5%, 10%, and 12.5%. All medium had their original pH set to 5.5. After that, each flask was autoclaved for 20 minutes at 121°C and 15 pressure to disinfect it. Following room temperature cooling, the media were infected with a 2% *Aspergillus niger* spore suspension and cultured for 10 days at 28°C and 160 rpm in a shaking incubator (Figure 3.4).

3.11.3 Effect of Inoculum Size in CA Production

To get a final substrate concentration of 10%, 20 ml of untreated sugarcane molasses was added to each of four 500 ml Erlenmeyer flasks, which were then diluted with 180 ml of distilled water. Prior to sterilization, the pH of the media was brought down to 5.5. Every flask was autoclaved for 20 minutes at 121°C and 15 psi of pressure. The media were infected with Aspergillus niger spore suspensions at different concentrations of 1%, 2%, 4%, and 8% after they had cooled to room temperature. After that, the flasks were incubated for 12 days at 28 °C and 160 rpm in a shaking incubator (Figure 3.4).

3.11.4 Effect of the Initial pH of the Culture Media for CA Production

To achieve a 10% substrate concentration, 20 ml of untreated sugarcane molasses was added to each of four 500 ml Erlenmeyer flasks and diluted with 180 ml of distilled water. The media in the flasks had their initial pHs changed to 2.0, 4.0, 6.0, and 8.0 on an individual basis. After adjusting the pH, the media were autoclaved for 20 minutes at 121°C and 15 psi of pressure to sterilize them. After the media had cooled to ambient

temperature, they were infected with a 4% Aspergillus niger spore suspension and incubated for 10 days at 28°C with continuous agitation at 160 rpm in a shaking incubator (Figure 3.4).

3.12 Precipitation of Citrate

CA was extracted using the traditional precipitation method outlined by Dhillon et al. (2011) after the fermentation process was finished. Fungal biomass and other suspended particles were first eliminated from the fermentation broth by filtering it with Whatman filter paper. The filtrate was then mixed with an equal amount of 10% calcium hydroxide [$Ca(OH)_2$] solution, and the combination was heated for one and a half to two hours at 60 to 70 °C. Tricalcium citrate tetrahydrate precipitated as a result of this treatment; it was subsequently filtered out and given a thorough water wash. After treating the precipitate with an equivalent volume of 20% sulfuric acid (H_2SO_4), it was heated once again for one and a half to two hours at 60 to 70 °C. Following the mixture's final filtering, the filtrate (mother liquor) containing CA was gathered[13].

3.13 Marier-Boulet (Pyridine-Acetic Anhydride) Method for CA Estimation

The Marier–Boulet method is a widely used colorimetric technique for estimating citric acid concentration in fermented liquor. The procedure begins by clarifying the fermented sample through centrifugation or filtration (0.2-micron PTFE syringe filter) to remove suspended solids and microbial cells. The clarified sample is then appropriately diluted with deionized water to ensure the citric acid concentration falls within the assay's detection range.



Figure 3.5. *Standard Citrate and Sample solution after adding Pyridine-Acetic Anhydride*

1.0 ml of the prepared sample is combined with 1.3 ml of pyridine for the test, and then 5.7 ml of acetic anhydride is quickly added (Figure 3.5). For 30 minutes, this mixture is incubated at 32 °C to enable the citric acid and the reagents to create a yellow-colored complex. Following incubation, the solution is allowed to cool to ambient temperature before being tested for absorbance using a spectrophotometer at approximately 405 nm in comparison to a reagent blank. By comparing the absorbance with a calibration curve made from known citric acid standards processed under the same conditions, the quantity of citric acid in the sample is then ascertained[113].

3.13.1 Preparation of the Standard Curve of Citrate

Tri-sodium citrate dihydrate (MERCK) was first used to create a citrate stock solution (5 mg/ml). To get different dilutions, aliquots of 50, 100, 150, 200, 250, and 300 µL of this stock were put into different test tubes and filled with 4.95, 4.90, 4.85, 4.80, 4.75, and 4.70 ml of distilled water, respectively.

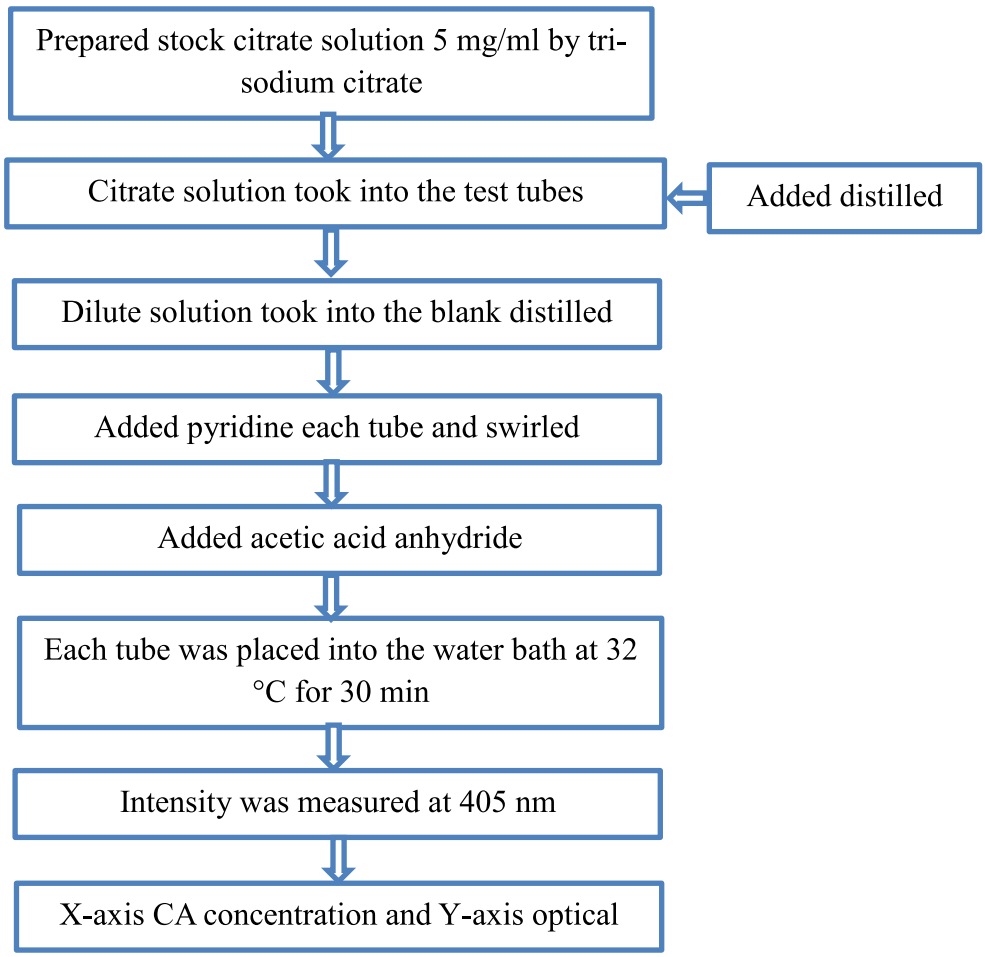


Figure 3.6. Overall CA estimation method (Marier-Boulet method)

One milliliter (1 ml) of each diluted solution was pipetted into a separate test tube, along with a blank tube that held one milliliter of distilled water. Each tube was then filled with 1.30 ml of pyridine (MERCK), which was swirled to mix it. After that, 5.70 ml of acetic anhydride (MERCK) was added, and the tubes were incubated for 30 minutes at a steady 32 °C in a water bath. A spectrophotometer was used to determine the intensity of the ensuing yellow tint at 405 nm following incubation, and absorbance values were noted. A standard calibration curve was then plotted, with citrate concentration on the X-axis and absorbance on the Y-axis[113]. The process showed in (figure 3.6)

3.13.2 Estimation of Citrate in Culture Filtrate

Following the previously mentioned process, the mother liquor was filtered and diluted 100 times. Simultaneously, a blank test was performed with 1 ml of distilled water in place of the culture filtrate. The developed standard calibration curve was then used to calculate the sample's citrate concentration.

3.13.3 Calculation for Estimation of CA Content

The amount of Citrate was measured in mg/ml using the standard curve by following the calculation[114]:

Citrate of concentration (mg/ml) × Dilution Factor/1000

Here, the dilution factor is 100

3.14 Statistical Analysis

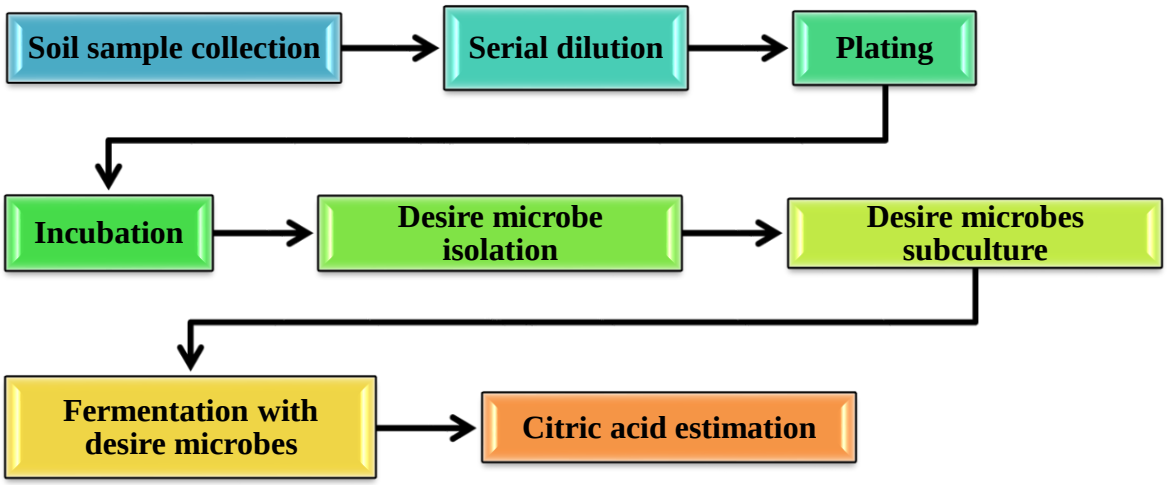
Software called GraphPad Prism, version 8.0.2, was used to perform statistical analyses. The significance of each test was assessed using a one-way ANOVA. The mean ± standard error of the mean (SEM) is how the data are displayed. Statistical significance was defined as a p-value of less than 0.05, and high significance as a p-value of less than 0.01.

Part II:

3.15 Background of Research Method

We aimed to enhance citric acid production by isolating novel microbial strains, specifically targeting Aspergillus species known for their citric acid fermentation capabilities. Soil samples were collected from diverse locations, as soil is a rich reservoir of varied fungal populations. These samples underwent serial dilution to reduce microbial density and facilitate the isolation of individual colonies. The diluted samples were then plated on selective growth media and incubated under optimal conditions to promote fungal growth. After incubation, colonies with potential for citric acid production were identified and carefully isolated. The selected colonies were subcultured to obtain pure strains. Once pure cultures were established, they were used to initiate fermentation under controlled laboratory conditions optimized for citric acid synthesis. Finally, citric acid concentration in the fermentation broth was quantified using appropriate analytical techniques to assess the production efficiency of each isolated strain.

The following steps were followed to conduct this study:



3.16 Collection of Soil Samples to Isolate of Fungus Species

For this experiment, over 14 soil samples were collected from various regions across Bangladesh. Specifically, samples were obtained from Curzon Hall and Mokarram Bhaban at the University of Dhaka, as well as from Suhrawardy Udyan and the Padma River bank near Shimulia Ghat. Additionally, soil was collected from Madai Khamar in Badarganj, Rangpur. From these samples, more than six fungal isolates were identified and selected for use in CAproduction through fermentation

3.17 Saline Solution Preparation for Serial Dilution of the Soil Sample

To weigh precisely 0.84 grams of sodium chloride (NaCl) using a precise balance in order to prepare 100 milliliters of a 0.84% saline solution. Pour the salt into a volumetric flask or sanitized beaker. Stir the container as you gradually add distilled water to make sure the NaCl dissolves completely. Once the mixture has completely dissolved, add more distilled water until it reaches 100 milliliters. Autoclave the produced saline for 15 minutes at 121°C to sterilize it if a sterile solution is needed for microbiological usage. When the solution has cooled, it can be used in the lab for dilution or microbiological suspension.

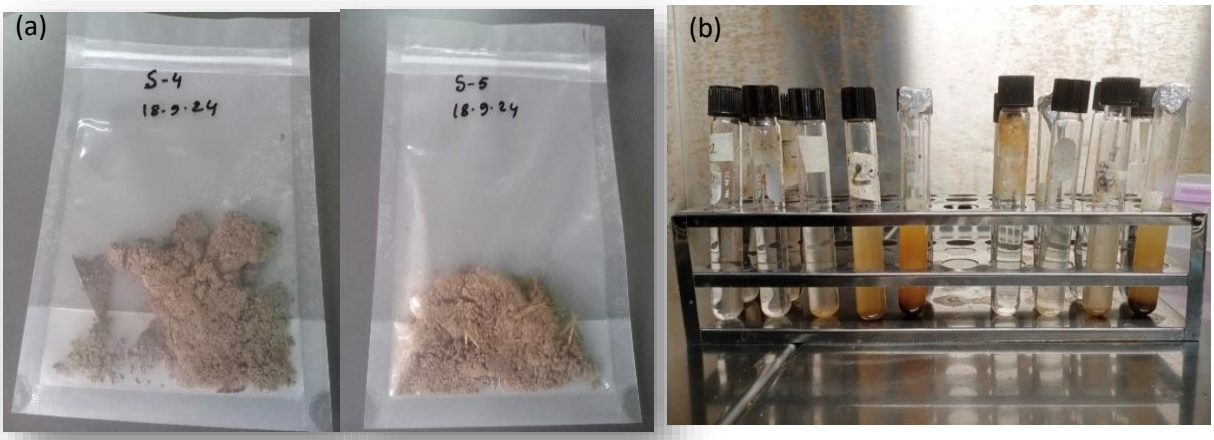


Figure 3.7*. (a) The collected Soil sample, (b) Saline solution with soil sample*

3.17.1 Serial Dilution Procedure to Isolate Fungal Strains

For the purpose of serial dilution, a 7-fold dilution series was performed using the collected soil samples to isolate microbial cultures. Initially, 1 gram of soil was aseptically taken from the polybag and transferred into a sterile test tube containing 9 ml of distilled water. A vortex mixer was used to properly homogenize the mixture in order to guarantee that the soil particles and microorganisms were suspended uniformly. A 10^{-2} dilution was obtained by transferring 1 ml of the well blended mixture into a second test tube that held 9 ml of brand-new, sterile distilled water. Up to the seventh dilution (10^{-7}), this serial dilution procedure was carried out step-by-step, moving 1 ml into subsequent tubes that each contained 9 ml of distilled water. Following the last dilution, 1 ml aliquots of specific dilutions were aseptically plated onto Czapek-Dox agar medium that had been sterilized. By lowering the microbial load to levels that could be counted, this technique made it easier to isolate and count different fungal colonies

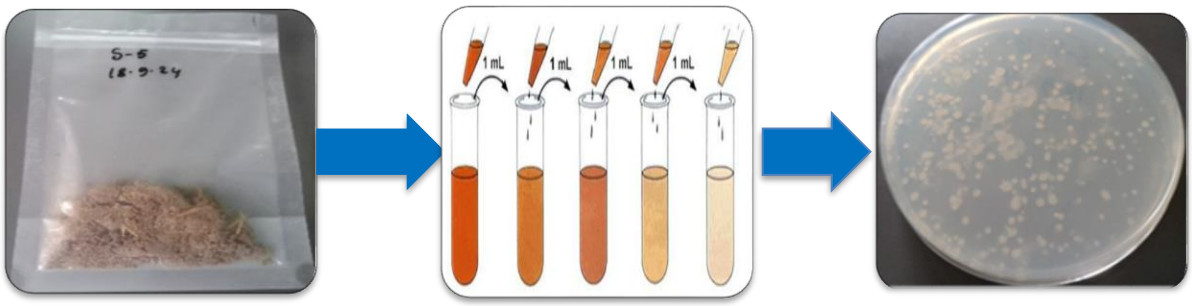


Figure 3.8. *Serial dilution for microbial growth of soil sample*

3.18 Preparation of Czapek Dox Agar Media for isolation of CA Producing Fungi

To promote robust microbial growth under controlled fermentation conditions, a defined solid medium was formulated with carefully balanced nutrients. Sucrose (30.00 g/L) served as the primary carbon source, providing readily metabolizable energy essential for microbial proliferation. Sodium nitrate (2.00 g/L) acted as the nitrogen source, supporting protein synthesis and cell growth. The inclusion of dipotassium phosphate (1.00 g/L) ensured adequate phosphate availability and helped stabilize the medium's pH.

