ADDIS ABABA UNIVERSITY

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SCHOOL OF CHEMICAL AND BIO-ENGINEERING

Statistical optimization of process parameters for citric acid synthesis from water hyacinth using solid-state fermentation

By Setty T/haimnot

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A Thesis Submitted to the School of Chemical and Bio Engineering in Partial Fulfillment of the requirement for the Attainment of Degree of Master of Science in Chemical and Bio Engineering

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This is to certify that the thesis is prepared by Setty T/haimanot, entitled: Statistical optimization of process parameters for citric acid synthesis from water hyacinth using solid-state fermentation and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Process Engineering complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Citric acid is a versatile organic compound widely employed across various industries, with its primary application residing in the food sector. This organic acid exhibits remarkable attributes such as high solubility, palatability, and low toxicity, rendering it invaluable in the realms of food, biochemistry, and pharmaceuticals. The principal objective of this study is to synthesize citric acid using the fungus Aspergillus niger, utilizing water hyacinth as the primary substrate, and to assess the resulting citric acid's quality and concentration. In the laboratory setting, we harnessed the solid-state fermentation method within 250 mL Erlenmeyer flasks to transform water hyacinth into citric acid. To systematically analyze and optimize the synthesis process, Design Expert 13.0.5.0 was employed, applying Response Surface Methodology (RSM) to study the concurrent impact of key variables: incubation time (3 days, 6 days and 9 days), fermentation temperature (set at 25^{o}C, 30^{o}C and 35^{o}C), and pH levels (2, 4 and 6). The study utilized a three-variable, three-level Box-Behnken design to establish a statistical model that elucidates the relationship between citric acid concentration and these independent variables while optimizing the production process. The statistical model proved significant ($p < 0.0001$) and demonstrated low standard deviation (0.3445). Notably, it exhibited no lack of fit (R^2=0.9959), and the predicted R-Squared value of 0.9907 closely aligned with the Adj R-Squared of 0.9514. Citric acid production showcased significance under moderate levels of incubation time, fermentation temperature, and pH. Analysis of the model's regression equation highlighted the positive influence of temperature and time, while pH exhibited a negative effect on the yield of the desired response. Through rigorous experimentation, we achieved a maximum citric acid yield of 22.205 g/L under the optimal conditions, which included an incubation time of 7.375 days, a fermentation temperature of 31.071 ^{o}C, and a pH level of 4.245. These settings yielded a combined desirability of high value. The validation process confirmed the statistical model's reliability, demonstrating an insignificant difference between experimental and model-predicted results.

Keywords: Citric acid, Aspergillus niger, water hyacinth, Substrate, Solid State Fermentation,

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

AAS	Atomic Absorption Spectroscopy
A. niger	Aspergillus niger
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
BBD	Box -Behnken Design
Df	Degree of Freedom
DOE	Design of Experiment
EPHL	Ethiopian public Health Institute
FAAS	Flame Atomic Absorption Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
HW	Water hyacinth
PDA	Potato Dextrose Agar
Ppm	Parts Per Million
rpm	Revolution per Minute
RSM	Response Surface Methodology
S. lipolytica	Saccharomycopsis Lipolytica
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TCA	Tricarboxylic Acid

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Citric acid ($C_6H_8O_7$) is classified as a weak organic acid and serves as an intermediate compound in the tricarboxylic acid cycle. It contains three carboxyl (R-COOH) groups and is commonly known as 2-hydroxy-propane-1,2,3-tricarboxylic acid ($C_6H_8O_7.H_2O$). This naturally occurring acid is abundantly found in citrus fruits, hence its name derived from the Latin word "citrus," referring to trees of the genus Citrus, such as lemon trees. In its pure form, citric acid is colorless and readily dissolves in water. It boasts biodegradability, environmental friendliness, cost-effectiveness, safety, and versatility, making it a widely used chemical. Citric acid finds extensive application in various sectors, including the production of medicinal citrates, confectionery, effervescent salts and soft drinks. Additionally, it is utilized in minor quantities for processes like silvering, engraving, dyeing, and calico printing [1].

Citric acid is available in two forms: anhydrous and monohydrate, with respective molecular weights of 192 g/mol and 210 g/mol. The anhydrous form crystallizes when citric acid is dissolved in hot water, while the monohydrate form is obtained from cold water crystallization. Converting monohydrate to anhydrous citric acid occurs at approximately 78°C. It is solid at room temperature and has a melting point of 153°C. It exhibits three distinct pKa values, occurring at pH 3.1, 4.7, and 6.4, attributed to the presence of three carboxylic acid functional groups within its molecular structure [2].

Fungal fermentation, primarily utilizing Aspergillus niger. The fungi has been the main method for commercial citric acid synthesis. The research that laid the foundation for the successful citric acid production was conducted by James Currie in 1916. He discovered that various strains of A. niger could yield significant volumes of citric acid. Key discoveries included A. niger's capacity to thrive at a pH range of approximately 2.5–3.5, thereby reducing the formation of gluconic and oxalic acids. Additionally, citric acid production was observed to rise with higher sugar concentrations [3].

Citric acid holds significant commercial value owing to its versatile applications. Primarily utilized in the food industry (70%), it serves as a pH regulator. In pharmaceuticals (12%), it adds flavor to medications and functions as an acidulant and anti-coagulant. Other sectors (18%) also benefit from its various uses [4]. The food and beverage sector extensively utilize citric acid for its pleasant taste, low toxicity, and sweetening characteristics. Within food formulation, it serves multiple roles such as stabilizing flavors, enhancing taste, and regulating acidity levels. In pharmaceutical and cosmetic fields, it finds extensive use as an acidifying agent, flavor enhancer, preservative, antioxidant, emulsifier, and chelating agent [3].

The annual global output of citric acid has reached 2.1 million tons in 2016 increasing steadily; it reached 0.9 and 1.7 million tons in 2000 and 2010, respectively [5]. About 70% of the total quantity of citric acid produced each year is used in the food sector, 12% in the pharmaceutical as a flavoring, anticoagulant, and preservative, and the remaining 18% in other industries such as detergent, cosmetics, textiles, paper and oil recovery [6]. Citric acid is used in the detergent industry as a phosphate substitute due to its less eutrophic effect, as well as in the cement industry to slow the hardening process [7].

1.2 Statement of the Problem

The Ethiopian Rift Valley lakes have grappled with the relentless spread of water hyacinth, a highly invasive aquatic weed of global concern. Among these lakes, Lake Koka, has fallen victim to this relentless infestation. Covering an extensive surface area of 255 square kilometers, this invasive weed has now claimed an alarming thirty-seven percent of the lake's total expanse [8]. The implications of this unchecked proliferation are diverse and far-reaching, including disruptions to vital sectors such as fishing, irrigation, transportation, and, notably, severe threats to the region's unique biodiversity.

In tandem with this environmental challenge, there has been a marked increase in the global and local demand for citric acid, a versatile chemical compound with wide-ranging applications. Local consumption of citric acid has historically relied on imports. However, an examination of import data from the country's Customs Authority reveals a rather inconsistent pattern in import quantities over the years, despite a discernible upward trajectory in demand. Notably, during the period of 2014-2015, there was a nearly twofold increase in the imported quantity compared to the years 2005-2006. Moreover, the annual

average import quantity during the period of 2015-2016 surpassed previous import levels, reaching approximately 77 tons.

In response to the increasing demand for citric acid and the fluctuating nature of imports, there has been a notable shift towards exploring renewable and sustainable sources for citric acid production. This transition aligns with global efforts to mitigate environmental impact and promote sustainable practices in the chemical industry. Crucially, this study seeks to contribute to this emerging paradigm by investigating the viability of using an environmentally problematic aquatic biomass—water hyacinth—as a primary source for citric acid production. The unique properties of water hyacinth, characterized by its lower lignin content and higher cellulose and hemicellulose content, render it an attractive candidate for bioconversion processes. Successfully harnessing the potential of water hyacinth for citric acid production could yield significant benefits across various industries, including food, pharmaceuticals, and others. Simultaneously, it holds the promise of addressing the pressing environmental challenges posed by water hyacinth infestation in the Ethiopian Rift Valley lakes.

1.3 Objectives

1.3.1 General objective

The general objective of the research is to statistically optimize the process parameters involved in the citric acid synthesis from water hyacinth using solid-state fermentation.