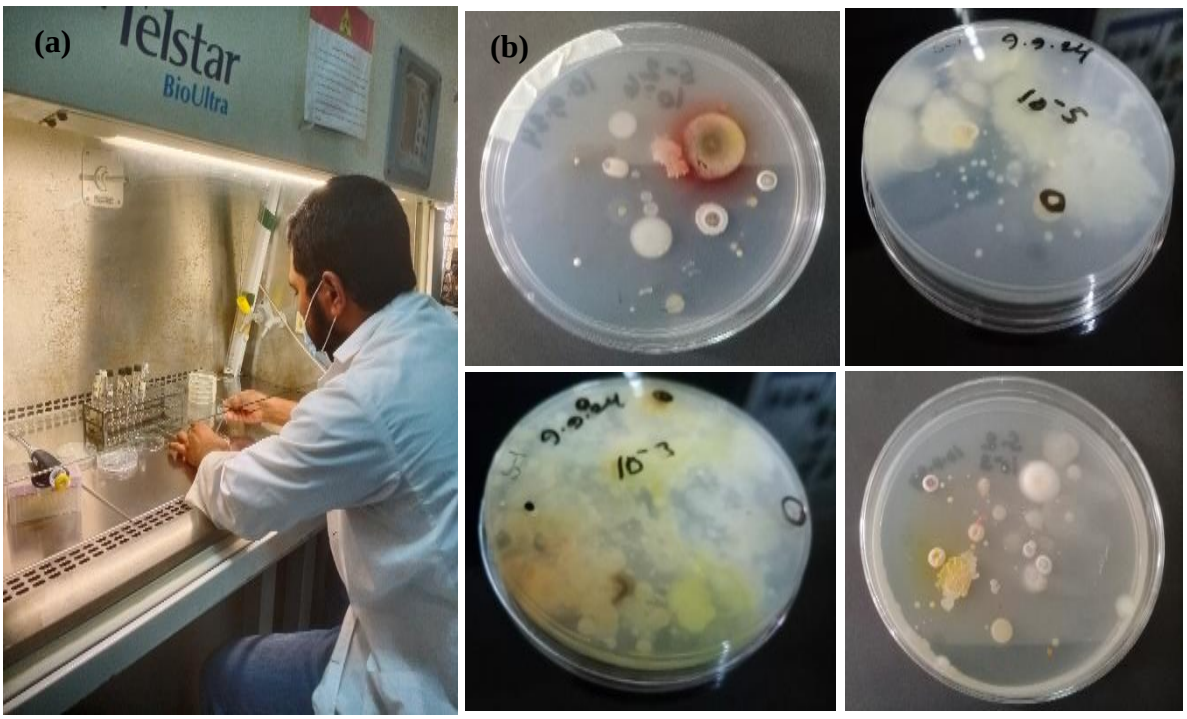


Figure 3.9. *(a) Sample (soil) plating in laminar air flow (b) microbial growth after few days*

Additionally, magnesium sulfate (0.50 g/L) and potassium chloride (0.50 g/L) supplied vital cations for enzymatic activity and osmotic regulation. A trace amount of ferrous sulfate (0.01 g/L) was added to fulfill the iron requirement for key metabolic processes.

To solidify the medium, 1.5 g/L of agar was incorporated, enabling the cultivation of microbial colonies on a solid surface. The medium's final pH was adjusted to 7.3 ± 0.2 at 25 °C, providing an optimal environment for the initial stages of microbial growth and physiological activity. This formulation supports selective growth, especially of fungi like Aspergillus niger, and is commonly used in primary screening and morphological studies[115].

Table 3.2. Czapek Dox Agar Medium Composition[115].

Ingredient	Amount of composition(g/L) in media preparation
Sucrose	30.00
Sodium Nitrate	2.00
Dipotassium Phosphate	1.00
Magnesium Sulfate	0.50
Potassium Chloride	0.50
Ferrous Sulfate	0.01
Agar	1.5
Final pH (at 25 °C) 7.3 ± 0.2	

3.18.1 Addition of Chloramphenicol into Czapek Dox Agar Media to Control Bacterial Growth

To support efficient microbial growth during fermentation, a nutritionally balanced solid medium was formulated with a combination of essential carbon, nitrogen, and mineral sources. The main carbon source, sucrose (30.00 g/L), supplied the energy required for biomass growth and microbial metabolism. The nitrogen supply, sodium nitrate (2.00 g/L), is essential for the production of proteins and nucleic acids. Dipotassium phosphate (1.00 g/L) was added to preserve phosphate availability and pH buffering. Additionally, ferrous sulfate (0.01 g/L) provided the trace iron needed for important metabolic processes, while

magnesium sulfate (0.50 g/L) and potassium chloride (0.50 g/L) were added to enhance enzyme activation and ionic equilibrium.

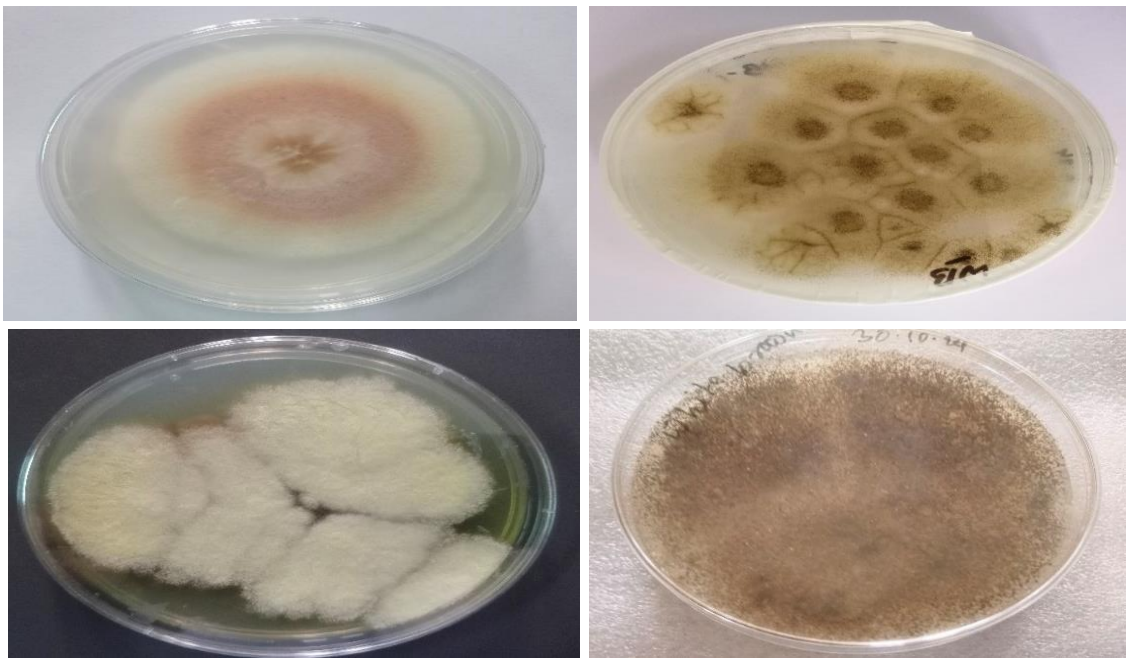


Figure 3.10. *Microbial strain after subculture of the isolated desire microbe from soil sample*

To solidify the medium, agar (1.5 g/L) was used, allowing for the isolation and surface growth of microbial colonies. To ensure selective growth and prevent bacterial contamination, chloramphenicol (0.15 g/L**)** was incorporated as an antimicrobial agent. The final pH of the medium was adjusted to 7.3 ± 0.2 at 25 °C**,** providing a favorable environment for microbial growth prior to secondary metabolite production such as citric acid.

3.19 Microscopic Characteristics of Isolated fungal Strains

The isolated Aspergillus strain was characterized under a microscope to look at its morphological characteristics, such as hyphal structure and spore production. Using a sterile needle, a tiny sample of the fungal colony was removed and placed on a sterile glass slide along with a drop of lactophenol cotton blue stain. After carefully covering the slide with a coverslip, it was examined at 400×magnification using a light microscope. Septate hyphae, conidiophores, vesicles, phialides, and conidia—all essential taxonomic features for recognizing Aspergillus species—were clearly visible thanks to the dyed preparation.



Figure 3.11. *Microscope (Boeco, Germany)*

Additionally, slide culture was performed on Czapek dox agar to preserve the natural arrangement of reproductive structures for more detailed observation. These microscopic features were compared with standard identification keys to confirm the genus-level identity of the fungal strain.

3.20 Selection of Fungal Strains for CA Production in Liquid Czapek Dox Media

For the production of CA through fermentation, a specifically formulated medium was prepared to support optimal microbial growth and metabolite synthesis. The medium composition included 2.00 g/L of sodium nitrate as the primary nitrogen source and 1.00 g/L of dipotassium phosphate to provide essential phosphate ions and buffering capacity. To support enzymatic activity and cellular stability, 0.50 g/L each of magnesium sulfate and potassium chloride were incorporated.

Trace iron, crucial for metabolic pathways, was supplied via 0.01 g/L of ferrous sulfate. Additionally, 0.15 g/L of chloramphenicol was included to inhibit bacterial contamination during fermentation. The final pH of the medium was adjusted to 7.3 ± 0.2 at 25 °C to maintain a stable environment conducive to CA biosynthesis [85].

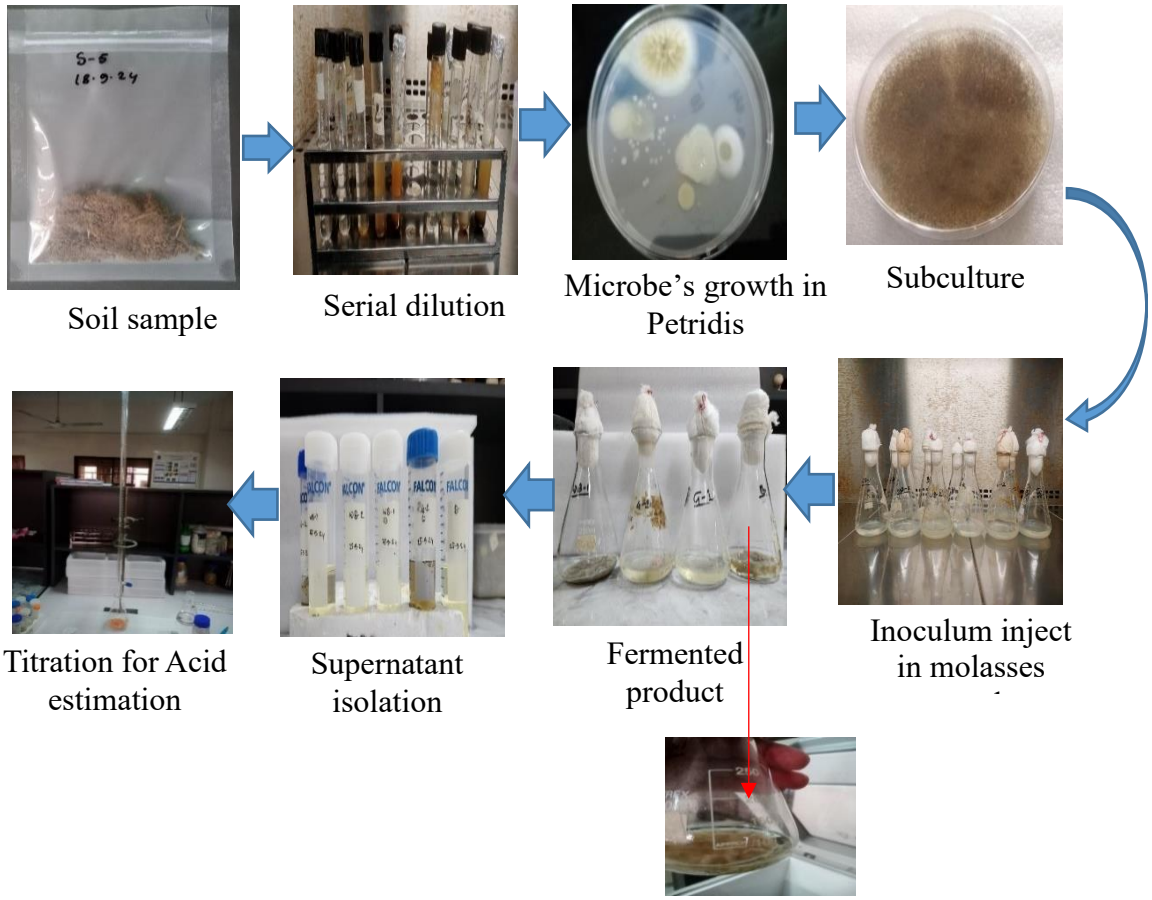


Figure 3.12. *Overall process diagram of CA production from isolated fungal strains (Aspergillus species)*

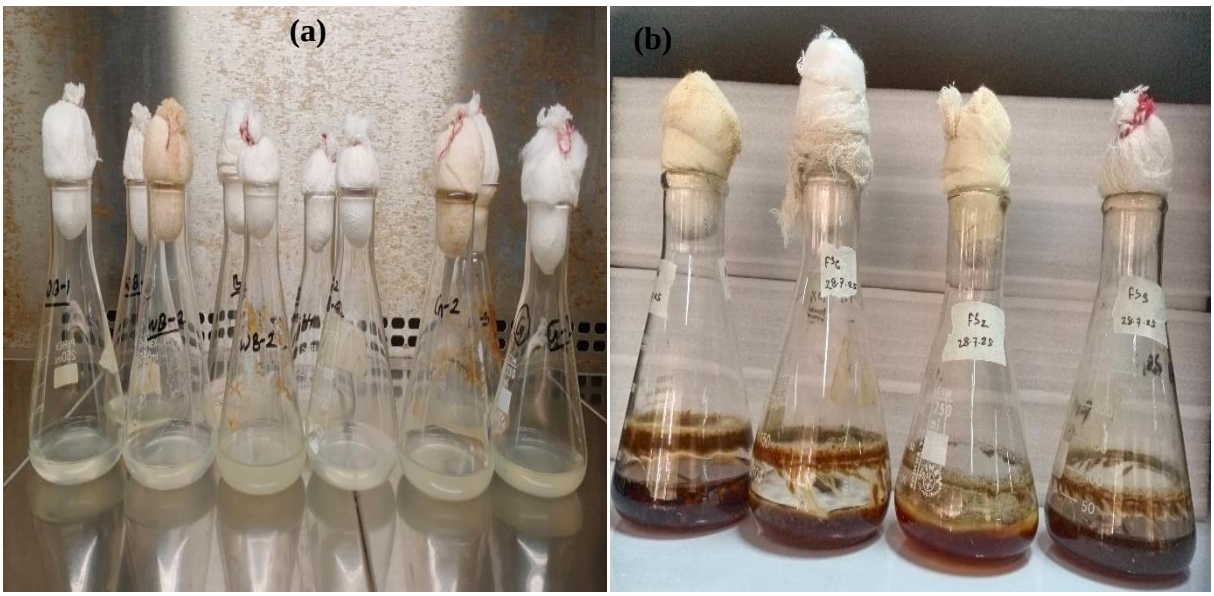


Figure 3.13. (A) Liquid Media Preparation (B) Fermented Broth



Figure 3.14. *Cell free supernatant for CA assay*

3.21 Gypsum Production Process

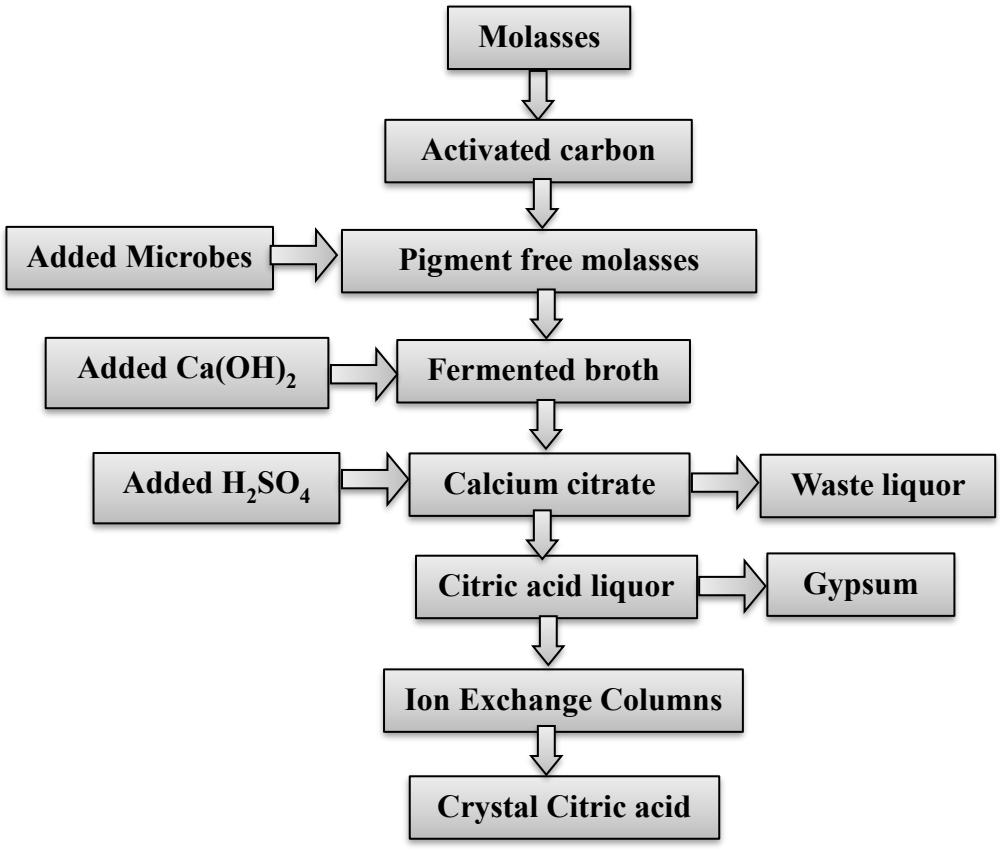


Figure 3.15*. Overall process for gypsum production*

First, raw molasses was passed through activated carbon (30% w/v) to remove the color. Next, the decolorized molasses was used for fermentation with the desired microbes. The fermentation process varied in duration 5, 7, and 10 days—in order to optimize

productivity. The environmental conditions were maintained at 37 °C and the initial pH was of 5.6. After fermentation, the separation of citrate and gypsum was required.

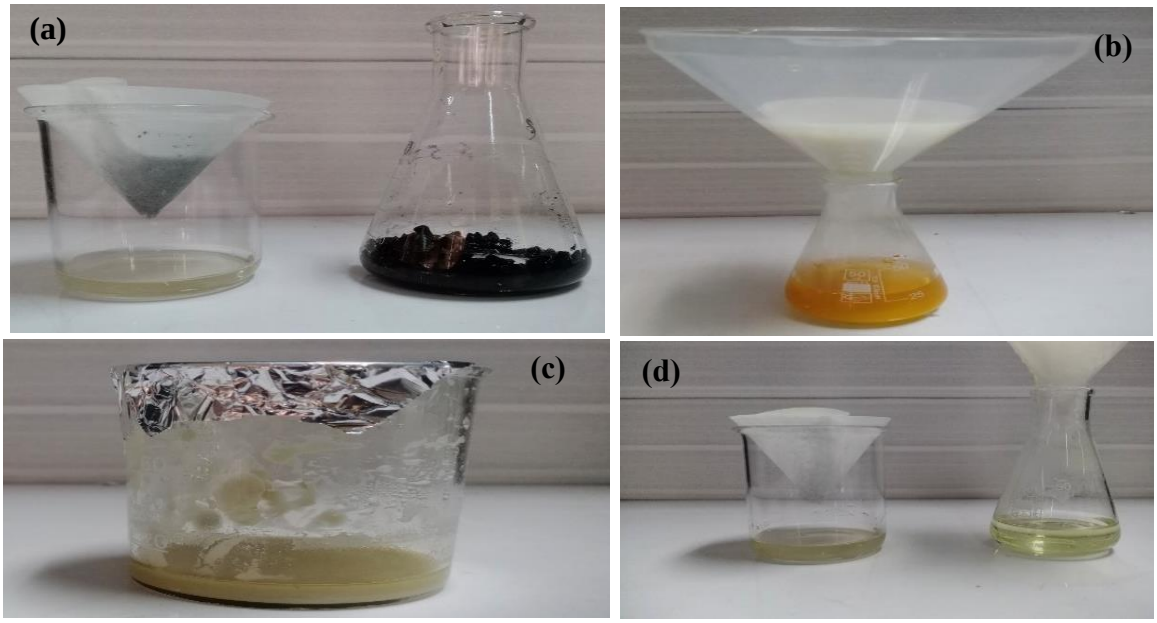


Figure 3.16. (a) Molasses after removing color pigment with activated carbon (b) fermented liquor (c) gypsum (d) fermented liquor after pretreatment with activated carbon

To achieve this, calcium hydroxide was added to the fermented broth, and the mixture was heated at 60 °C for 60 minutes, resulting in the precipitation of calcium citrate. Then, sulfuric acid was added to the calcium citrate, and the mixture was again heated at 70 °C for 60 minutes. This process yielded CA liquor and gypsum as by-products. The CA liquor was then purified by passing it through an ion exchange column. Finally, it was dried in an oven at 105 °C for 30 minutes to obtain crystalline citric acid (Figure 2.13) Showed the overall process.

3.22 Compositional analysis

3.22.1 FTIR

The compositional analysis of the citric acid and gypsum were carried out by examining its functional groups, and the characteristics of the molecule were determined from its absorbance spectrum. The functional group composition of the citric acid and gypsum were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) to identify the fundamental compositional groups. The IR spectra of the citric acid and gypsum were

obtained using a standard FTIR instrument (model SHIMADZU FTIR 4500) and recorded with an online pen plotter (Figure 3.17).



Figure 3.17. *FTIR instrument (model SHIMADZU FTIR 4500)*

3.22.2 UV-Vis Spectrophotometer

The UV-Vis spectrophotometer is an analytical instrument used to measure the absorbance of light by a sample at specific wavelengths, providing valuable information about the sample's concentration and composition. The process begins with the preparation of the sample solution, which must be clear and free of particulates to ensure accurate measurement. A blank solution containing all components except the analyte is also prepared to calibrate the instrument and account for background absorbance. After setting the desired wavelength, the instrument is first calibrated using the blank, establishing a baseline. The sample is then placed in the spectrophotometer, where it is exposed to light at the selected wavelength.

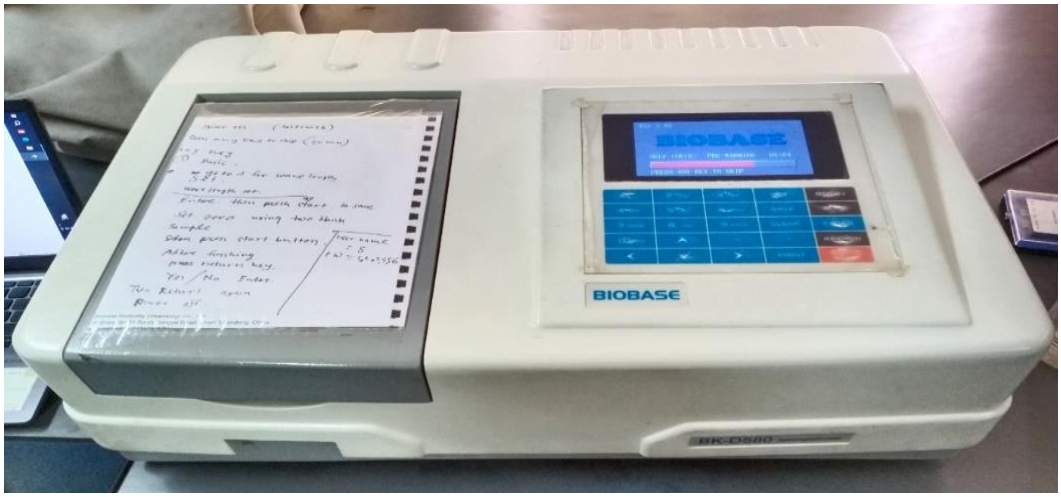


Figure 3.18*. UV-vis spectrophotometer.*

The instrument measures the amount of light absorbed by the sample, which is recorded as absorbance. Using the Beer-Lambert Law and a calibration curve generated from standards of known concentration, the absorbance data is used to determine the concentration of the analyzed in the sample.

3.22.3 Titration for CA estimation

To estimate the concentration of CA through titration, a standard procedure was followed using 0.1N sodium hydroxide (NaOH) as the titrant and phenolphthalein as the pH indicator.

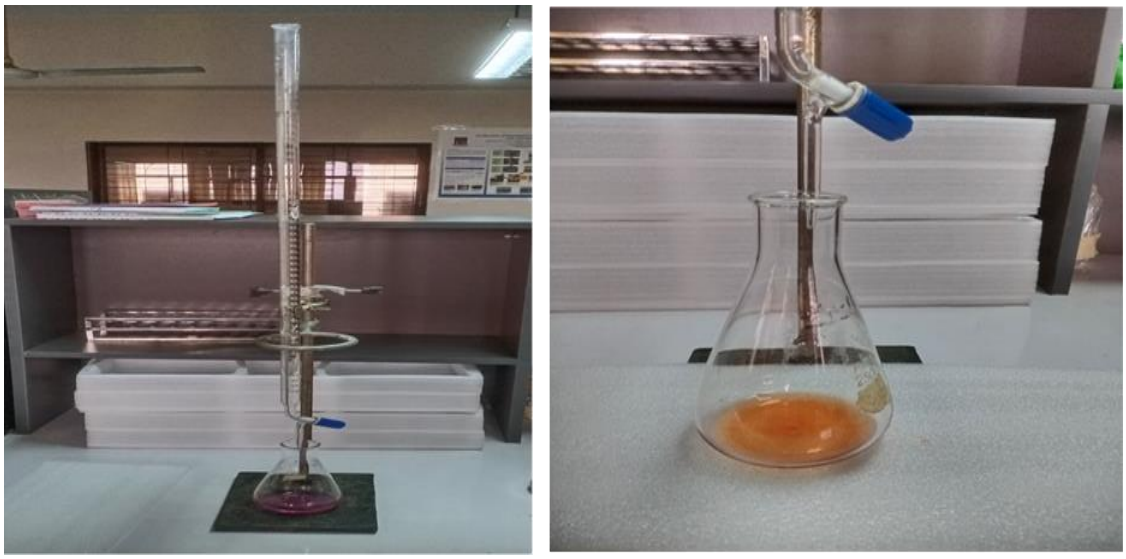


Figure 3.19. *CA estimation by titration*

First, the burette was carefully rinsed and filled with the NaOH solution, ensuring there were no air bubbles in the tip. Using a pipette, 25 ml of the CA sample was transferred into a clean conical flask. A few drops of phenolphthalein were then added to the flask, which remained colorless in the acidic solution. The flask was placed beneath the burette, and NaOH was added drop by drop while gently swirling the contents. The titration continued until a persistent pale pink color appeared, signaling the endpoint. The volume of NaOH consumed was noted, and the process was repeated multiple times for accuracy. The amount of CA present was calculated using the titration formula based on the volume of NaOH used and the known normality.

CHAPTER 4.

RESULTS

Part-I

4.1 Growth kinetics of *Aspergillus niger* F-81

Aspergillus niger is a filamentous fungus known for its ability to tolerate high temperatures, making it highly thermotolerant. It reproduces asexually through spores, which allows it to grow rapidly and adapt to a wide range of environmental conditions when favorable factors such as temperature, moisture, and nutrients are present. Because of this adaptability, Aspergillus niger is considered an opportunistic organism, capable of thriving in diverse habitats. It grows particularly well in nutrient-rich media like Potato Dextrose Agar and Malt Extract Agar, which provide the sugars and nutrients necessary to support its robust growth and development.

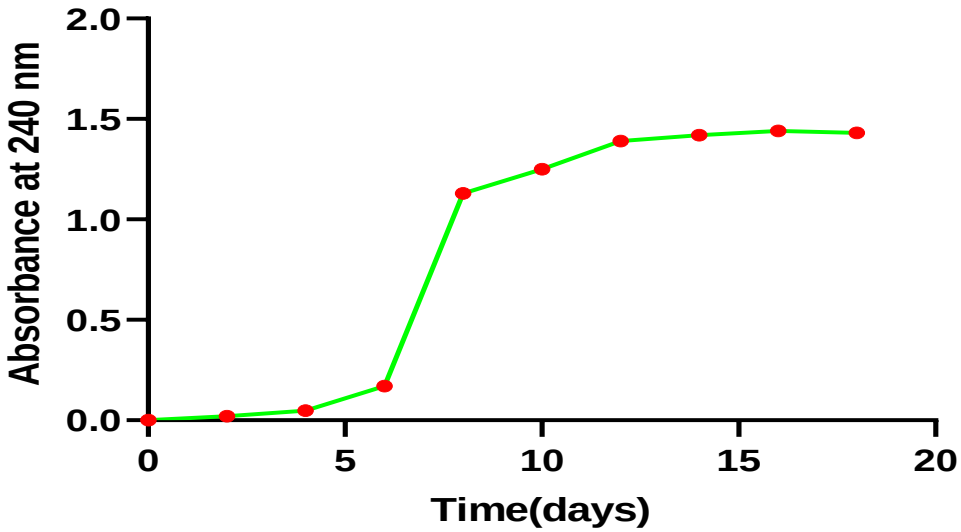


Figure 4.1 Growth curve of Aspergillus niger F-81.*100 ml of PDB media was inoculated with 2% spore(1.5*10^6/ml) of A.niger and placed into a shaking incubator at Room Temperature,160 rpm.The optical density was measured at 240 nm after every 2 days.*

4.2 Specifications of Sample (Sugarcane Molasses)

The cane molasses sample collected from the local sugar factory was analyzed to determine its quality by measuring key components. The total sugar content, which includes both reducing and non-reducing sugars, was found to be 53.5%. This indicates that the molasses contains a moderate to high amount of fermentable sugars, making it suitable for applications such as fermentation, food sweetening, and industrial use. The moisture content was measured at 24.7%, which is within the typical range for molasses

and suggests a balanced consistency that is neither too dry nor too wet. Proper moisture levels are important to ensure good handling properties and to prevent microbial spoilage during storage. The ash content, representing the mineral and non-organic residue left after burning, was relatively high at 16.3%. This reflects the presence of essential minerals but also indicates some impurities, which is common in cane molasses. Finally, the pH value of 5.6 shows that the molasses is slightly acidic, a natural characteristic that helps preserve it and contributes to its distinct flavor profile. Overall, these results suggest that the molasses sample is of typical quality for cane molasses, with good sugar content and balanced moisture, suitable for various commercial and industrial purposes.