1.3.2 Specific objectives

The specific objectives of this study are as follows::

- Characterize the physical properties of water hyacinth through proximate analysis (moisture content, total solids, volatile solids and ash content).
- Determine the lignocellulosic content of water hyacinth.
- To synthesize citric acid from water hyacinth by fermentation process using Aspergillus niger.
- To examine the effect of factors such as incubation time, fermentation temperature and initial pH on citric acid yield.
- To identify the optimal combination of process parameters that maximizes citric acid yield.

1.4 The scope of the study

The research was concentrated on Lake Koka as the geographical scope, water hyacinth being the carbon source for citric acid synthesis was characterized. Aspergillus niger as microorganism. Process parameters, including temperature, pH, fermentation time was investigated. Statistical methods and optimization techniques was utilized to determine the optimal combination of process parameters for maximizing citric acid yield. The final product citric was characterized by assessment product purity by melting point determination and UV Visible spectroscopy.

1.5 Significance of the Study

This study holds a pivotal role in addressing the pressing issue of water hyacinth infestation within the country. Water hyacinth, known for its low lignin content and higher cellulose and hemicellulose content, presents a unique opportunity for effective utilization as a carbon source. By exploring the potential of water hyacinth as a viable substrate for citric acid production, this study contributes significantly to ongoing research and development efforts aimed at mitigating the challenges posed by water hyacinth biomass disposal.

Moreover, the implications of this research extend beyond the realms of environmental problem-solving. Citric acid, a major focus of this study, serves as a critical input for various industries within the country, including pharmaceuticals, beverage production, and detergent manufacturing, among others. Consequently, the findings of this study are poised to provide valuable insights into the factors influencing citric acid production. By uncovering these factors, the research can play a vital role in enhancing the efficiency and effectiveness of citric acid production processes, thereby bolstering the competitiveness and sustainability of these crucial industries.

In summation, this study transcends the confines of environmental research and encompasses economic and industrial significance. It not only addresses the water hyacinth challenge but also contributes to the optimization of citric acid production, thereby fostering a more sustainable and prosperous future for the country.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 History of citric acid production

Commercial production of citric acid began in England in 1826, initially relying on imported Italian lemons, which typically contain 7-9% citric acid. This was before the invention of the microbial method [9]. For nearly a century, Italian manufacturers held a monopoly on its production, selling it at a high price. As a result, there were widespread efforts worldwide to discover alternative methods for citric acid production, utilizing both chemical and microbial techniques. The initial exploration into the feasibility of citric acid production via fermentation involved a sugar medium enriched with inorganic salts and utilized Penicillium glaucum [10]. Following this investigation, Wehmer achieved a milestone by isolating two strains capable of synthesizing citric acid, subsequently identified as Citromyces spp. (Penicillium). Despite these advancements, the industrial application of Citromyces spp. for citric acid production faced limitations due to contamination issues and prolonged fermentation durations [11].

Figure 2-1: Citric acid

In 1916, James Currie conducted pivotal research that revolutionized the economic industrial-scale citric acid production using Aspergillus niger. Currie's breakthrough discoveries highlighted the significant citric acid yields achievable from different strains of A. niger. Particularly noteworthy were the observations regarding A. niger's ability to thrive within a pH range of approximately 2.5–3.5, thereby inhibiting the formation of gluconic and oxalic acids. Furthermore, Currie demonstrated a direct correlation between increased sugar

concentration and heightened citric acid production. The pharmaceutical corporation Pfizer pioneered the modern day industrial citric acid manufacturing in the United States in 1923 [3].

2.2 Micro-organisms used for citric acid production

Numerous microorganisms, such as bacteria, fungi, and yeasts, have been utilized for synthesis of citric acid. Nevertheless, the majority of the microorganisms fail to yield commercially viable quantities. This limitation can be attributed to the fact that citric acid is a byproduct of energy metabolism, accumulating significantly only when there are substantial imbalances in conditions. Table 2-1 displays the strains used to produce citric acid. Although numerous microorganisms can be employed to synthesis citric acid but only A. niger and certain yeasts such as Saccharomycopsis sp. are used for commercial production. The downside of fermentation using yeasts results in the production of large quantities of isocitric acid as an unwanted byproduct [9]. The dominant organism for commercial production, has continued to be the fungus A. niger [10]. Since it is simple to handle, can ferment a variety of inexpensive raw materials, and produces a large output [12].

Table 2- 1: Micro-organisms used in citric acid production

Micro-organisms	Strains	References
Fungi	Aspergillus niger	[13], [14], [15], [16], [17]
	A. aculeatus	[18]
	A. awamori	[12]
	A. carbonarius	[18]
	A. wentii	[19]
	A. foetidus	[20], [21]
	Penicillium janthinelum	[12]

The frequently utilized method to enhance citric acid producing strains involves inducing mutations in parental strains using mutagens. Among physical mutagens, gamma radiation Gunde-Cimerman, Islam et al., 1986 [22], [23], [24] and UV-radiation [25], [26] have often used. To produce hyperproducer strains, UV treatment is often coupled with various chemical mutagens such as aziridine, N-nitroso-N-methylurea, or ethyl methane-sulfonate [27]. Employing a suitable selection method on a model medium containing non-specific carbon

sources allows for the isolation of strains that produce significant quantities of citric acid from unconventional substrates, derived from the resulting mutants.

2.3 Fermentation process in citric acid production

The evolution of citric acid fermentation processes can be delineated into three distinct phases. Initially, citric acid production relied on species of Penicillium and Aspergillus, cultivated under stationary or surface culture conditions. The second phase saw the introduction of submerged fermentation methods, mainly utilizing Aspergillus species. In the third and more recent stage, solid-state culture, continuous culture, and multistage fermentation techniques have been developed for citric acid production.

Fermentation-based synthesis remains the most cost-effective and prevalent method for producing citric acid. Over 90% of the global citric acid supply is derived through fermentation, offering several advantages. Fermentation processes are characterized by simplicity and stability, with relatively straightforward plant operations that require less complex control systems. Moreover, they demand lower technical expertise, result in reduced energy consumption, and are less susceptible to disruptions from power failures, ensuring consistent plant functionality.

2.3.1 Surface fermentation

The primary method employed for industrial-scale citric acid production was surface fermentation, also known as liquid surface culture (LSC). This technique was pioneered by Société des Produits Organiques in Belgium in 1919, and later adopted by Chas Pfizer & Co. in the US in 1923 [13]. Despite the increasing popularity of submerged fermentation in recent years, surface fermentation remains in use among small and medium-scale industries. In this conventional process, the culture solution is contained in shallow trays with a capacity ranging from 50 to 100 liters, allowing the fungus to develop as a mycelial mat on the surface of the medium.

These fermentation processes occur within dedicated chambers equipped with trays made from various materials, including special-grade steel, high-purity aluminum, or polyethylene. However, stainless steel trays are preferred due to their resistance to deformation over prolonged usage [4]. Effective air circulation is maintained within the fermentation chambers

to regulate temperature and humidity, ensuring optimal growth conditions. The chambers are maintained under aseptic conditions, particularly during the initial two days when spores germinate, to prevent contamination.

Contamination risks primarily arise from organisms such as Penicillin, other Aspergillus species, yeast, and lactic bacteria. Carbon sources utilized in the process typically include refined or crude sucrose, cane syrup, or beet molasses. When employing molasses, it is diluted to a concentration of 15-20% and treated with hex cyanoferrate (HFC) [12].

2.3.2 Submerged Fermentation

The technique of submerged fermentation (SmF) is commonly employed in the manufacturing of citric acid. It is estimated that about 80% of world production is obtained by submerged fermentation [28]. Submerged fermentation is a method that entails the cultivation of microorganisms within a liquid medium. Nutrient substrates are consumed at a rapid rate, necessitating a continuous supply of replenishment. This fermentation approach is most suitable for moisture-dependent microorganisms, particularly bacteria. This fermentation process requires more sophisticated installations to be employed in large scale and rigorous control. Conversely, it offers numerous advantages, including higher yields, lower capital, maintenance, and labor costs, as well as reduced contamination risks, making it more conducive to citric acid production [5]. Typically, submerged fermentation can be executed using batch, fed-batch, or continuous systems, with the batch system being the most commonly employed method for citric acid production. In batch-fed fermentation, sterilized growth nutrients are added to the culture. This method is prevalent in bio-industries, typically during biomass growth in the fermenter [29].