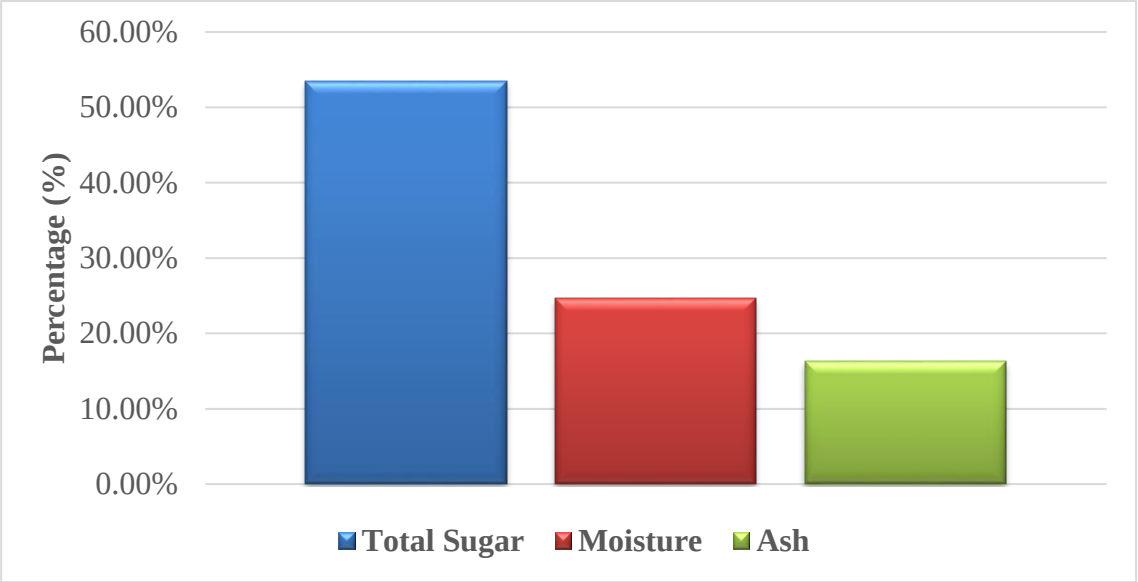


Figure 4.2. *Percentage of Total sugar, moisture, and Ash of Cane Molasses*

4.3. Validation of Marier-Boulet Method for Citric Acid Estimation

The accuracy of the Marier-Boulet Method (Pyridine-Acetic Anhydride Method) for determining and quantifying citric acid was assessed using three independent, non-pretreated culture batches grown in molasses media at concentrations of 5% and 7.5%. Each batch was inoculated with 2% inoculum containing 1.47×10^6 spores/ml, and the initial pH of the media was maintained at 5.6. Cultures were incubated in a shaking incubator at 160 rpm and room temperature for 8 days. Statistical analysis revealed no significant differences in citric acid concentrations among the three batches within each molasses concentration group. These consistent results confirm the reliability and validity of the Marier-Boulet Method for accurately quantifying citric acid under the described experimental conditions.

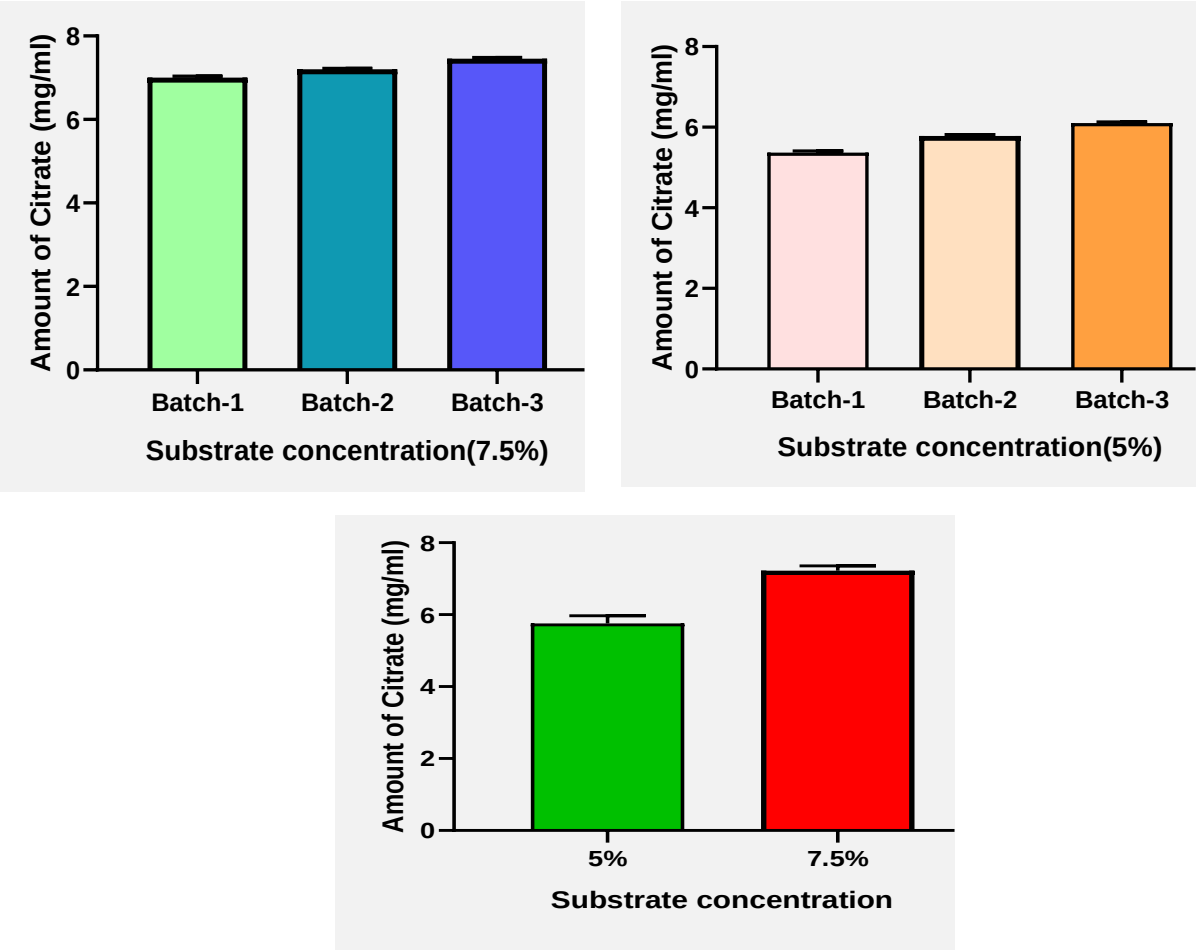


Figure 4.3 Validation of Marier-Boulet Method for Citric Acid Estimation. T-test was done. Difference between means of each pair of culture batches was observed. The data shows mean ± SEM, n=3.

4.4 Optimum Fermentation Conditions

4.4.1 Effect of Fermentation Time on Citric Acid Production

In this study, 250 ml of untreated 5% molasses medium was prepared and inoculated with a 2% spore suspension containing 2.1×10^6 spores/ml. The initial pH of the medium was adjusted to 5.6 before incubation. Fermentation was carried out in a shaking incubator at 160 rpm under ambient temperature to evaluate the influence of fermentation time on citric acid (CA) production. The results showed that citric acid synthesis increased progressively with time, reaching its maximum yield of 15.92 mg/ml on the 16th day of fermentation. This suggests that, under the given conditions, the 16th day represents the optimal fermentation duration for maximizing citric acid production (Figure 4.4).

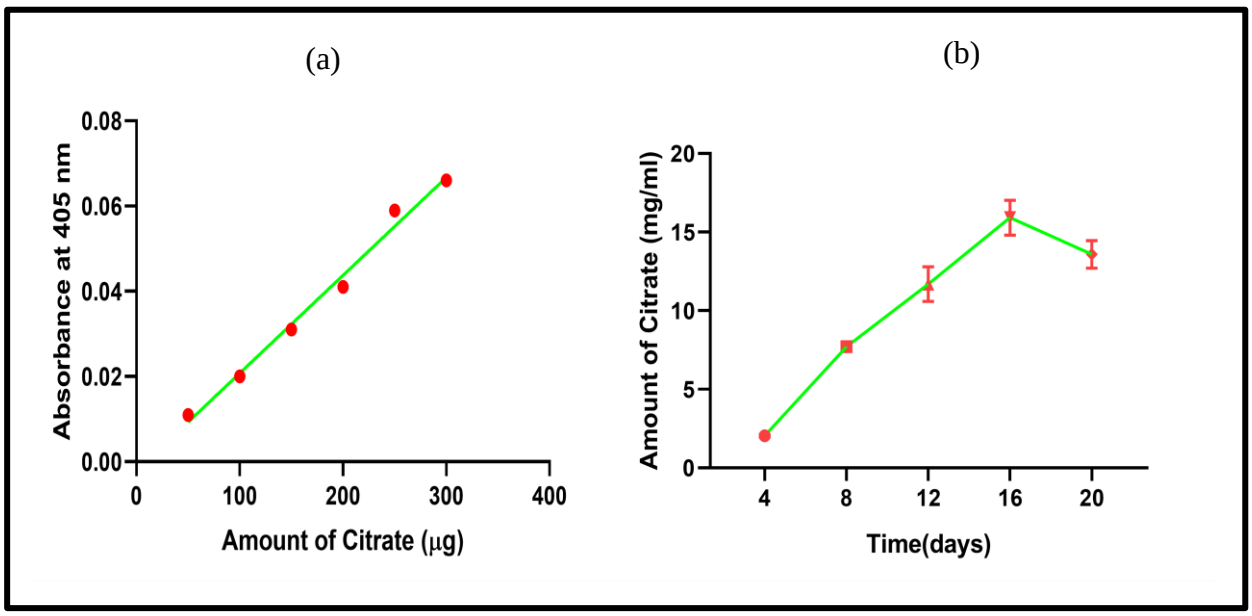


Figure 4.4. (a) Standard curve to determine CA concentration. (b) Effect of Fermentation Time on CA Production by Submerged Fermentation. Fermentation conditions maintained: Substrate concentration 5%, Inoculum size 2%, Initial pH 5.6, Temperature-RT and 160 rpm. The data shows mean ± SEM, n=3.

4.4.2 Effect of Pretreatment on Citric Acid Production

In this study, the effect of substrate pretreatment on citric acid (CA) production was evaluated using molasses as the fermentation medium. A 2% inoculum of *Aspergillus niger*, containing 1.81 × 10^6 spores/ml, was introduced into 100 ml of molasses medium prepared under two conditions: untreated (non-pretreated) and pretreated with potassium ferrocyanide ($K_4Fe(CN)_6$). The molasses concentration in both cases was adjusted to 5%. Before inoculation, the pH of the medium was standardized to 5.6, and the flasks were subsequently incubated at room temperature for 10 days in a shaking incubator at 160 rpm to ensure adequate aeration and nutrient distribution.

The pretreatment step using $K_4Fe(CN)_6$ was designed to remove heavy metals and other impurities present in molasses that are known to interfere with citric acid biosynthesis by *A. niger*. The results demonstrated that substrate pretreatment had a significant positive effect on citric acid production. The pretreated molasses medium yielded the highest citric acid concentration, reaching 12.49 mg/ml, which was considerably greater than the amount produced in the non-pretreated medium (Figure 4.5).

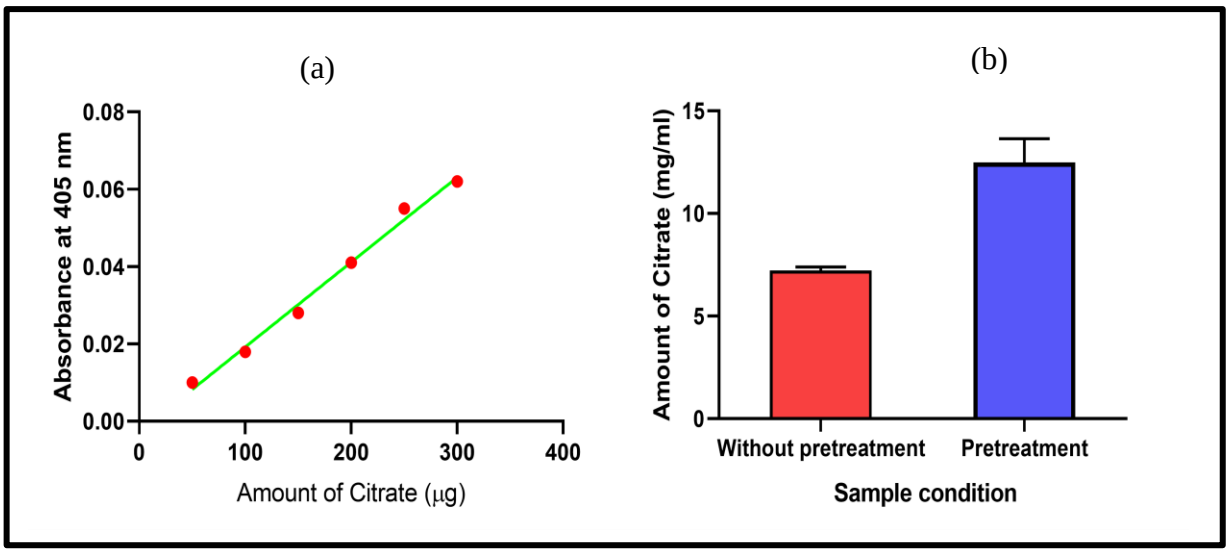


Figure 4.5. (a) Standard curve to determine CA concentration. (b) Effect of Pretreatment on CA Production by Submerged Fermentation. Fermentation conditions maintained: Substrate concentration 5%(one pretreated & other non-pretreated), Inoculum size 2%, Initial pH 5.6, Temperature-RT and 160 rpm, for 10 days. The data shows mean ± SEM, n=3.

4.4.3 Effect of Substrate Concentration on Citric Acid Production

In figure 4.6, the effect of varying substrate concentrations on citric acid production by Aspergillus niger was investigated using molasses at four levels: 5%, 7.5%, 10%, and 12.5% (v/v). Fermentation was performed under controlled and uniform conditions, including 2% inoculum size, an initial pH of 5.6, and agitation at 160 rpm, room temperature, and an incubation period of 10 days.

The results indicated a clear influence of substrate concentration on citric acid yield. Among the tested concentrations, the maximum citric acid production (12.09 mg/ml) was observed at 10% (v/v) molasses. At lower concentrations, yields were considerably reduced, with 5.18 mg/ml and 6.36 mg/ml of citric acid obtained from 5% and 7.5% molasses, respectively. Interestingly, further increasing the substrate concentration to 12.5% did not enhance production; instead, a decline in yield (7.48 mg/ml) was recorded.

This trend suggests that substrate availability plays a critical role in citric acid biosynthesis, with 10% molasses providing an optimal balance between nutrient sufficiency and metabolic efficiency of *A. niger*. At lower concentrations (5% and 7.5%), insufficient carbon availability may have limited biomass growth and secondary metabolite formation, thereby restricting citric acid accumulation.

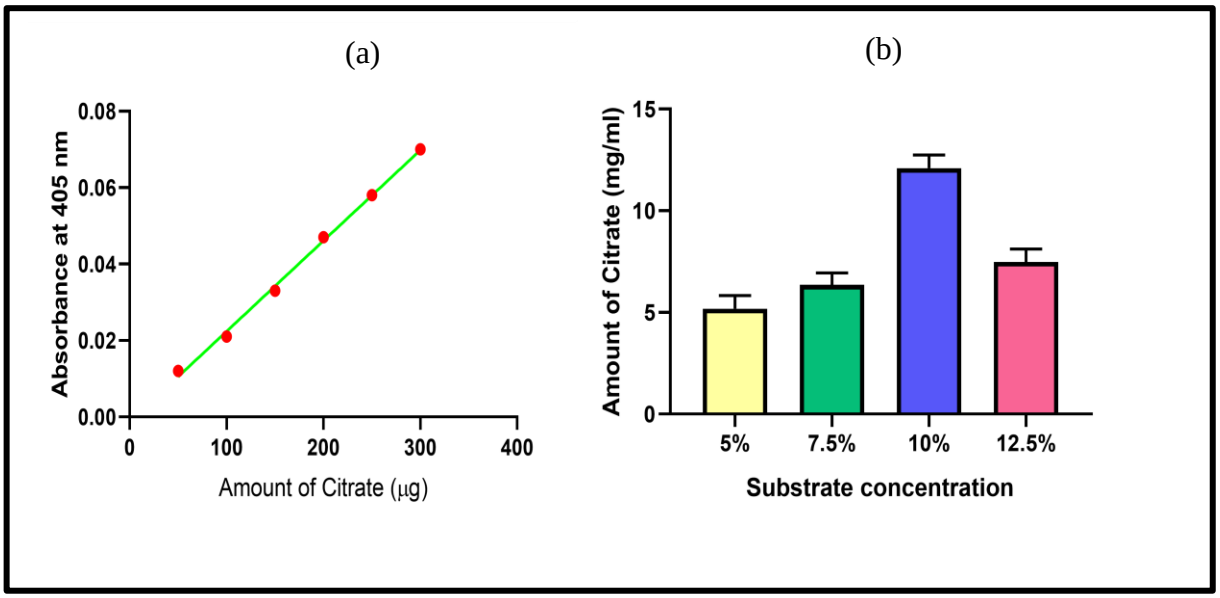


Figure 4.6. (a) Standard curve to determine CA concentration. (b) Effect of Substrate concentration on CA Production by Submerged Fermentation. Fermentation conditions maintained: Substrate concentrations 5%,7.5%,10% and 12.5%.Inoculum size 2%,Initial pH 5.6,Temperature-RT and 160 rpm for 10 days. The data shows mean ± SEM, n=3.