2.3.3 Solid State Fermentation

Solid-state fermentation (SSF), also known as the Koji process, emerged as an alternative approach for citric acid synthesis using agro-industrial residues. Initially developed in Japan, SSF capitalized on the abundance of raw materials such as fruit wastes and rice bran, offering a straightforward method for citric acid synthesis [18]. A key advantage of SSF lies in its higher yield and its ability to utilize inexpensive agro residues, making it a more environmentally sustainable option compared to submerged fermentation [19]. However, the success of SSF hinges on selecting suitable substrates, managing process variables, and

choosing the right microbial strain. Therefore, the availability of high-quality substrates in ample supply and at low cost is essential for ensuring cost-effectiveness and economic viability of the process [20].

Typically, the substrate in question is moistened to approximately 70% moisture content, this is adjusted based on its the absorption capacity. The initial pH is usually set within the range of 4.5 to 6.0, and the fermentation temperature can vary between 28 to 30°C. The most commonly utilized organism for solid-state fermentation (SSF) is usually Aspergillus niger, although there have been instances involving yeasts as well.

Strains which are well-suited for SSF have high nitrogen and phosphorus content due to the lower diffusion rate of nutrients and metabolites in the solid-state process, which occurs at lower water activity levels. One significant advantage of SSF is that the presence of trace elements may not negatively impact citric acid production as severely as it does in submerged fermentation (SmF). As a result, substrate pre-treatment is often unnecessary [30].

Solid-State Fermentation (SSF) employs solid substrates such as bran, bagasse, and paper pulp. A significant benefit of utilizing these substrates is their capacity to recycle nutrient-rich waste materials as fermentation substrates. Within this fermentation method, substrates undergo gradual and consistent utilization, enabling prolonged fermentation periods with the same substrate. SSF is characterized by its simplicity, low energy consumption, and minimal capital investment requirements. Consequently, this technique facilitates the controlled release of nutrients. Many domestic, industrial, and agricultural wastes can be effectively repurposed in SSF processes. SSF is best suited for fermentation process involving fungi and microorganisms that require less moisture content [31].

However, solid-state fermentation (SSF) also has its limitations. It requires microorganisms that can tolerate low moisture levels. Additionally, precise monitoring of moisture content, oxygen (O_2), and carbon dioxide (CO_2) levels is challenging. Furthermore, microorganisms tend to grow slowly in solid-state fermentation, which can limit product formation.

Table 2- 2: Raw material for different fermentation process.

Fermentation Process	**Raw Materials**
Surface	Brewery waste, tubers of Asphodelus aestivus, turnip whey (enriched with molasses), carob sugar, cotton waste
Submerged	Bagasse hydrolysates, beet molasses, cane molasses, black strap molasses, coconut oil, corn starch, date syrup, glycerol, hydrolysate starch, n-paraffin, , soya bean oil, rapeseed oil, spent grain liquor, wood hemicellulose, whey permeate, xylan hydrolysate, yam bean starch, carob pod extract, brewery wastes, palm oil, olive oil
Solid state	Pineapple waste, apple pomace, carrot waste, coffee husk, cassava bagasse, corncob, deoiled rice bran, grape pomace, kumara, molasses, mussel processing wastes, okara, orange waste, rice bran, sucrose, sugarcane-press mud, wheat bran, carob pod, cellulose hydrolysate, kiwi fruit peel

Whereas the drawbacks for this type of fermentation process are that it is suitable for microorganisms that can tolerate low moisture content. Precise monitoring of SSF (e.g., O_2 and CO_2 levels, moisture content) is not possible. The microorganisms exhibit slow growth, resulting in a constraint on product formation.

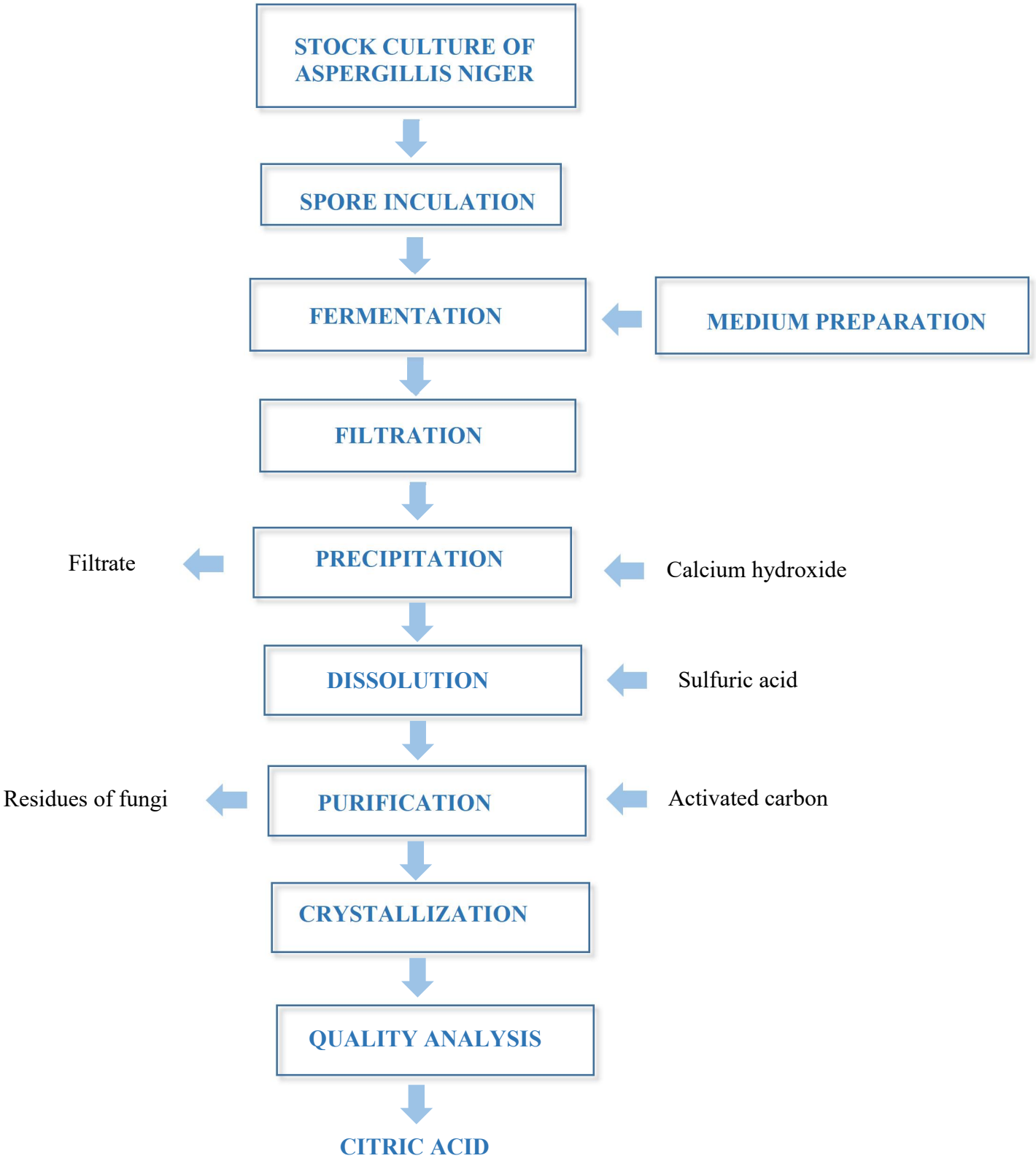

Figure 2- 2: Schematic diagram of solid-state fermentation

2.4 Raw Material Used in Citric Acid Production

While the predominant method for citric acid production involves liquid fermentation using sucrose-based or starch media, a diverse range of substrates, including molasses, various starchy substances, and hydrocarbons, have also found application. Soccol et al. (2006) provides examples of various raw materials used in citric acid synthesis, including beet, blackstrap and cane molasses, carob pod extract, n-paraffin, glycerol, , hydrolyzed starch, , wood hemicellulose, rapeseed oil, palm oil, olive oil, corn starch, yam bean starch and soybean oil [4].

Common solid substrates often include cereal grains like wheat, rice, corn and barley, along with wheat bran, and various lignocellulosic materials such as straws, sawdust, or wood shavings, as well as diverse plant and animal materials. The majority of these compounds consist of polymeric molecules that have limited solubility or are insoluble in water. However, they are generally cost-effective and easily available, making them a rich source of nutrients for microbial growth.

The substrates used for citric acid synthesis were classified into two groups. Substrates that possess a low ash content, from which cations can be extracted using conventional methods. (e.g. cane or beet sugar, crystallized dextrose and dextrose syrups) and substrates with a high ash content and high amounts of other non-sugar substances (e.g. cane and beet molasses, crude unfiltered starch hydro-lysates) [2].

To lower the production costs of citric acid and promote environmental sustainability, inexpensive substrates can be utilized. One such option is the utilization of non-crystallizable effluents, or molasses, which are produced as byproducts after sucrose isolation in sugar refineries. Molasses presents advantages such as reduced cost and a high sugar content ranging from 40% - 55%, comprising fructose, glucose, and sucrose [32].

Numerous research studies have been conducted to manufacture citric acid using molasses as a preferred choice because of its affordability and elevated sugar content ranging from 40% to 55%. The quality of molasses can fluctuate depending on its origin, pretreatment process, cultivation approach of crops, types of fertilizers and pesticides used during plantation, storage and handling,,conditions production procedures, etc. Both cane and beet molasses are

stable for citric acid production. Nonetheless, research indicates that beet molasses is the preferred choice, mainly because of its lower trace metal content. Cane molasses, on the other hand, contains calcium, iron, zinc, magnesium and manganese. All of which can hinder the synthesis of citric acid. As a result, some form of pre-treatment is necessary to eliminate or reduce the presence of these trace metals. However, cane molasses present challenges in attaining favorable fermentation yields [33].

Numerous agro-industrial byproducts, including banana peel, apple pomace, coffee husk, sugar beet cosset, cassava bagasse, wheat straw, pineapple waste, kiwi fruit peel, and more, have been explored in the context of solid-state fermentation techniques for their potential as substrates in citric acid production. These residues are particularly well-suited for solid-state cultures due to their cellulose and starch content. Despite the fact that these solid residues offer abundant nutrients for microorganisms and serve as favorable substrates for microbial growth and activity, there is still considerable work required to establish economically viable processes utilizing these residues [4].

2.4.1 Water hyacinth as a substrate

Water hyacinth, scientifically known as Eichhornia crassipes, belongs to the perennial, herbaceous, and aquatic plant species within the Pontederiaceae family [34]. This species, along with other members of the Eichhornia genus, primarily thrives in aquatic environments. However, E. crassipes stands out as a significant weed due to its rapid growth rate. Capable of producing numerous seeds annually, each plant has the potential to generate thousands of seeds, which can remain viable for over 28 years. The proliferation of water hyacinth leads to the formation of dense mats that cover open water surfaces. This dense growth impedes water flow, obstructs sunlight from reaching native aquatic plants, and diminishes oxygen levels in the water, thereby posing a threat to aquatic fauna such as fish [35].

Figure 2- 3: Water hyacinth.

It is a widely recognized invasive weed in lakes across the world and poses a threat to the aquatic ecosystem. The weed is a plant that is native to Brazil, Amazon basin and Ecuador region. It spreads rapidly across numerous tropical and subtropical regions, including Latin America, the Caribbean, Africa, Southeast Asia, and the Pacific [36]. In Ethiopia, this weed was initially reported around 60 years ago in Koka Lake and the Awash River. The emergence and swift proliferation of this weed in various regions, including the Awash River Basin (specifically in areas like Koka Lake and Koka Dam), Abbay River Basin (encompassing Lake Tana and the Blue Nile), Baro-Akobo River Basin (covering Sobate, Baro, Gillo, and Pibor rivers), and Rift Valley Basins System (encompassing Lake Ellen, Lake Abaya, and Lake Elltoke), have posed significant challenges for utilizing water resources in these areas [37].

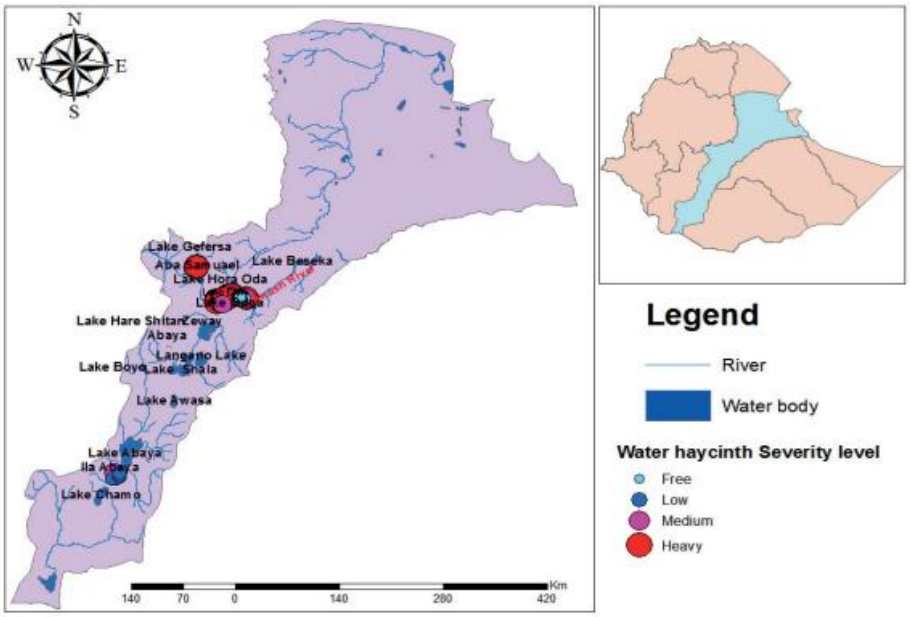

Figure 2- 4: Distribution of water hyacinth across the Rift Valley water bodies of Ethiopia.
*Source: [38][39]

Lake Tana stands as Ethiopia's largest lake, containing half of the nation's freshwater reserves. Additionally, it serves as the primary source of the Blue Nile, contributing as much as 60% of the total water flow in the Nile River [40]. Beyond its critical role as a water source for over 123 million people residing in the Nile Basin, the lake plays a significant role in providing sustenance through its fish population. Recognized for its ecological significance, Lake Tana

has been included in the top 250 lake regions globally for biodiversity. Remarkably, it hosts a diverse array of aquatic life, boasting a total of 28 fish species.