4.4.4 Effect of Inoculum Size on Citric Acid Production

The effect of inoculum size on citric acid production was systematically evaluated by employing inoculum levels of 1%, 2%, 4%, and 8% under controlled fermentation conditions using 5% sugarcane molasses as the substrate, an initial pH of 5.6, room temperature, and agitation at 160 rpm for a period of 12 days. The results clearly indicated that inoculum size had a significant impact on the yield of citric acid, with 4% inoculum producing the highest concentration of 17.67 mg/ml.

At lower inoculum sizes, such as 1% and 2%, citric acid yields were considerably reduced, reaching only 3.76 mg/ml and 10.80 mg/ml, respectively. Conversely, an increase in inoculum size to 8% did not enhance production further but rather caused a decline in yield to 13.98 mg/ml. These findings suggest that an optimal inoculum size is critical for maximizing citric acid output, with 4% representing the most favorable balance between microbial growth, substrate availability, and metabolic efficiency under the tested conditions (Figure 4.7).

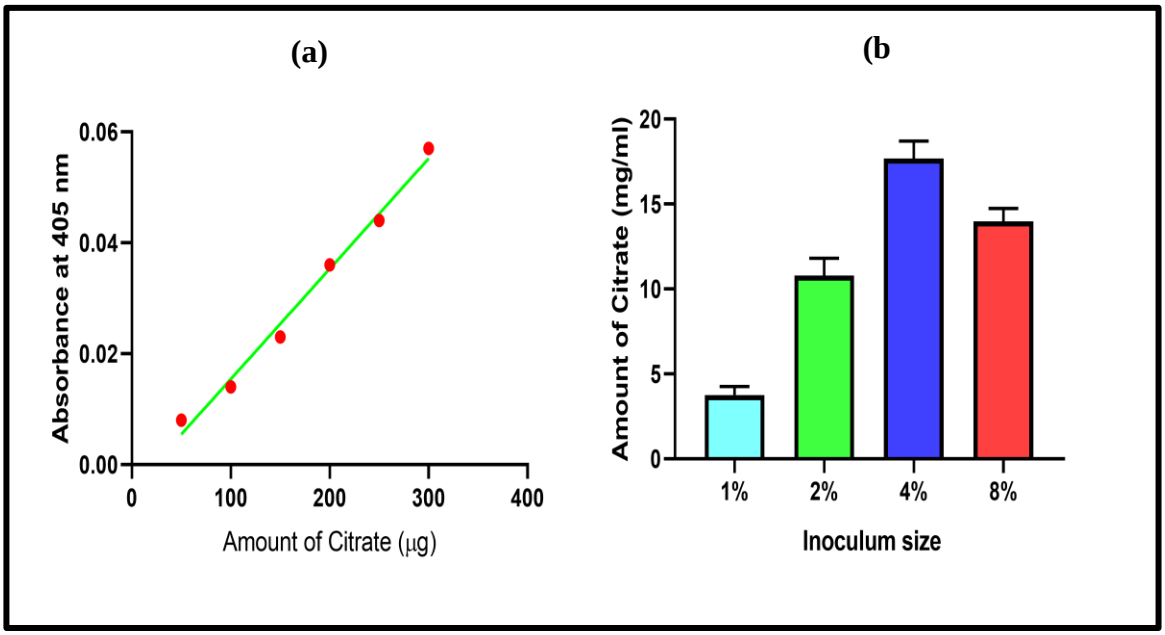


Figure 4.7. (a) Standard curve to determine CA concentration. (b) Effect of Inoculum size on Citric Acid Production by Submerged Fermentation. Fermentation conditions maintained: Substrate concentrations 5% and Inoculum size 1%, 2% 4% and 8%, initial pH 5.6, Temperature-RT and 160 rpm for 12 days. The data shows mean ± SEM, n=3.

4.4.5 Effect of Initial pH on Citric Acid Production

A 5% molasses medium was separated into four flasks, each holding 100 ml, in order to evaluate the impact of the starting pH on the generation of citric acid. Each flask's media's initial pH was set to 2, 4, 6, and 8, correspondingly.

For ten days, each flask was shaken at room temperature with agitation at 160 rpm after being inoculated with 2% *A. niger*. At a starting pH of 6, the maximum citric acid output of 9.70 mg/ml was recorded. In contrast, 3.24 mg/ml, 6.62 mg/ml, and 5.85 mg/ml of citric acid were produced at pH values 2, 4, and 8, respectively.

These results suggest that initial pH plays a significant role in citric acid biosynthesis. An initial pH of 6 appears to provide optimal conditions for *A. niger* metabolism and enzyme activity involved in citric acid production, while extremely acidic (pH 2) or alkaline (pH 8) conditions likely inhibit fungal growth or enzymatic efficiency, resulting in lower yields.

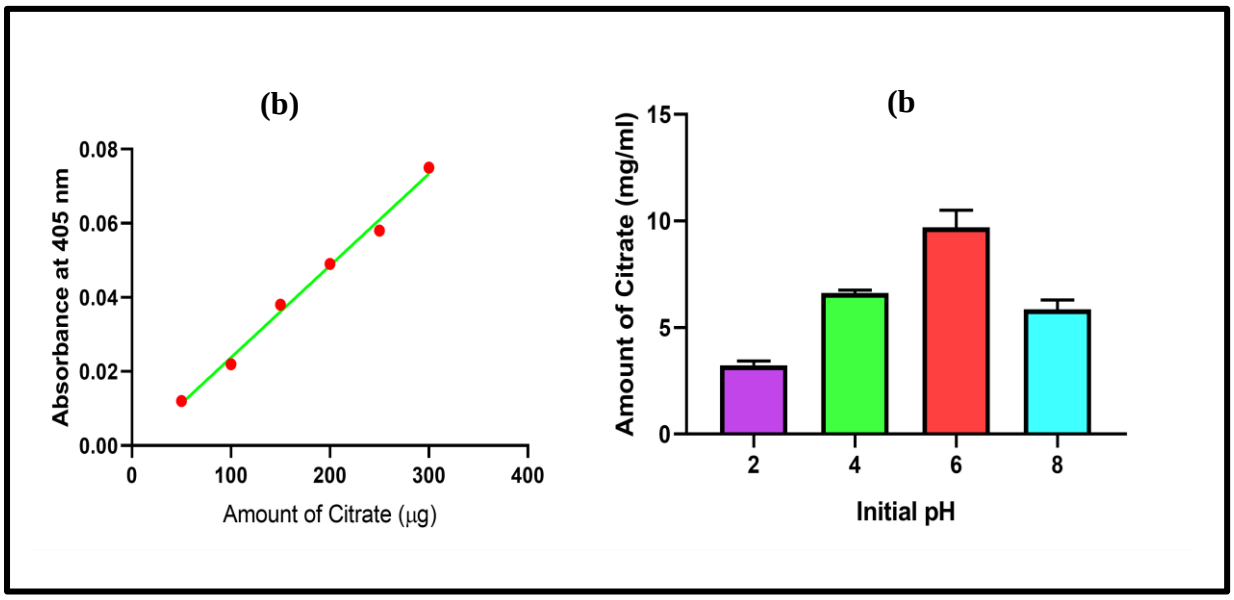


Figure 4.8. (a) Standard curve to determine CA concentration. (b) Effect of Initial pH on Citric Acid Production by Submerged Fermentation. Fermentation conditions maintained: Substrate concentrations 5%.Inoculum size 2%, Initial pH 2,4,6 and 8.Temperature-RT and 160 rpm for 10 days. The data shows mean ± SEM, n=3.

4.4.6 Citric Acid Production on All Optimized Parameters

After optimizing individual fermentation parameters, their combined effect was evaluated to determine the overall efficiency of citric acid production (Figure 4.9). The final fermentation was performed using 10% molasses concentration, an initial pH of 6, a 4% inoculum size, and incubation at room temperature with shaking at 160 rpm for 16 days. Under these optimized conditions, Aspergillus niger produced a maximum citric acid concentration of 28.25 mg/ml, which represents a substantial increase compared to non-optimized fermentations. This enhanced yield reflects the synergistic influence of all factors working together, as substrate concentration ensured sufficient carbon availability without inhibitory effects, the slightly acidic pH favored metabolic flux toward citric acid rather than by-products, the optimized inoculum size promoted rapid colonization and sustained production, and extended fermentation with constant aeration provided favorable oxygen transfer and nutrient distribution. Collectively, these conditions created an ideal biochemical environment that improved fungal growth kinetics, maximized carbon utilization, and enhanced citric acid biosynthesis, underscoring the critical role of systematic optimization in industrial fermentation processes.

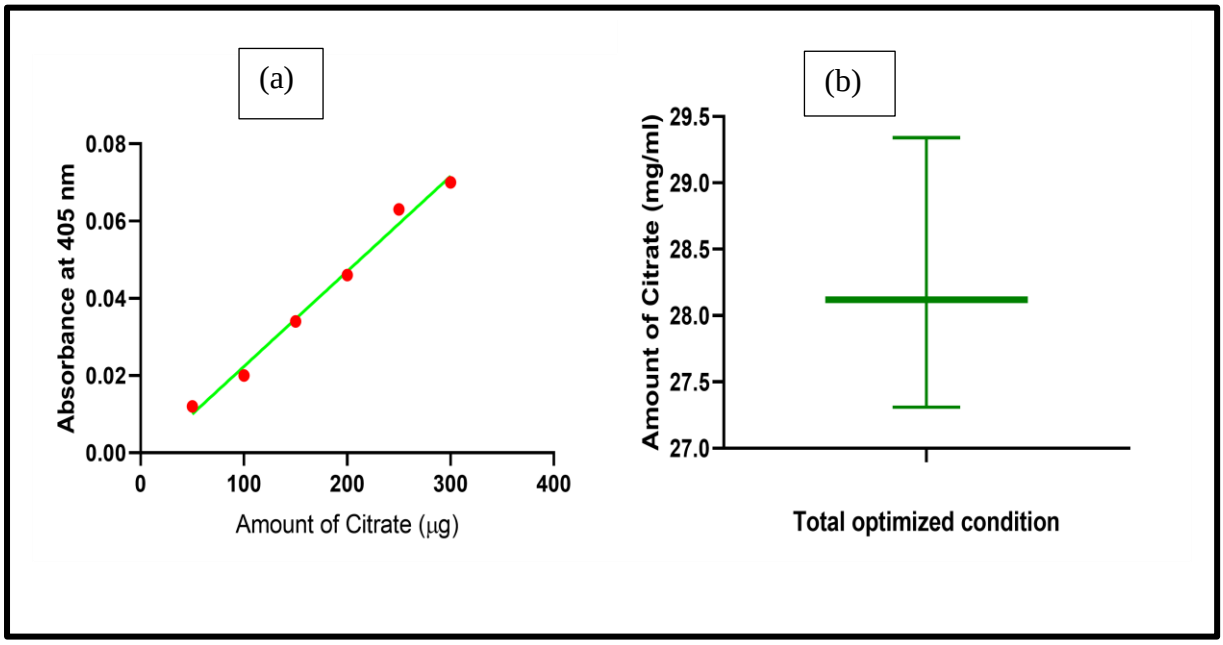


Figure 4.9. (a) Standard curve to determine CA concentration. (b) Citric Acid production on all optimized parameters by Submerged Fermentation. Fermentation conditions maintained: substrate concentration 10%, Initial pH 6, Inoculum size 4%, Temperature-RT and 160 rpm for 16 days. The data shows mean ± SEM, n=3.

Part-II

4.5 Enumeration of Fungal Strains and Selection for CA Production

A study was conducted to isolate and presumptively select fungal strains capable of citric acid (CA) production from various environmental locations of soil sample in Bangladesh using Czapek Dox Agar supplemented with chloramphenicol to inhibit bacterial growth and promote selective fungal proliferation (Table 4.1). Presumptive fungal isolation was achieved in most samples, with the highest growth observed in Sample S1 (6 isolates), followed by S3 (5 isolates) and S5 and S10 (4 isolates each), while S4 and S7 showed no visible fungal growth.

Presumptive selection of CA-producing fungi was recorded in several samples, with S3 yielding the highest number of potential CA producers (2 isolates), closely followed by S6 (2 isolates), and S1, S2, S8, and S10 each producing one isolate with CA production potential. Sampling sites demonstrated a wide diversity of fungal presence, with positive CA-producing isolates obtained from Curzon Hall, University of Dhaka (S1: FS2, FS4), Mokarram Bhaban, University of Dhaka (S4: FS1), Padma River Bank, Shimulia Ghat,

Munshiganj (S6: FS5, FS6), and Madai Khamar, Badarganj, Rangpur (S9: FS3), whereas Suhrawardy Udyan, Dhaka (S12) showed fungal growth but no CA producers.

Table 4.1. Presumptive Isolation and Selection of CA-Producing Fungi from Soil Samples

Sample	Location	Presumptive Isolation as a Fungal	Subculture in Czapex Dox Agar with Chloramphenicol	Presumptive Selection of CA Production Fungal
S1	Curzon Hall, University of Dhaka.	6	1	FS2, FS4
S2		3	1	
S3		5	2	
S4	Mokarram Bhaban, University of Dhaka.	0	0	FS1
S5		4	2	
S6	Padma River Bank, Shimulia Ghat, Munshiganj	3	2	FS5, FS6
S7		0	0	
S8		3	1	
S9	Madai Khamar, Badarganj, Rangpur	3	0	FS3
S10		4	1	
S11		3	0	
S12	Suhrawardy Udyan, Dhaka	2	0	0
S13		3	0	
S14		1	0	

These findings highlight the presence of promising CA-producing fungal strains across diverse ecological niches, from urban academic settings to riverbanks and rural agricultural regions, suggesting that locations such as Curzon Hall and the Padma River Bank may serve as valuable sources for further biotechnological research and citric acid production.

4.6 Citric Acid Production from 5% Substrate (Molasses) Concentration with Six Types of Microbial Strain (*A. niger*)

Citric acid production was evaluated using six different microbial strains (FS1 to FS6) in under uniform fermentation conditions. The fermentation process was carried out using a

5% molasses concentration as the carbon substrate, with an initial pH of 6.0, and conducted at room temperature. A standardized inoculum size of 1 cm^2 was applied for each strain. The production of citric acid was measured at three different time intervals—5, 7, and 10 days—to assess how production varied over time. The figure 4.10 showed, that in the Strain FS2 stood out as the most efficient producer of citric acid. It achieved a production level of 0.93 mg/ml at day 5, which further increased to 1.09 mg/ml on day 7, and slightly rose to 1.11 mg/ml by day 10. This consistent and significantly higher yield across all three time points indicates that FS2 possesses strong metabolic capabilities for citric acid biosynthesis under the given environmental conditions. Moreover, Strains FS5 and FS3 showed moderate productivity. FS5 reached 0.55 mg/ml at day 5 and gradually increased to 0.61 mg/ml by day 10. Similarly, FS3 exhibited a production of 0.51 mg/ml at day 5 and reached 0.61 mg/ml by day 10. Although these strains did not match the yield of FS2, they still demonstrated stable and progressive acid production.