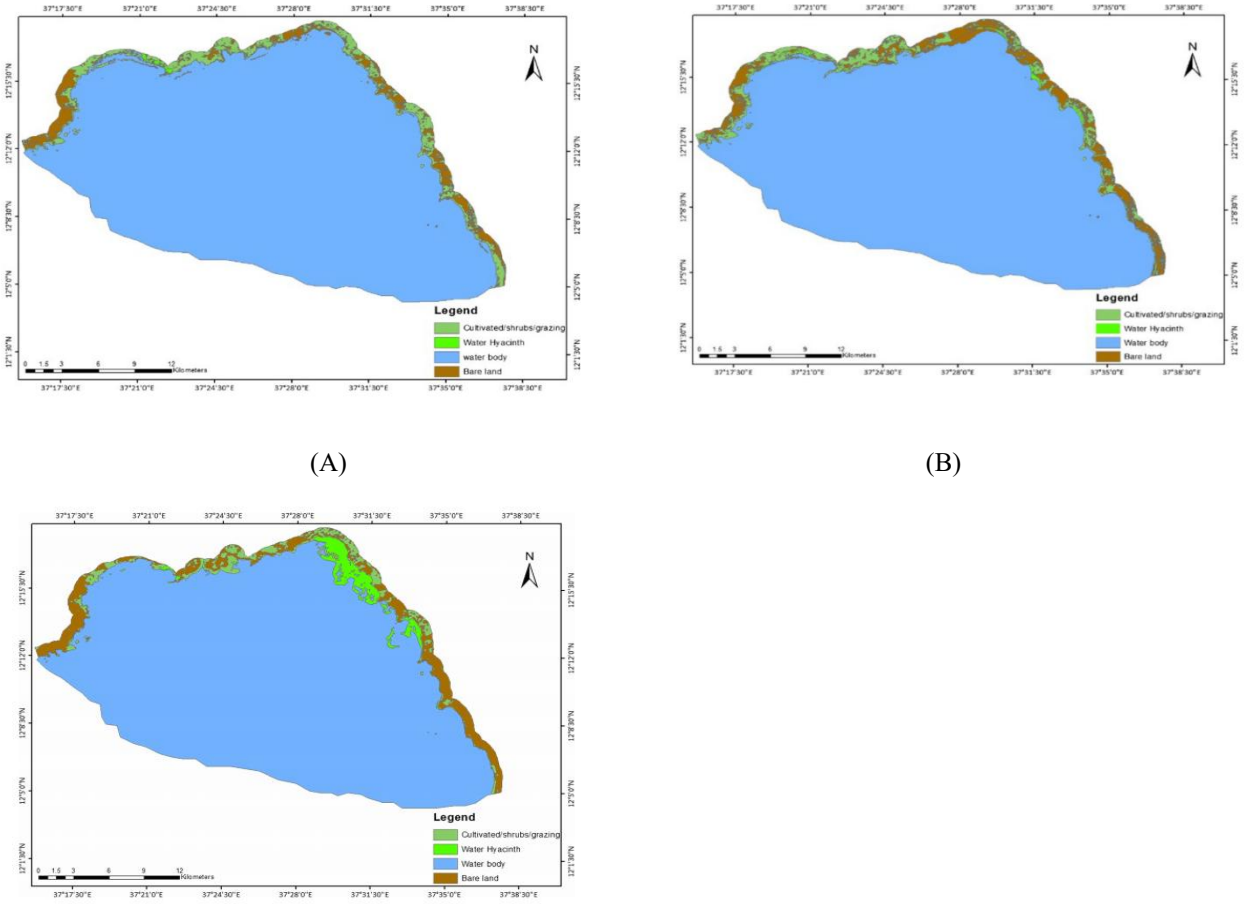

Figure 2- 5: Water hyacinth infestation on water bodies in Ethiopia (A) 2013, (B) 2015, and (C) 2017 respectively.
*Source:[41]

In 2011, the Regional Environmental Bureau officially classified water hyacinth as the most perilous weed impacting Lake Tana. At that time, approximately 20,000 hectares of the lake's northeastern shoreline had succumbed to the infestation. Subsequently, in 2014, Ethiopian researchers discovered that roughly one-third of the lake's shoreline, spanning approximately 128 kilometers, had fallen under the invasion of water hyacinth.

Lake Koka, situated at Latitude 08° 24'0"N and Longitude 39° 35'0"E, originated from the damming of the Awash River during the late 1960s to facilitate hydropower generation. With an area spanning 255 square kilometers, the lake boasts a maximum depth of 14 meters and a minimum depth of 9 meters. Its dimensions include a maximum length of 20 kilometers and a

maximum width of 15 kilometers, with a shoreline extending between 195 and 205 kilometers. The water temperature averages around 20°C [42]. Notably, approximately thirty-seven percent of the lake's surface area is covered by water hyacinth [43].

This invasive plant species poses various challenges, including obstructing water transportation, disrupting hydroelectric operations, clogging canals and rivers, leading to flooding, and posing health risks to humans. Moreover, it contributes to increased evapotranspiration, interferes with fishing, irrigation, navigation, and livestock watering, and diminishes biodiversity. Although water hyacinth presents significant challenges, it also possesses beneficial properties. Despite its high water content, its fibrous tissues and rich energy and protein content render it suitable for diverse applications. These include wastewater treatment, composting, ethanol and glucose production, and biogas generation.

2.4.2 Physical composition of water hyacinth

The composition of the water hyacinth in water content, chemical and structural compositions is shown in table 2-3. The advantage of water hyacinth as biomass source for citric acid production is, its low lignin content and higher cellulose and hemicellulose content which is desirable.

Table 2- 3: Chemical compositions of water hyacinth.

	[44]	[45]
Moisture content %		90
Organic matter (% dry basis)		
Cellulose + Hemicellulose	46.74	24
Lignin	27.69	16
Ash (% dry basis)	18.20	20
Elemental composition (% dry basis)		
C	42.18	38.4
H	6.5	5.85
O	27.52	28.1
N	4.25	2.9

2.5 Factors Affecting Citric Acid Production

2.5.1 Medium and Its Components

2.5.1.1 Carbon source

The nature of the carbon source plays a important role in the synthesis of citric acid. It has been observed that the presence of easily metabolizable carbohydrates is crucial for achieving high citric acid production. Sucrose has been found to be the most favorable source of carbon followed by glucose, fructose, and galactose. Notably, galactose leads to minimal fungal growth and does not promote citric acid accumulation [46]. Other carbon sources such as sorbose, ethanol, cellulose, mannitol, lactic acid, malic acid, and α-acetoglutaric acid support limited growth and result in low citric acid production. Starch, pentoses (xyloses and arabinoses), sorbitol, and pyruvic acid, on the other hand, impede growth, leading to minimal production. Cellulose and hemicellulose are polysaccharides that can degraded into simple sugar to produce citric acid.

Cellulase enzymes possess the remarkable ability to break down cellulose into fermentable sugars like glucose. These sugars serve as valuable raw materials for various products including ethanol, biofuel, and other useful chemicals such as lactic acid, acetic acid, and propionic acid, derived from cellulosic feedstocks. [47].

The initial sugar concentration plays a crucial role in citric acid production as well as the formation of other organic acids by A. niger. According to Xu et al. (1989), A. niger strains demonstrated optimal citric acid production when the initial sugar concentration ranged from 10% to 14%. Citric acid production was found to be negligible when the sugar concentration fell below 2.5% [48].

2.5.1.2 Nitrogen source

Regarding the nitrogen source, it has a direct impact on citric acid production. The concentration of nitrogen is a significant factor influencing citric acid production. Physiologically, ammonium salts, such as urea, ammonium sulfate, ammonium nitrate, peptone, and malt extract, are preferred nitrogen sources. Nitrogen consumption leads to a decrease in pH, which is a crucial factor in citric acid fermentation. However, it's essential to maintain pH levels during the initial day of fermentation before a certain amount of biomass is produced.The nitrogen source concentration necessary for citric acid fermentation

generally falls within the range of 0.1 to 0.4 N per liter. Higher nitrogen concentrations promote fungal growth and the consumption of sugars but lead to a reduction in the amount of citric acid produced [49].

2.5.1.3 Phosphorous source

Phosphate presence significantly influences citric acid yield, with potassium dihydrogen phosphate being identified as the optimal phosphorus source. Shu and Johnson (1948) found that a phosphorus concentration ranging from 0.5 to 5.0 g/L in a chemically defined medium is required for maximum citric acid production by the fungus [50]. Phosphate is essential for A. niger's growth and metabolic processes [51]. Lower phosphate levels favor citric acid production, but excess phosphate can lead to the formation of specific sugar acids, reduced CO2 fixation, and increased fungal growth. Phosphates primarily affect enzyme activity rather than gene expression [52]. Interestingly, different strains exhibit varying nitrogen and phosphorus concentration requirements in the medium, with nitrogen and phosphorus limitations being critical factors in citric acid production due to their complex interplay.

2.5.1.4 Trace elements

The nutritional availability of trace elements appears to be the primary factor influencing the citric acid yield. Several divalent metals, including zinc, manganese, iron, copper, and magnesium, have been identified as having an impact on citric acid production by A. niger. However, it's important to consider the interplay among various components in the medium, both in Submerged Fermentation (SmF) and potentially in Solid-State Fermentation (SSF). When added together with KH2PO4, zinc has been found to promote citric acid production. Conversely, the presence of manganese ions, iron, and high concentrations of zinc could lead to reduced citric acid yields, particularly in a phosphate-free medium. Shankaranand and Lonsane (1994) observed that A. niger exhibited few differences in its response to metal ions and minerals in SSF compared to SmF systems. SSF systems appeared to be capable of mitigating the adverse effects of high concentrations of these components in the medium. Consequently, the addition of chelating agents like potassium ferrocyanide to the medium was found to be ineffective [51].

Copper was discovered to complement iron's capacity to enhance citric acid biosynthesis at its optimal level. Manganese deficiency led to the inhibition of anaerobic and TCA cycle enzymes, with the exception of citrate synthetase. This resulted in the overproduction of citric

acid as a byproduct of glycolysis [52]. Even a minor deficiency in manganese (in parts per million) could reduce citric acid yield by as much as 10%. The addition of iron was found to decrease citric acid accumulation and also had some impact on mycelial growth. Benuzzi and Segovia (1996) emphasized the significance of different copper concentrations in the medium for pellet formation, as it played a crucial role in establishing a suitable cellular structure related to citric acid production physiology. The optimal initial concentration of $CuSO_4.5H_2O$ was determined to be 78 mg/L [53]. Magnesium is essential for both growth and citric acid production, with the optimal concentration of magnesium sulfate falling within the range of 0.02% to 0.025%.