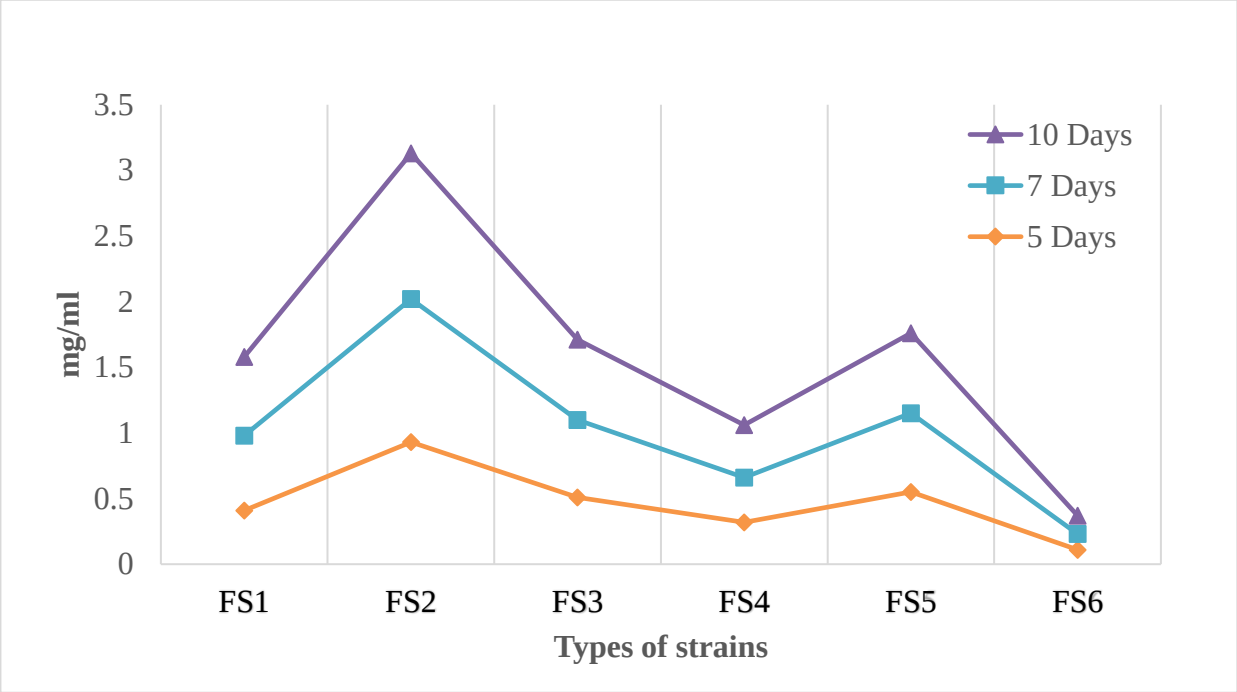


Figure 4.10. *Citric acid production in terms of 5 % substrate concentration and six varieties of microbial strains*

In Strain FS1 began with a relatively low citric acid yield of 0.41 mg/ml at day 5 and increased to 0.6 mg/ml by day 10. While this reflects improvement over time, the final concentration remained lower compared to FS2, FS3, and FS5. What is more, Strain FS4 produced even lower levels of citric acid, starting at 0.32 mg/ml, and reaching only 0.4

mg/ml by day 10. This suggests that its ability to convert molasses into citric acid was less efficient than other strains. On the contrary, Strain FS6 was the least effective, with minimal production starting at 0.11 mg/ml on day 5 and increasing only slightly to 0.14 mg/ml by day 10. Such low productivity implies that this strain might lack the enzymatic or metabolic capability required for citric acid production under these conditions.

This data clearly indicates that strain FS2 is the most promising candidate for citric acid production under the tested fermentation parameters. Its high and consistent yield demonstrates strong potential for industrial-scale production. In contrast, strains like FS6 and FS4 may not be suitable due to their low productivity. Monitoring citric acid production at multiple time intervals also helps identify not just the most productive strain but also the optimal fermentation duration for maximizing yield.

4.7 Citric Acid Production from 7% Substrate (Molasses) Concentration with Six Types of Microbial Strain (*A. niger*)

The data clearly demonstrates considerable variation in the citric acid production capacity of the six tested microbial strains under identical fermentation parameters. Figure 4.11 showed that the strain FS2 significantly outperformed the others, producing citric acid concentrations exceeding 1 mg/ml at all-time points and reaching 1.17 mg/ml by day 10. This suggests that FS2 possesses a highly efficient metabolic pathway for converting molasses into citric acid, likely with optimized enzyme activity and substrate uptake mechanisms. Its production curve indicates that it rapidly adapts to the fermentation environment and reaches near-maximum yield relatively early, implying good suitability for industrial processes where shorter fermentation times are economically beneficial. In contrast, strains FS5, FS1, and FS3 exhibited moderate citric acid synthesis, with values gradually increasing from around 0.5 mg/ml on day 5 to approximately 0.6–0.66 mg/ml on day 10. This steady upward trend suggests ongoing metabolic activity and substrate utilization, although their efficiency is substantially lower than FS2. These strains might benefit from further optimization of fermentation parameters such as nutrient concentration, pH adjustments, or inoculum size to enhance their productivity.

Strain FS4's relatively low production (0.38 to 0.45 mg/ml) indicates limited capacity to metabolize the substrate efficiently under these conditions. Meanwhile, FS6's very low output (only 0.19 mg/ml by day 10) reveals minimal citric acid biosynthesis potential,

which could be due to poor adaptation to the fermentation environment, weaker enzymatic machinery, or competition with other metabolic pathways.

Overall, this analysis highlights FS2 as the prime candidate for large-scale citric acid production, thanks to its superior yield and early peak production.

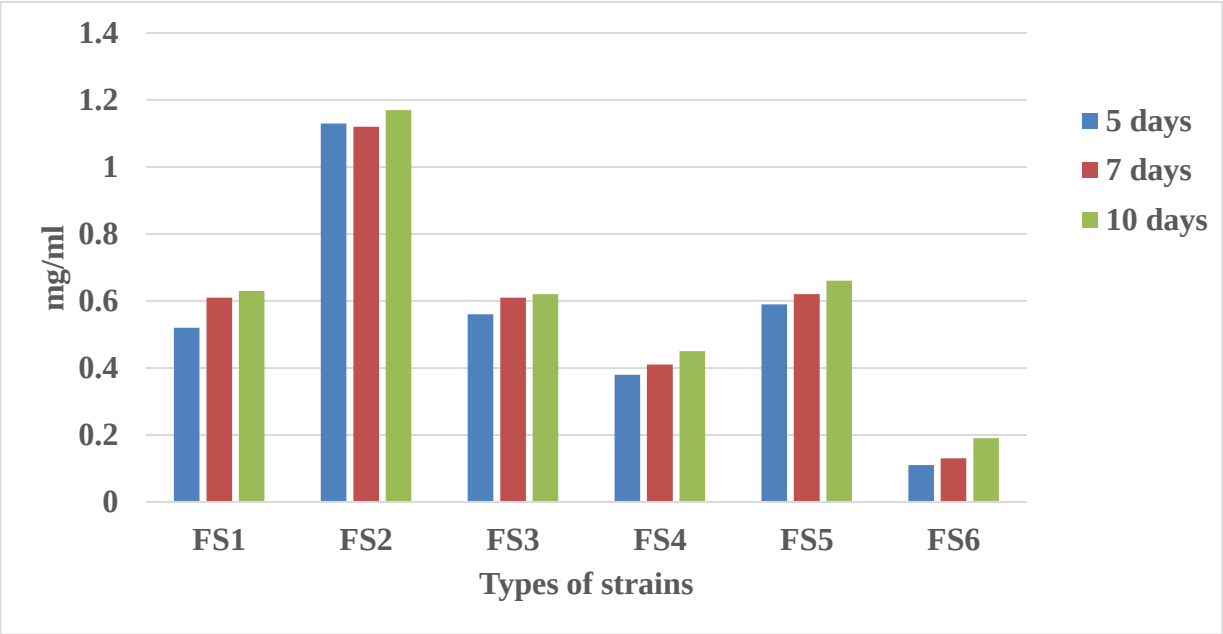


Figure 4.11 *Citric acid production in terms of 7.5 % substrate concentration and six varieties of microbial strains*

For other strains, tailored optimization strategies—such as adjusting environmental factors, prolonging fermentation time, or genetic improvement—may be necessary to improve their citric acid output to economically viable levels. Understanding these strain-specific differences is crucial for designing efficient fermentation processes in industrial biotechnology.

4.8. Characteristics of Isolated Six Microbial Strain

Microscopic examination of the isolated fungal strains was performed using lactophenol cotton blue staining, which allowed detailed observation of hyphal and spore morphology. The study revealed a diverse range of fungal structures, highlighting the morphological variation among the collected isolates. The isolated strains were characterized by septate hyphae and prominent conidial heads (Figure 4.13). Conidiophores were long, erect, and terminated in swollen vesicles, each supporting radiating chains of conidia. This

distinctive arrangement of conidial heads is a key feature for identifying members of the Aspergillus genus.

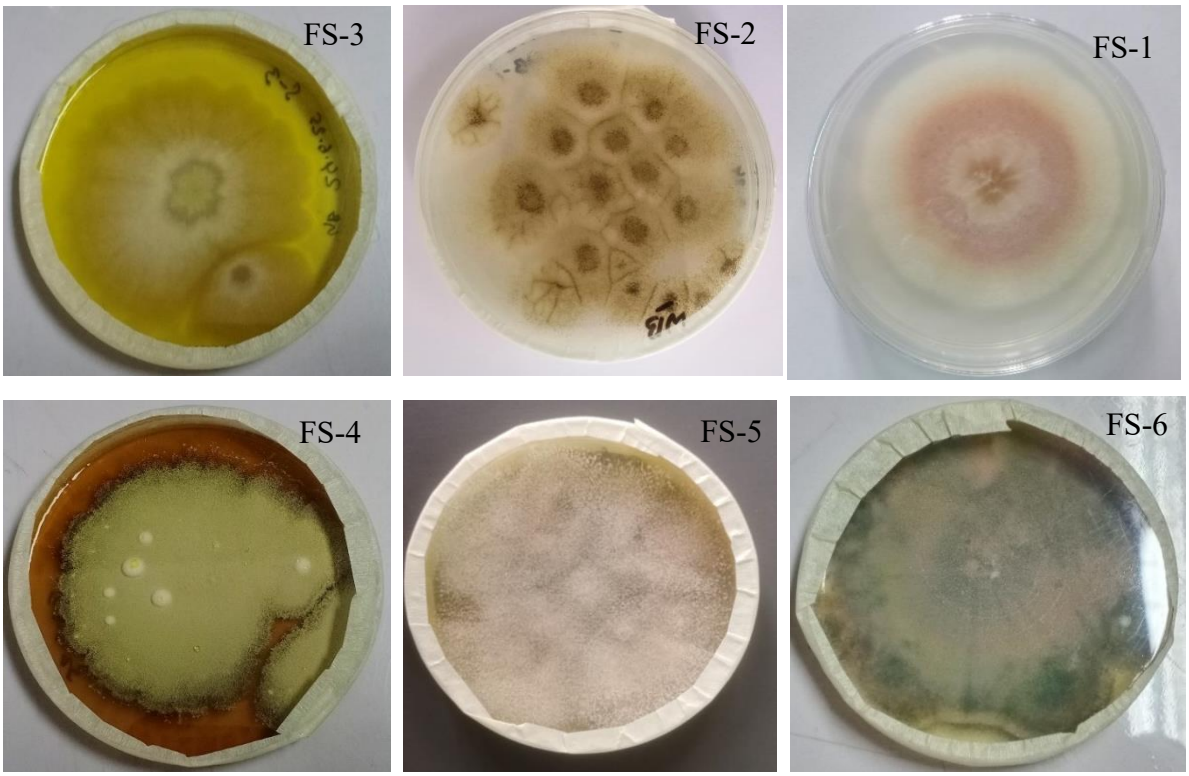


Figure 4.12. *The isolated desired six subcultural microbes*

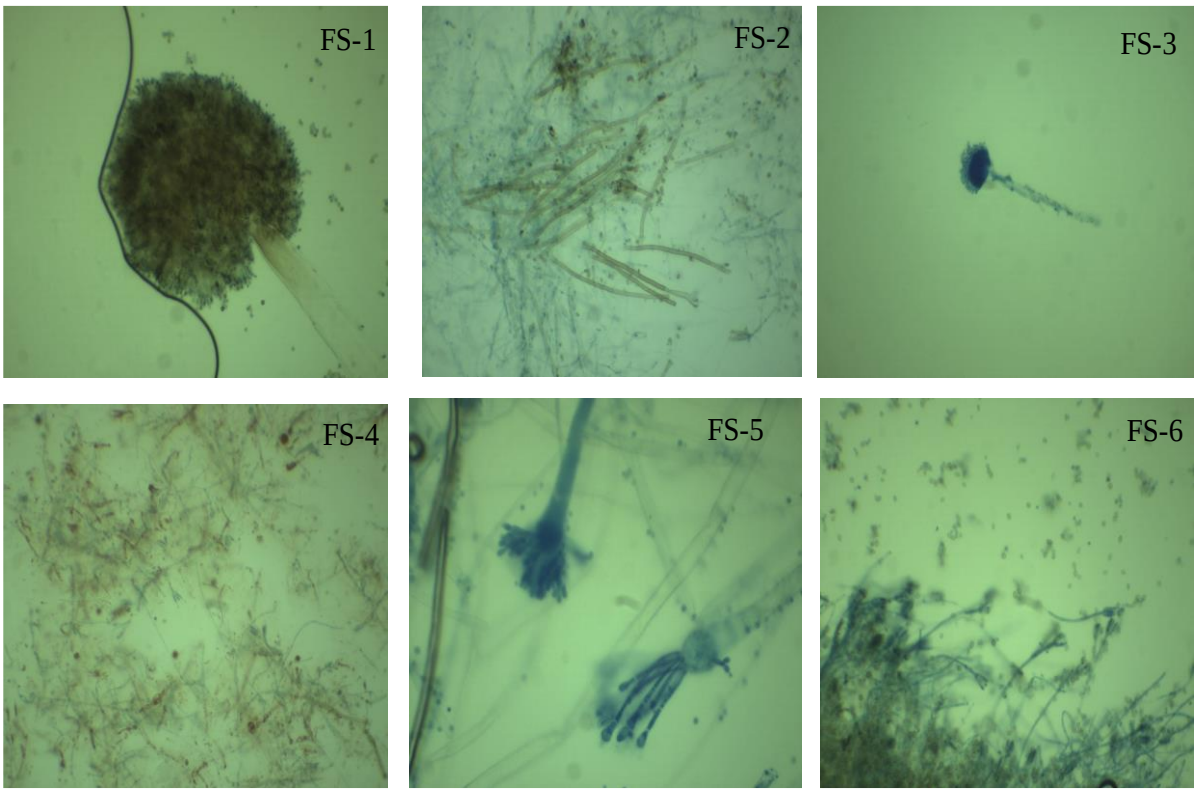


Figure 4.13. *Microscopic figure of isolated six microbes from the soil sample*

4.9 FTIR analysis of the Standard Citric Acid with Sugarcane Molasses Fermented Production Citric Acid

FTIR-based comparative composition of citric acid from cane molasses, standard sodium citrate, and commercial citric acid Fourier transform infrared (FTIR) spectroscopy was used to compare the chemical identity and purity of citric acid obtained from sugarcane molasses fermentation with a standard sodium citrate sample and a commercial citric acid reference.

The Fourier-transform infrared (FTIR) spectra of citric acid produced through fermentation of cane molasses (samples FS1–FS6) were analyzed and compared with standard sodium citrate and commercial citric acid (table 4.2). In figure 4.14**,** the broad O–H stretching band observed around 3338–3339 cm^{-1} in FS samples is characteristic of carboxylic acids. However, the presence of an additional peak near 2115 cm^{-1} in FS samples suggests the presence of impurities or side products.

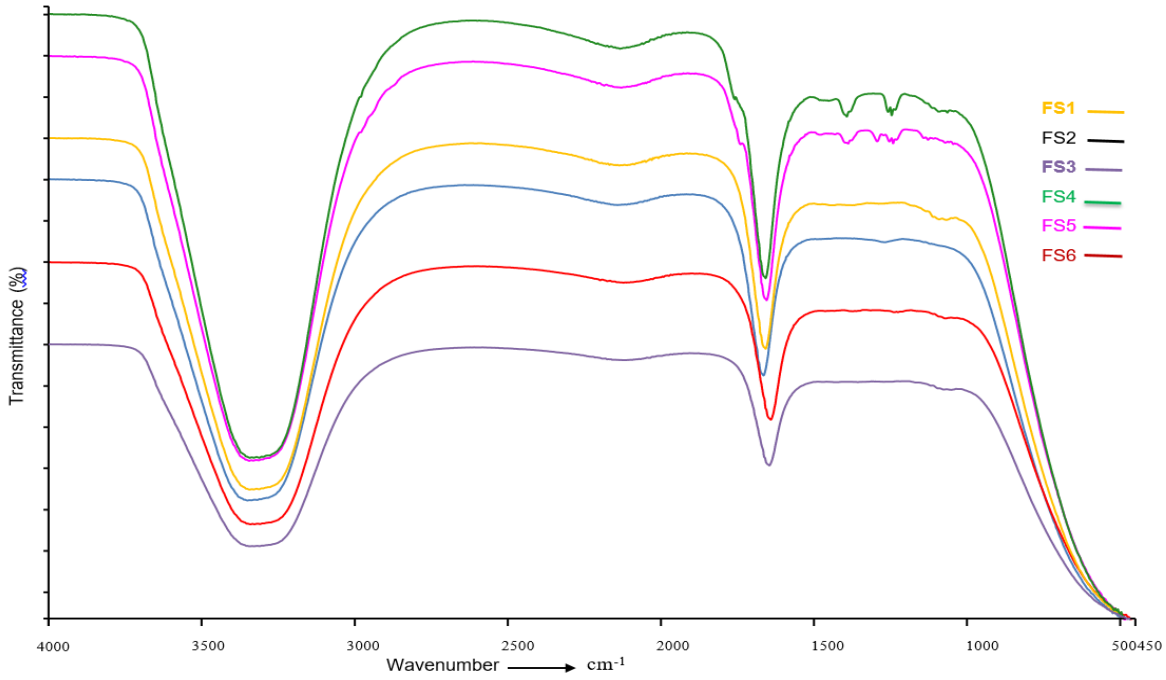


Figure 4.14. *FTIR analysis report of the fermented liquor from sugarcane molasses with (FS1-FS6) microbial strain*

The C=O stretching vibration around 1634 cm^{-1} in FS samples indicates hydrogen bonding, typical of citric acid in aqueous solutions. In contrast, in figure 4.15 & 4.16**,** standard sodium citrate and commercial citric acid exhibit sharper C=O stretches around

1720 cm^{-1}, reflecting their more crystalline and pure forms. The fingerprint region below 1500 cm^{-1} in FS samples shows fewer distinct peaks, suggesting lower purity compared to the well-defined patterns observed in standard and commercial citric acid. These FTIR analyses confirm that citric acid produced from cane molasses fermentation contains characteristic citric acid functional groups but also includes impurities, highlighting the need for purification to match the purity levels of standard and commercial citric acid.

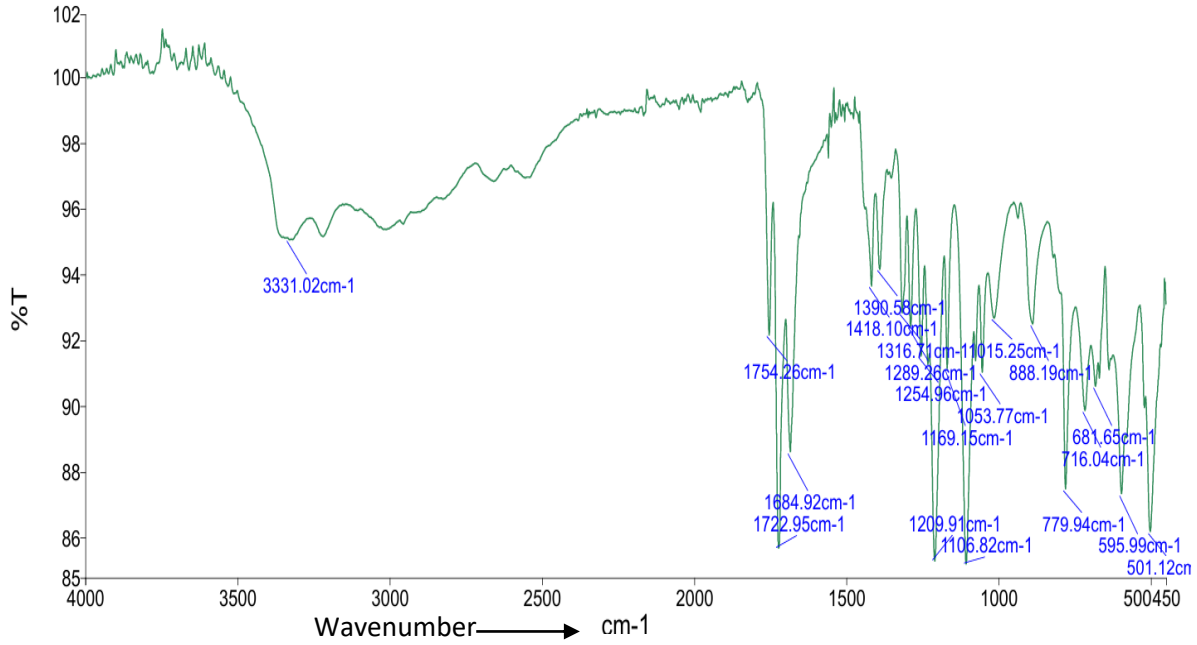


Figure 4.15. *FTIR analysis report of the commercial citric acid*

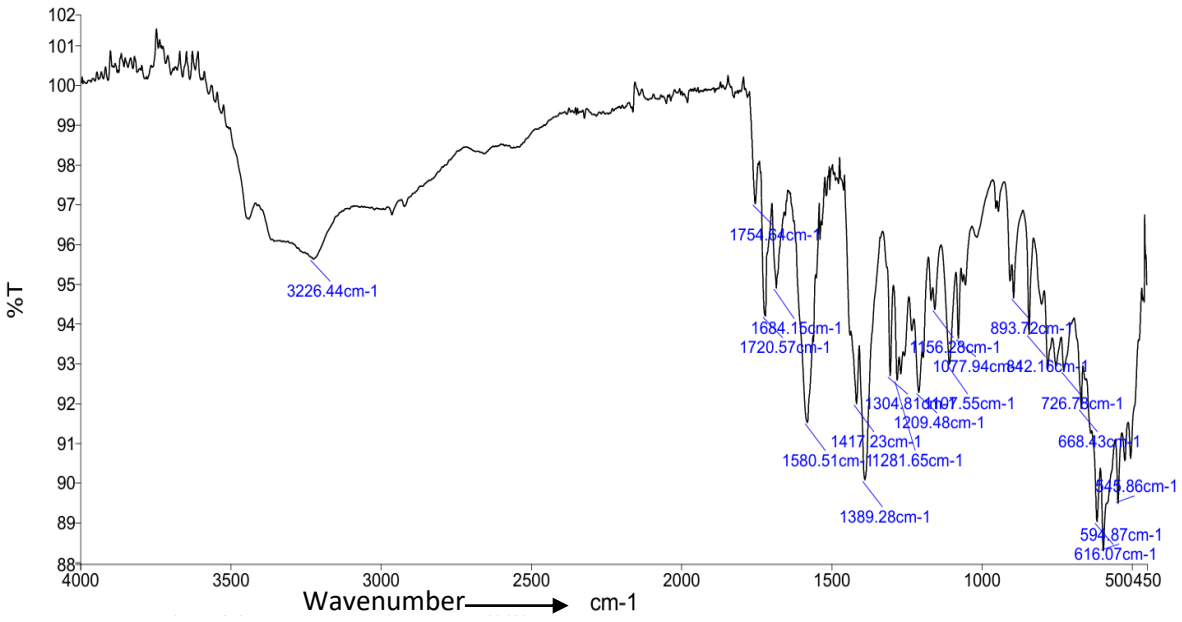


Figure 4.16. *FTIR analysis report of the standard sodium citrate*

Table 4.2. FTIR analysis of the fermented liquor from sugarcane molasses by different microbial strains (FS1-FS6) with commercial citrate and standard sodium citrate

Wavenumber (cm^{-1})	Functional Group	FS1–FS6 (Fermentation)	Standard Sodium Citrate	Commercial Citric Acid	Interpretation
3338–3339	O–H stretching (carboxylic acid)	Present	3226	3331	Broad peak indicates –OH group; slightly lower for sodium citrate due to salt formation
2110–2117	C≡C / C≡N or overtone	Present	–	–	Likely impurities or combination bands; citric acid normally does not have triple bonds
1634	C=O stretching (hydrogen-bonded carboxyl)	Present	–	–	Lower than pure citric acid, indicates hydrogen bonding or aqueous form
1720–1723	C=O stretching (free carboxyl)	–	Present	Present	Sharp peak in standard and commercial citric acid confirms free carbonyl groups
1365–1389	COO^- asymmetric stretching	Present in FS4, FS5	Present	Present	Symmetric/asymmetric carboxylate vibrations
1217–1269	C–O stretching	Present in FS4, FS5	Present	Present	Characteristic C–O vibration of carboxylic acids or salts
463–1304	Fingerprint region (C–C, C–O, O–H bending)	Sparse peaks	Present	Present	Less defined in FS samples, indicating lower purity

4.10 FTIR Analysis of Gypsum

The FTIR analysis of gypsum reveals characteristic absorption peaks at 3356.04, 1627.78, 1109.41, 1042.92, 877.38, 664.66, and 576.00 cm^{-1} (Figure 4.17), which align well with the known vibrational modes of calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$).

Table 4.3. FTIR analysis of Gypsum

Wavenumber (cm^{-1})	Assignment	Interpretation
3356.04	O–H stretching	Broad peak typical of water in gypsum (crystalline water / hydrogen bonding).
1627.78	H–O–H bending	Indicates the bending vibration of water molecules (H_2O) – confirms presence of dihydrate form.
1109.41	S=O asymmetric stretching	Strong band due to sulfate ion (SO_4^{2-}); typical for gypsum.
1042.92	S=O symmetric stretching / SO_4 deformation	Another sulfate-related band, possibly overlapping with O–H bending.
877.38	O–H deformation or S–O bending	Could be due to water deformation or sulfate lattice vibrations.
664.66	S–O bending / lattice vibration	Typically assigned to sulfate bending vibrations in gypsum structure.
576.00	S–O bending / skeletal vibrations	Lower frequency sulfate vibration; confirms SO_4^{2-} presence.

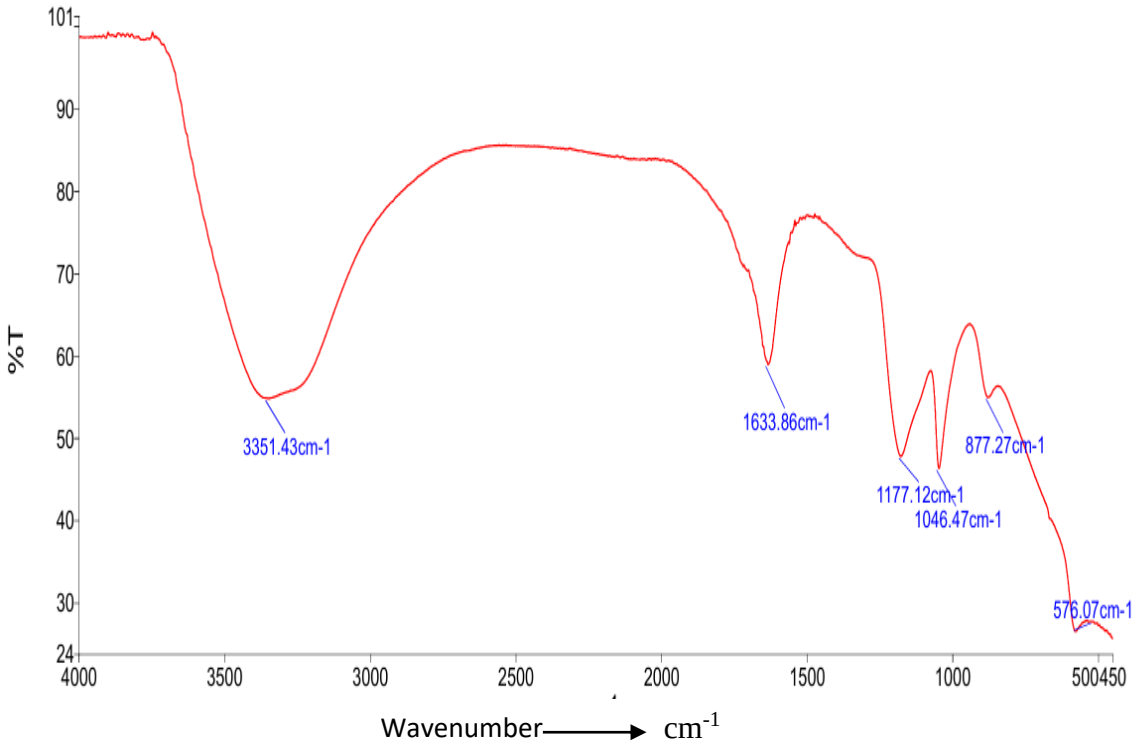


Figure 4.17. *FTIR analysis report of gypsum*

The broad band at 3356.04 cm^{-1} corresponds to O–H stretching vibrations, while the peak at 1627.78 cm^{-1} is attributed to H–O–H bending, both indicating the presence of structurally bound water molecules—a defining feature of gypsum. The intense bands at 1109.41 and 1042.92 cm^{-1} arise from asymmetric and symmetric stretching vibrations of the sulfate (SO_4^{2-}) groups, which are central to the gypsum crystal structure. Peaks at 877.38, 664.66, and 576.00 cm^{-1} are associated with bending modes of the sulfate ion and lattice vibrations.

CHAPTER 5. DISCUSSION

Part I

The food and pharmaceutical sectors depend on citric acid, the most abundant organic acid. Since imports account for nearly all of Bangladesh's demand, which is increasing at a rate of 3.5–4% per year, local manufacturing techniques are required. Following the discovery of the TCA cycle and glycolytic pathway in the 1950s, our understanding of its synthesis improved, resulting in innovations like the enhanced submerged fermentation technique created in the United States[38].

Although there are several fermentation techniques for producing citric acid, including Surface Fermentation (SF), Solid-State Fermentation (SSF), and Submerged Fermentation (SmF), this study used Submerged Fermentation since it was straightforward and simple to utilize. Molasses is the best medium for generating citric acid because it is less susceptible to changes in the medium's composition, which also allows for greater control over the use of a variety of substrates[79]. Numerous microorganisms, such as bacteria, yeasts, and fungi, have been examined for their ability to produce citric acid over time. However, *Aspergillus niger* strains are still the most dependable and efficient option. Their great production efficiency, simplicity of culture, and ability to employ a variety of raw materials as substrates are the main reasons for this[13]. Numerous studies have shown that fermentation duration significantly influences citric acid production. This organism primarily synthesizes citric acid during its exponential growth phase. In our investigation, the maximum citric acid production was recorded on the 16th day, corresponding to the early stationary phase of *A. niger.* As fermentation progresses and the organism enters the late stationary phase, citric acid levels begin to decline. This reduction is likely due to the depletion of available sugars in the medium. Similar trends have been reported by other researchers under varying fermentation conditions[116]. Furthermore, some studies have indicated that the pretreatment of raw substrates significantly impacts citric acid production during submerged fermentation. This conclusion is corroborated by our data, which show that pretreated substrates produced greater citric acid yields than untreated ones. Traces of iron, calcium, zinc, manganese, and, magnesium found in cane molasses, a frequent substrate, are known to prevent the production of citric acid. To mitigate this, potassium ferrocyanide is often applied as a chemical pretreatment agent, effectively precipitating iron and zinc. Additionally, enzymatic hydrolysis has been employed to pre-treat starchy materials like corn, wheat, and potatoes, helping maintain heavy metal concentrations below inhibitory levels and thus enhancing citric acid generation[7]. The

accumulation of CA and the concentration of sugar have a direct relationship on CA fermentation. In our study we found that maximum yield of CA was produced at substrate concentration of 10%. To Reduce the concentrations of sugar in the medium used for fermentation frequently cause oxalic acid to build up, which hinders the formation of citric acid and lowers yields[117].