2.5.1.5 Lower alcohols

The introduction of lower alcohols has been shown to enhance citric acid production when using commercial glucose and other crude carbohydrates. Suitable alcohols for this purpose include methanol, ethanol, isopropanol, or methyl acetate. The optimal quantity of methanol or ethanol varies depending on the specific strain and medium composition, generally falling within the optimal range of 1% to 3%. Several authors have extensively investigated the effects of methanol or ethanol addition [15], [49], [54]. Reports indicate that the addition of ethanol led to a twofold increase in citrate synthetase activity and a 75% decrease in aconitase activity, while the activities of other enzymes in the TCA cycle showed slight increases [55]. It has been observed that alcohols stimulate citric acid production by influencing both growth and sporulation. This effect is attributed to changes in cell permeability, spatial organization of the membrane, or alterations in the lipid composition of the cell wall.

2.5.1.6 Miscellaneous

Certain compounds known to inhibit metabolism, such as calcium fluoride, sodium fluoride, and potassium fluoride, have been discovered to expedite citric acid production. Conversely, the presence of potassium ferrocyanide has been found to reduce citric acid yields. Numerous compounds have various mechanisms that favor the accumulation of citric acid. Some of these compounds can interfere with the action of metal ions and counteract the influence of toxic substances on growth during the initial phase. Among these compounds are 4-Methylumbelliferone, 3-hydroxi-2-naphtoic, benzoic acid, 2-naphtoic acid, iron cyanide, quaternary ammonium compounds, amine oximes, starch, EDTA, vermiculite, and others.

Table 2- 4: Factors affecting citric acid production

Factor	Positive effect	Level	Negative effect
Carbon source	Sucrose Glucose Fructose Galactose	14-22%	Starch Xylose Arabinose Sorbitol Pyruvic acid
Phosphorous Source	Potassium Dehydrogen phosphate	0.5-50 g/L	
Nitrogen Source	Ammonium nitrate Ammonium sulphate Peptone Malt extract Urea	Under 25% 0.1-0.4 gN/L	
Trace elements	Zinc Copper Magnesium sulfate	Low level 0.02-0.025%	Manganese 1ppm
Low alcohols	Methanol Ethanol n-propanol Iso-propanol Methylacetate	1-4% volume per mass	
Oils and fats		0.05-0.3%	
Other compounds	Calcium fluorite Sodium fluoride Potassium 3-hydroxy-2-naphtic acid		Potassium ferrocyanide Quaternary ammonium compound Amine oxides

*Source: [4]

2.5.2 Process parameters

2.5.2.1 pH

The pH level within a culture can undergo changes due to microbial metabolic activities. One prominent reason for this is the secretion of organic acids like citric, acetic, or lactic acids, which tends to lower the pH. It's important to note that the kinetics of pH changes are highly dependent on the specific microorganism involved. For microorganisms like Aspergillus sp., Penicillium sp., and Rhizopus sp., the pH can drop rapidly, sometimes reaching levels below 3.0. In contrast, other groups of fungi such as Trichoderma, Sporotrichum, and Pleurotus sp.

tend to maintain a more stable pH, typically within the range of 4 to 5. Additionally, the characteristics of the substrate also play a role in pH kinetics.

In general, for optimal citric acid production, a pH level below 2.0 is typically required. A lower initial pH offers the advantage of preventing contamination and inhibiting oxalic acid formation. Some studies have reported an optimal pH of 2.2 for both mold growth and citric acid production, while in molasses medium, higher pH values, such as 5.4 and 6.0-6.5, have been found to be optimal for citric acid production.

2.5.2.2 Aeration

Aeration plays a vital role in citric acid fermentation, as demonstrated in various studies [56]. Increased aeration rates have been shown to enhance yields and reduce fermentation time [12]. The concentration of dissolved oxygen also affects citric acid production, with levels above 25% saturation being crucial to maintain [57]. To meet the oxygen demand, suitable aeration devices are utilized, chosen based on the viscosity of the fermentation broth. This preference for compact mycelial pellets during A. niger fermentation is partly due to their favorable impact on aeration [58]. However, filamentous growth, possibly induced by metal contamination, can significantly reduce dissolved oxygen tension even without a substantial increase in dry weight.

Aeration is maintained consistently throughout fermentation, typically at rates of 0.5 to 1.5 vvm. Initially, lower aeration rates ranging from 0.1 to 0.4 vvm are commonly employed for economic reasons. High aeration rates can lead to excessive foam formation, necessitating the use of mechanical defoamers. Although sparging with pure oxygen has been shown to increase citric acid yield, its costliness limits its practical application [59].

2.6 Product recovery

Citric acid recovery from fermentation generally employs three primary methods: precipitation, extraction, and adsorption/absorption, often utilizing ion exchange resins.

Precipitation has traditionally been the primary method for citric acid recovery, suitable for various processes. However, it necessitates the removal of micelles from the fungus, fermentation broth, and suspended materials through filtration. An optimized precipitation

method involves a temperature of 50°C for 20 minutes, resulting in efficient precipitation of calcium citrate and subsequent citric acid recovery [60]. Although the precipitation rate is relatively slow, it is easy to filter. The medium is enriched with calcium oxide hydrate, commonly known as milk lime, which subsequently undergoes precipitation to form tricalcium citrate tetrahydrate. Following this, the precipitate is subjected to filtration and rinsed with water. Subsequently, it is processed in an acidulator using calcium sulfate, resulting in the formation of gypsum through the reaction with sulfuric acid.

Solvent extraction emerges as an eco-friendly option for citric acid purification and crystallization, offering advantages over traditional precipitation methods. Utilizing substances like n-octyl alcohol and tridodecylamine for extraction, this approach avoids gypsum generation, thus minimizing environmental impact. Notably, it has gained approval from the US FDA for use in food and pharmaceutical sectors [12]. Electrodialysis, on the other hand, employs charged membranes to separate ionic and aqueous solutions, proving cost-effective for transparent fermentation media. However, it comes with a higher price tag compared to precipitation and other methods [61]. In laboratory settings, citric acid analysis typically involves spectrophotometry, chromatography, nuclear magnetic resonance spectroscopy, and titration.

2.7 Applications of citric acid

Citric acid is widely recognized as a safe and versatile food additive, meeting the standards of the Joint FAO/WHO Expert Committee on Food Additives [62], [63], [64]. Its extensive use in the food and pharmaceutical industries, comprising about 60% of its total utilization, is attributed to its safety, pleasant taste, water solubility, and chelating and buffering properties. Additionally, it finds applications in cosmetics, toiletries, agriculture, and other sectors due to its multifunctionality as a buffer and chelating agent.

Table 2- 5: Applications of citric acid

Sector	Industry	Application
Cosmetics and toiletries		Acts as pH adjuster, antioxidant and buffering agent
Food and beverage	Animal fats and oils	it boosts the antioxidant properties when combined, also used as a sequestrant.
	Dairy products	Used as an emulsifier in ice creams and processed cheese, aiding in the smooth blending of ingredients. It also functions as an acidifying agent in various cheese products, contributing to their characteristic tanginess.
	Fruits and vegetable juices	Acts as stabilizer in commercially prepared juices, helping to maintain the freshness and consistency of the juice over time.
	Gelatin desserts	Adjusts pH to the desired levelsand contributes to the proper setting of the gelatin for the formation of the dessert.
	Jellies and jams	Gelling agent,helping to achieve the desired consistency, while also imparting the ideal level of tartness, tanginess, and flavor.
	Soft drinks and syrups	As acidulant in carbonated and sucrose-based beverages, enhancing their natural fruit flavor while imparting a desirable tartness..
	Frozen fruits	Neutralizes the residual lye, effectively lowering the pH to deactivate oxidative enzymes. Additionally, it safeguards ascorbic acid by deactivating trace metals present in the solution.
	Candies	Acts as acidulant. Provides tartness. Minimizes sucrose inversion. Produces dark color in hard candies. Prevents crystallization of sucrose.
Pharmaceuticals		It is commonly used in tablets, syrups, and effervescent formulations. It serves various purposes such as enhancing solubility when combined with bicarbonates, acting as an antioxidant in vitamin preparations, providing acidity in mild astringent formulations, and serving as an anticoagulant. Moreover, it adds flavor to oral solutions and oral disintegrating tablets.

CHAPTER THREE

3 MATERIALS AND METHODS

The laboratory work was conducted in both the laboratory of the School Chemical and Bio-Engineering, Addis Ababa Institute of Technology, Addis Ababa University, Addis Ababa and College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa.