According to Ali et al. (2002), insufficient mycelial growth at lower sugar concentrations is linked to low citric acid synthesis. They discovered that sugar levels of 15%, 18%, and 21% (w/v) produced citric acid yields of around 11% (w/v); nevertheless, these yields need long incubation periods[118]. Citric acid synthesis rises with increased carbon concentrations in the fermentation medium, according to Show et al. (2015). However, prior research has shown that maintaining sugar concentrations between 14% and 22% usually results in the best yields[31]. The size of the inoculum has a major impact on the production of citric acid. Numerous studies indicate that an inoculum size of 5% (v/v) of *A. niger* can produce the best citric acid yield under submerged fermentation circumstances. Furthermore, according to Ali et al. (2004), optimal citric acid generation was also achieved with an inoculum size of 4% (v/v), which is in close agreement with the results of this investigation.[119][87].

The study investigated the use of Parkia biglobosa fruit pulp to aid in the production of citric acid by *A. niger*. Although pH 2 produced the best citric acid yield (1.15 g/L), other factors like as inoculum size, concentration of substrate, temperature, and time of fermentation had a major impact on the final product. The results show that P. biglobosa pulp is a useful and affordable substrate for citric acid synthesis[120]. In this study, statistical optimization significantly enhanced the amount of citric acid produced by submerged fermentation. The ideal medium composition was determined to be 240.1 g/L beet molasses, 10.5 g/L maize steep liquor, and an inoculum density of 10^8 spores/ml using Response Surface Methodology using a Box–Behnken design. The yield was 14 times greater than that of the unoptimized medium, at 87.81%. Ideal process parameters of pH 4.0, an aeration rate of 6500 ml/min, and a temperature of 31.5 °C were determined by additional refining using a Central Composite Design. These results show that systematic statistical optimization can lead to significant increases in the efficiency of citric acid production, opening the door to large-scale, economically viable manufacture [121]. This work provides a comprehensive overview of citric acid biosynthesis, linking its physicochemical properties to its wide-ranging applications in food and medicine. By

examining key fermentation variables—such as fungal morphology, pH, aeration, carbon sources, nutrient limitations, and trace elements—it highlights their collective role in optimizing yields. Detailing both surface and submerged fermentation processes with *Aspergillus niger* and *Yarrowia lipolytica,* alongside the underlying metabolic pathways, the study underscores the importance of process control in industrial production. Moreover, its focus on recovery techniques and the valorization of agro-industrial wastes, like orange peels, reinforces citric acid's potential as a model product for sustainable, circular bio-economy initiatives[7].

Temperature significantly influences citric acid (CA) production, with the optimal range reported to be 25–30 °C for submerged fermentation. However, due to equipment limitations, this study could not investigate the effect of temperature. Instead, the fermentation was conducted at ambient room temperature, which stands as a limitation of the study and may have affected the maximum yield potential[58]. Citric acid production using two different date varieties as substrates resulted in high yields within a short incubation period. Optimal production was achieved at an initial pH of 3.0, a temperature of 30 °C, and an 8-day incubation period. The addition of 4% methanol further enhanced citric acid output in both media. These findings suggest that date-based media are cost-effective and support robust growth of *Aspergillus niger,* making them suitable for citric acid fermentation[122].

Enhancing citric acid (CA) production requires converting wild microbial strains into industrially efficient ones, a process not yet completed in this study. One widely used method for strain improvement is mutagenesis, where agents like gamma rays, UV radiation, and chemical mutagens are applied to induce favorable genetic changes. Recently, combining ultraviolet and chemical mutagens has proven effective in developing high-yielding strains for industrial applications. For selecting improved mutants, both passage and single-spore isolation techniques are employed, with the passage method being generally favored.

Part II

In present study, After 10 days of fermentation with 5% molasses at an initial pH of 6.0 at room temperature, strain FS2 revealed the greatest citric acid output of the six investigated strains in this study, yielding roughly 1.11 g/L. Even if it shows promise under less-than-ideal circumstances, this yield pales in comparison to findings from well-established

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studies. For example, Ali et al. (2002) used Aspergillus niger GCBT7 in a stirred fermenter with 150 g/L molasses sugar, a regulated pH (6.0), and a temperature of 30 °C to describe the generation of citric acid up to 99.6 g/L in just 6 days[118]. Similarly, Guc and Erkmen 2017, observed yields between 19–35 g/L within 4 days from beet molasses at 160–200 g/L sugar concentrations under comparable acidic and thermal conditions[123]. The significantly lower yield in the current study can be attributed to factors such as the low substrate concentration (5%)**,** lack of temperature regulation**,** and absence of acidic pH optimization**,** all of which are known to critically influence the biosynthesis of citric acid. Nevertheless, the ability of strain FS2 to produce citric acid steadily under mild environmental parameters suggests that it could serve as a promising candidate for further optimization, especially under controlled fermentation settings that mimic industrial processes.

Another present study reveals significant differences in citric acid production among six microbial strains under identical fermentation conditions when substrate concentration was 7.5%. Strain FS2 showed the highest and fastest citric acid output, reaching 1.17 mg/ml by day 10, making it the most promising candidate for industrial use due to its efficiency and early peak production. Strains FS1, FS3, and FS5 produced moderate levels (around 0.6–0.66 mg/ml by day 10), indicating potential for improvement through process optimization**.** Strain FS4 had low production **(**0.38–0.45 mg/ml**),** while F**S6** showed minimal productivity (0.19 mg/ml), likely due to poor metabolic adaptation.

From twenty soil samples, 96 Aspergillus niger isolates were identified, showing varying abilities to produce citric acid in Czapek-Dox broth. The most efficient local isolate, A. niger MH368137, was selected for optimization studies alongside the reference strain EMCC132. Optimal conditions for maximum citric acid production included 5 days of incubation at 28°C, pH 6.5, inoculum size of 3.0×10^{6} spores/ml, 100 rpm shaking, maltose (40 g/L) as the carbon source, peptone (3 g/L) as nitrogen source, sodium phosphate, and 2% methanol as an enhancer. Under these optimized conditions, citric acid yield increased 14-fold (13.33 mg/ml). When tested with agricultural wastes, sugarcane molasses proved to be the best substrate, yielding up to 256.94 mg/ml of citric acid using the MH368137 strain.[87].

According to Kariptas et al. (2009), this study used Aspergillus niger in shaking cultures to examine the effects of temperature, pH, and starting sugar concentration on the

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synthesis of citric acid from beet molasses. A maximum yield of 52.3 g/L of citric acid was obtained under ideal circumstances (pH 5.0, 30°C, and 140 g/L sugar). Citric acid production rose from 14.3 to 33.2 g/L in the treated medium. The findings show that beet molasses is a useful substrate and that the medium conditions have a significant impact on the synthesis of citric acid by *A. niger*[124].

Another study focused on enhancing citric acid production from cane molasses using mutant strains of *Aspergillus niger*. Through UV and chemical mutagenesis, strain GCMC-7 was developed, producing up to 96.1 g/L citric acid in 168 hours. Magnesium sulfate addition improved productivity further to 113.6 g/L by promoting favorable mycelial morphology and reducing biomass. Kinetic analysis confirmed the mutants' superior growth and citric acid yield, highlighting GCMC-7's strong commercial potential[119].

The comparatively lower citric acid yield observed in this study can be attributed to several influencing factors, including the strains we used were isolated from soil and may not be genetically optimized. Furthermore, many research studies use mutant or genetically engineered strains that are selectively improved for high citric acid yield. In addition, Geographical conditions—such as ambient temperature, humidity, altitude, and even local microbial strain adaptation—can directly affect microbial behavior, particularly in citric acid (CA) production by *Aspergillus niger* or similar organisms.

Moreover, molasses is a cost-effective but complex carbon source**;** it contains **i**mpurities**,** metal ions, and variable sugar content, which can inhibit the desired microbial growth in fermentation broth. Therefore, it declined the acid production rate in our studies**.** Some studies use purified glucose or sucrose**,** or pre-treated molasses to remove inhibitors and improve substrate availability. What is more, the controlled of pH, temperature, and aeration, using refined substrates to enhance microbial efficiency. Additionally, the inoculum size and lack of active aeration may have further limited microbial metabolism and citric acid synthesis. These variables collectively account for the reduced productivity compared to values reported in more optimized fermentation systems.

Gypsum ($CaSO_4 \cdot 2H_2O$) is a versatile mineral used in construction, agriculture, and industry for its fire resistance, workability, and soil-enhancing properties. It is found in drywall, plaster, and cement additives, while industrial byproducts like desulfurization gypsum and phosphogypsum offer reuse potential but require careful environmental

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management. It uses in construction has steadily increased, leading to a rise in gypsum-containing construction and demolition waste. In the context of a circular economy, recycling this waste is gaining importance. To assess the environmental viability of recycled gypsum, a life cycle assessment (LCA) was conducted using industry data and LCA databases. Two recycling scenarios with varying transportation distances were analyzed and compared to gypsum sourced from coal-fired power plants (FGD gypsum) and natural gypsum. The study found that recycled gypsum offers environmental advantages—particularly in terms of land use and resource depletion—when compared to FGD and natural gypsum. However, transportation distance plays a significant role in the overall environmental impact[125].

According to the FTIR spectra of citric acid made from fermented cane molasses, these samples show distinctive O–H stretching bands at about 3339 cm^{-1} and C–O stretching vibrations at 1300–1200 cm^{-1}, which are suggestive of citric acid or citrate derivatives. However, unlike the commercial citric acid standard, they do not have the characteristic carbonyl doublet at ~1754 cm^{-1} and ~1723 cm^{-1} that is usually detected in pure, protonated citric acid. This lack indicates that the fermentation products, like citrate salts, are probably hydrated or partially neutralized.

A study by Mirghani et al. (2019) and Tsioptsias et al. (2024) on the production of citric acid from cane molasses using Aspergillus niger reported FTIR analyses that verified the presence of distinctive citric acid absorption bands, comparable to those seen in the commercial standard, confirming this interpretation. This implies that, with the right optimization, the fermentation process can produce citric acid with spectrum characteristics similar to the commercial product[126][127]. Furthermore, a study by Khattab et al. (2022) examined how cane molasses enhanced the generation of citric acid by gamma-irradiated Aspergillus niger. The extracted citric acid's molecular structure was preserved during the fermentation process, as demonstrated by FTIR analysis, which showed that the primary characteristic absorption bands stayed consistent with those of the standard citric acid[128]. These results highlight the potential of cane molasses as a fermentation substrate for the synthesis of citric acid. Although citric acid is produced throughout the fermentation process, the observed FTIR spectra indicate that additional purification or crystallization may be required to produce a product with spectral properties identical to the commercial standard.

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The FTIR analysis of gypsum shows characteristic peaks at 3356, 1628, 1109, 1043, 877, 665, and 576 cm^{-1}, corresponding to key vibrational modes of calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$). The bands at 3356 and 1628 cm^{-1} indicate O–H stretching and H–O–H bending from water molecules, while the peaks near 1109 and 1043 cm^{-1} arise from sulfate group stretching. The lower wavenumber peaks (877, 665, and 576 cm^{-1}) relate to sulfate bending and lattice vibrations, confirming gypsum's structural features. These findings are consistent with published reference spectra. For example, Elert et al. (2016) and Rowe & Kuebler (2007) report similar peak positions for gypsum, with O–H stretching near 3400 cm^{-1}, H–O–H bending near 1620 cm^{-1}, and strong sulfate-related bands between 1100 and 500 cm^{-1}[129][130]. The close agreement between the observed and reference values confirms the material as gypsum and supports its identification through FTIR spectroscopy.

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CHAPTER 6.

CONCLUSIONS

Part I

Microbial citric acid (CA) has a lot of promise for the economy because of its many applications, especially in the food, beverage, detergent, cosmetic, and pharmaceutical sectors. The majority of CA in Bangladesh is imported to meet demand. Additionally, its annual use is rising gradually (3.5–4%), which suggests that better manufacturing options are needed. High production yields must be maintained while the manufacturing process is environmentally friendly by using easily accessible and reasonably priced agro-industrial waste materials to support the enormous scale of production. With Aspergillus niger, microbial fermentation is the main method used to create CA. The purpose of this study is to identify the ideal circumstances for the submerged fermentation of cane molasses with *Aspergillus niger (F-81*) and the byproduct gypsum in order to produce CA. The concentration of CA in fermentation broth was estimated and determined using the Marier-Boulet method. In the pretreated sample, the maximum CA generation was 12.49 mg/ml, while in the non-pretreated sample, it was 7.3 mg/ml. The inoculum sizes and pH values for both fermentation times were 2% and 5.6, respectively, and they lasted 10 days. Future research will examine a number of variables that affect the production rate of CA, including *A. niger* regulatory genes, pH, temperature, inoculum size, substrate concentration, and heavy metal concentrations.

Part II

The comparatively lower citric acid yield observed in this study can be attributed to several influencing factors, including the strains (FS_1 to FS_6) we used were isolated from soil and may not be genetically optimized. Furthermore, many research studies use mutant or genetically engineered strains that are selectively improved for high citric acid yield. In addition, Geographical conditions—such as ambient temperature, humidity, altitude, and even local microbial strain adaptation—can directly affect microbial behavior, particularly in citric acid (CA) production by *Aspergillus niger* or similar organisms. Moreover, molasses is a cost-effective but complex carbon source**;** it contains impurities**,** metal ions, and variable sugar content, which can inhibit the desired microbial growth in fermentation broth. Therefore, it declined the acid production rate in our studies. Some studies use purified glucose or sucrose**,** or pre-treated molasses to remove inhibitors and improve substrate availability. What is more, the controlled of pH, temperature, and aeration, using refined substrates to enhance microbial efficiency. Additionally, the inoculum size and

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lack of active aeration may have further limited microbial metabolism and citric acid synthesis. These variables collectively account for the reduced productivity compared to values reported in more optimized fermentation systems.

In addition the by-product of gypsum has numerous used like the construction sector remains the largest consumer of gypsum, using it extensively in drywall, plaster, and ceiling boards due to its lightweight, fire-resistant, and sound-dampening qualities. Demand is rising globally, driven by sustainable building trends, rapid urbanization in Asia-Pacific and the Middle East, and infrastructure growth in Africa. In agriculture, gypsum serves as a soil conditioner, improving structure, water infiltration, and providing calcium and sulfur—especially valuable for reclaiming sodic soils. Its adoption is expanding in precision and sustainable farming in countries like the U.S., India, and Brazil. Medically, gypsum is vital for orthopedic casts and dental molds, with steady demand supported by advances in prosthodontics and the growth of dental tourism. Increasing gypsum use in construction has led to more gypsum-rich demolition waste, making recycling an important part of the circular economy. Life cycle assessments show that recycled gypsum outperforms natural and FGD gypsum in reducing land use and resource depletion, though transport distance remains a key factor in its environmental footprint.

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CHAPTER 7.

RECOMMENDATION

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For future research, the exploration of the regulatory genes of *Aspergillus niger* involved in citric acid biosynthesis to better understand and enhance production pathways. Additionally, systematic investigation of fermentation parameters, such as, pH, temperature, inoculum size, substrate concentration, and heavy metal levels should be conducted to optimize yields further. Studying the interactions between these variables, especially in large-scale setups using agro-industrial wastes and gypsum byproducts, will also be valuable to develop efficient, sustainable, and cost-effective production processes. In addition, investigating sustainable management and recycling of gypsum byproducts is also crucial, with an emphasis on minimizing environmental impact through improved logistics and circular economy approaches. Integrating these strategies will help create more efficient, cost-effective, and eco-friendly processes for both citric acid production and gypsum utilization. In future, research on gypsum should prioritize developing cost-effective purification methods to improve byproduct quality for broader high-value uses. Establishing standardized quality criteria will boost industry trust and market acceptance. Exploring innovative applications like bio-composites and carbon capture can further enhance its value. It is also need to asses the integration of gypsum recycling into circular economy models and assessing supportive policies will promote sustainable industrial practices and maximize environmental and economic benefits.

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