3.1 Material

Aspergillus niger was the fungus strain used for this research. The strain was procured from mycology laboratory, College of natural science, Addis Ababa University.

Water hyacinth is used as substrate. The samples were collected from Lake Koka and brought to the Addis Ababa institute of technology (AAiT), School of Chemical and Bio-engineering department's environmental laboratory.

3.2 Chemicals and Analytic Reagents

The chemicals and analytic reagents grades used were 0.1 M of sodium hydroxide, 0.1 N sulfuric acid, phenolphthalein as indicator, distilled and deionized water, ethyl alcohol at 95% (v/v), and 70% (v/v), activated carbon, potato dextrose agar, 0.1 M hydrochloric acid, sucrose, tween 80 solution, magnesium sulfate, calcium chloride, potassium dihydrogen phosphate, ammonium sulphate magnesium heptahydrate, calcium chloride.

3.3 Equipment

The equipment used for this study includes Erlenmeyer flasks 250 mL, volumetric flask 200 mL conical flasks and beakers 50 mL, 100 mL, 250 mL, measuring cylinders 50 mL, 100 mL, 250 mL, whatmann №.1 and № .42 , micro membrane filter paper 45 µm, test tubes 15 mL, burette 50 mL, autoclave, magnetic stirrer, shaker incubator , balance (precision, ±0.0001), drying oven, different size of centrifuge, pH meter, incubator, water bath, aluminum foil, furnace, porcelain crucibles, crucible furnace, dryer, flat-bottomed balloon flask with roughened neck, melting point apparatus, condensation unit for flask, funnel, filtration

crucible, rubber cones, gloves, Petri dish, thermometer, vacuum filter, hemocytometer, Atomic Absorption Spectroscopy (AAS), plastic bag.

3.4 Characterization of water hyacinth

In this section the characteristics of water hyacinth was studied by using different techniques. Moisture content analysis, ash content, volatile analysis solid, and TGA were carried out.

3.4.1 Determination of Moisture Content

The samples were placed inside polyethylene bags containing water to maintain their freshness and prevent wilting, ensuring a consistent water content that mimics field conditions. These samples were promptly transported to the laboratory to determine their initial moisture content. Before drying, water hyacinth samples were thoroughly cleaned to remove any foreign matter such as stones, dust, and plant materials.

To measure the moisture content, 10 grams of water hyacinth sample was taken and oven-dried in a crucible at a temperature of 103±2°C. The sample was weighed every 30 minutes until a constant weight was achieved. After drying, the sample was allowed to cool in a desiccator for 30 minutes before being re-weighed. This method, known as "Method B – Oven-Drying Secondary," is detailed in ASTM D4442-16. The procedure was repeated three times for precision [65]. Finally, the results were calculated using the following formula:

$$\text{Moisture content (\%)} = \frac{w_1 - w_2}{w_1} * 100 \quad (3\text{-}1)$$

Where: W_1 = original weight

W_2 = weight after oven dried

3.4.2 Determination of ash content

Dry crucibles containing 10g samples were placed in a muffle furnace set at 550°C for 2 hours. Afterward, the samples were cooled in a desiccator and weighed. The ash content was determined using the equation described in ASTM E1755-01 (2015). The results are reported relative to the 105°C oven-dried mass of the sample [66]. The ash content was calculated using the following formula:

$$\text{Ash (\%w/w)} = \frac{M_2 - M_1}{M_3} * 100 \quad (3\text{-}2)$$

Where- M_1 - mass of crucibles, g

M_2 - mass of crucibles with ash, g

M_3 - mass of oven dried sample, g

3.4.3 Volatile Matter Content

Oven dried samples was put in a known weight of dry crucibles. The samples and the crucibles were placed in a furnace for 7 min at 950 ℃. The crucible was removed from furnace and placed in a desiccator for cooling, then was reweighed. It was determined using ASTM E872-82 (2011) [67]. The process was be repeated until constant weight was obtained. Then,

$$\text{Volatility content (\%)} = \frac{w_1 - w_2}{w_1} * 100 \quad (3\text{-}3)$$

Where: W_1 = oven dried weight of sample

W_2 = weight after heating in furnace

3.4.4 Crude Fibre Determination

Crude Fiber serves as a measure of indigestible cellulose, lignin, and other similar components within a sample, representing the residue left behind after undergoing acidic and alkaline digestions.

In the described procedure, a sample weighing 3 grams was sequentially treated with boiling solutions of sulfuric acid (0.25 N) and potassium hydroxide (0.25 N). The resulting residue was then separated via filtration, washed, dried, weighed, and subsequently ashed at a temperature of 500°C. The loss in weight observed during ashing corresponds to the amount of crude fiber present in the sample [68]. This loss in weight was calculated using the following formula:

$$\text{\% Crude fibre} = \frac{C_1 - C_2}{Cs} \text{ x } 100 \quad (3\text{-}4)$$

Where

C_1= weight of the crucible with dry residue

C_2 = weight of the crucible with ash

Cs= weight of sample

3.4.5 Crude Lipid Determination

A clean and dried 500 cm^3 round bottom flask, equipped with a few anti-bumping granules, was weighed (W1). Then, 300 cm^3 of petroleum ether (40-60°C) was added to the flask for extraction, which was subsequently poured into a Soxhlet extraction unit. A twenty-gram extractor thimble was placed into the Soxhlet unit. The round bottom flask, along with a condenser, was connected to the Soxhlet extractor, and cold-water circulation was initiated. The heating mantle was turned on, and the heating rate was adjusted until the solvent refluxed steadily. Extraction was conducted for 6 hours.

Following the extraction, the solvent was recovered, and the oil was dried in an oven set at 70°C for 1 hour. The round bottom flask, along with the dried oil, was then reweighed (W2). The lipid content was calculated as a percentage. This analysis was performed according to the guidelines outlined in AOAC (2000) [69].

$$\%\ \text{Crude Lipid(dry mass basis)} = \frac{W_2 - W_1}{W_s} \text{ x } 100 \qquad (3\text{-}5)$$

Where

W_1 = initial sample weight in grams and W_2 = weight of beaker and fat residue in grams

3.4.6 Protein Content Determination

For this task, Kjeldahl's method was used to evaluate the total nitrogen content of the sample [70].

Three steps were carried out: digestion, distillation, and titration.

I. **Digestion**: The sample under analysis was weighed into a digestion flask and subjected heat in the presence of sulfuric acid, an oxidizing agent which digests the food. Additionally, anhydrous potassium sulfate which is used to speed up the reaction by raising the boiling point. This process resulted in the conversion of any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia and other organic matter to CO_2 and H_2O. Ammonia gas is not liberated in an acid solution because

the ammonia is in the form of the ammonium ion (NH_4^+) which binds to the sulfate ion (SO_4^{2-}) and thus remains in solution. In this study, 1.6355 g of dry sample was weighed out into 300cm^3 Kjeldahl flask; then 1 g potassium sulfate, 0.7 g mercuric oxide, and 20 mL concentrated sulphuric acid were added. The sample was digested at a temperature slightly above the boiling point of sulfuring acid (340 ºC) until a clear green colour was obtained. The digest was cooled and diluted with 100 cm^3 with distilled water.

II. **Distillation**: The second stage followed was the distillation process. To change the ammonium ions to ammonia the pH of the sol was raised by adding 40 mL of 40% NaOH until the solution becomes highly alkaline. To facilitate smooth boiling during digestion and minimize the risk of bumping, fine bubbles of hydrogen gas were evolved. 0.5 g of zinc dust was added to each flask. The Kjeldahl flask was attached to a water condenser then heated to boil off the NH3 gas from the digest. To trap the distilled NH_3 in 26 receiving solution the tip of the condenser was submerged in a flask of receiving boric acid solution.

III. **Titration**: Distillation collecting distillate was carried out in volumetric flask containing 50 cm^3 of 2% boric acid and about 150 cm^3 of distillate was collected. Boric was used a receiving solution to capture the ammonia gas, resulting in the formation of an ammonium-borate complex. As the ammonia accumulated, the color of the receiving solution was changed to slight whitish hue. Three drops of mixed indicator (methyl red-bromocresol green) indicator were added to this distillate. Finally, 20 mL of 0.1 N HCl until the solution has a slightly violet color. A 4.45 mL of HCl was also used to the reagent blank. Then protein content determination was then carried out by the following formula:

$$\%N_2 = \frac{(V_{standard\_acid} - V_{blank}) \times N_{acid} \times 14}{\text{weight of sample}} \times 100 \qquad (3\text{-}6)$$

Where

N = normality

$V_{standard\_acid}$ = the titration volume of the acid

V_{blank} = volume of standard acid needed to titrate a reagent blank

14 is the molecular weight of nitrogen in g

Then, to determine protein content, a nitrogen-to-protein conversion factor of 6.25 (most proteins contain 16% of nitrogen, and thus 1/0.16 = 6.25)

% Crude protein= $\%N_2 * 6.25$

3.4.6.1 Carbohydrate determination

The total carbohydrate of water hyacinth was determined by calculating the sum percentage of moisture, ash, crude lipid, crude protein and crude fibre and by subtracting it from 100 as shown on equation (3-8).

$$\%\text{Total carbohydrate} = 100 - (\%\text{Ash} + \%\text{Lipid} + \%\text{Protein} + \%\text{Fiber}) \quad (3\text{-}8)$$

3.4.6.2 Fiber content determination

The research experiment is conducted at leather institute using therrmal gravimetric analyzer instrument (SDT Q600 V20.9 Build 20). The chemical composition of cellulose, hemicellulose, lignin, and the inorganic ash content in water hyacinth was carefully analyzed. 10.6700 mg of oven-dried water hyacinth was subjected to pyrolysis in a nitrogen at a certain flow rate in temperature range of 24°C to 710.00 °C, with increasing rate of 10.00 °C/min. The weight loss vs. temperature curve generated was then analyzed to determine the water hyacinth's chemical compounds based on the degradation of individual compounds as specified temperature range [71].

3.5 Raw material pretreatment

The water hyacinth was washed to remove various impurities and dirt from the surface of the sample. It was shade-dried for several days, then further dried in the drying oven for 2 hrs. at 60oC. It was powdered mechanically with a centrifugal mill with a 2mm sieve at an acceptable size range mechanical unit operation laboratory. The desired substrate particle size was less than 2mm for easier accessibility of substrates components and mycelial penetration. To acquire desired particle, size the substrate was farther sieved with a vibratory sieve machine in the mechanical unit operation laboratory at the same department. The powdered substrate was stored in a tight sealed polyethylene bag (Ziploc) until fermentation step.

The major equipment used for sample preparation were analytical balance, crucible, desiccator, drying oven, centrifugal mill, laboratory sieve and sieve shaker.

3.6 Microorganism and Inoculum Preparation

Aspergillus niger strain was procured from mycology laboratory at college of natural science, Addis Ababa University. The culture was further sub cultured on (potato dextrose agar) PDA slants. The PDA media was prepared by dissolving 4g of PDA in 80 ml of distilled water, then shaken to dissolve and raised to 100ml. the PDA media was cooked for 15 minutes with constant stirring until clear solution was observed. The media was decanted equally into test tubes, each test tube was cotton plugged, then sterilized in an autoclave for 15 minutes at 121^{o}C. After sterilization was complete, the test tubes were transferred to the thoroughly disinfected safety hood. The test tubes were positioned at the 30^{o} angle slant in order to increase surface area after the PDA solidified. It was inoculated by carefully transferring small amount of Aspergillus niger using a sterile loop. The test tubes were incubated at 28^{o}C for 5 days. After 5 days the culture was refrigerated at 4^{o}C for fermentation process.

3.7 Experimental Design

The data from laboratory experiment was analyzed with Design Expert software using response surface method (RMS), it determines optimum operation factor the program finds the suited model for the yield as a function of the three variables. The chosen three independent process variables which were fermentation temperature, incubation time, and pH.

The results underwent statistical analysis using one-way analysis of variance (ANOVA) to ascertain the optimal conditions to set for citric acid production. To minimize the impact of unexplained variability in the observed responses caused by systematic errors, all experiments were conducted randomly. This approach helps ensure that any observed differences in the results are more likely attributable to the experimental conditions rather than any systematic biases.

3.7.1 Fermentation Conditions Preparation and Settings

The experiments conducted to produce citric acid were carried out on Design Expert 13 software based on the response surface methodology (RMS) a three-variable Box-Behnken Design (BBD). The three-factors with three levels for each variable were incubation temperature, incubation time and pH as shown in the above Table 3-2. The number of an

experimental run for this design was 17 with five center point. Citric acid yield expressed in g/L.

Table 3-1: Experimental Range and Level of the Independent Variables

Variables	Units	Levels		
Temperature	ºC	25	30	35
Incubation Time	days	3	6	9
pH		2	4	6

3.7.2 Fermentation Process Parameter

The experiment was executed according to the details provided in Table 3-2. A series of 17 experiments were conducted utilizing a three-variable Box-Behnken design as part of the response surface methodology.

Table 3-2: Box-Behnken Design for Citric Acid yield

Run	**Incubation Time**	**Fermentation Temperature**	**pH**
1	3	35	4
2	9	30	6
3	3	30	6
4	9	35	4
5	6	25	2
6	9	25	4
7	6	30	4
8	3	30	2
9	6	30	4
10	6	35	6
11	6	30	4
12	6	30	4
13	6	30	4
14	6	35	2
15	3	25	4

16	6	25	6
17	9	30	2

3.7.2.1 Fermentation Temperature

Incubation temperature is one of the major factors influencing the microbial growth and citric acid formation. The impact of temperature on citric acid production was investigated by conducting fermentations at varying temperatures, including 25°C, 30°C, and 35°C, while also varying the incubation time and pH. These specific temperature levels were chosen because most filamentous fungi, which are commonly used in citric acid production, are mesophilic and thrive within an optimal temperature range of 25°C to 35°C. By examining citric acid production across different temperature conditions, the study aimed to elucidate the temperature's influence on the fermentation process and optimize conditions for enhanced citric acid yield.

3.7.2.2 Fermentation Time

Fermentation time was also a factor studied in citric acid synthesis. It was observed at different temperature and pH. The optimum incubation time for maximizing citric acid yield varies both with the organism and fermentation conditions. The influence of incubation time was investigated at different days ranging from 3, 6, and 9 days.

3.7.2.3 pH

The influence of pH on citric acid yield during fermentation was investigated by conducting experiments at different initial pH levels, including pH 2, pH 4, and pH 6, while also varying temperature and incubation period. These specific pH levels were chosen based on previous studies that demonstrated successful citric acid production by Aspergillus niger species within a pH range of 2 to 6. By exploring citric acid production across different pH conditions, the study aimed to understand how pH affects the fermentation process and optimize conditions for maximizing citric acid yield.

3.8 Preparation of Fermentation Media

Synthesis of citric acid in solid state fermentation was conducted by using 10g of pre-treated water hyacinth as the substrate. Each substrate portion was allocated to individual 250 mL Erlenmeyer flasks, with a working volume of 150 ml. Following this, a nutrient-rich medium was prepared and added to each flask. The composition of the nutrient media was NH_4SO_4 0.3 g, KH_2PO_4 0.23 g, $MgSO_4.7H_2O$ 0.077 g, 3%(v/w) ethanol and 80% moisture content. Sucrose was added to the medium (100 g/1000 g) to enhance citric acid production by boosting carbon source. The contents were thoroughly mixed and the pH of the mixture was adjusted to approximately to 3.5 with NaOH or HCl, then cotton plugged and autoclaved at 121 ºC for 15 minutes.

Spores' suspension of the fungus was prepared by rinsing a 5 days old culture in potato dextrose agar slant with sterilized 0.1% Tween 80 solution. The tween 80 solution was made by dissolving 50µL of polysorbate in sterilized distilled water and was vigorously shaken for a minute. Spores were quantified by a hemocytometer to adjust the count to approximately 1×107spores/ml.

After cooling, the autoclaved fermentation media underwent inoculation with 1 ml of inoculum, followed by incubation under varied conditions in a shaker incubator, with a constant shaking speed of 200 rpm. The pH, time, and temperature were adjusted as per the experimental design. Upon completion of fermentation, the medium was diluted with sterilized distilled water (in a ratio of 1:4 w/v) and agitated for 60 minutes at 121 rpm to ensure thorough mixing. Subsequently, the mixture was filtered through filter paper, and the resulting filtrate was utilized for analysis.

3.9 Separation mechanism

Among different separation mechanisms used to separate citric acid from fermentation media, precipitation recovery method was used in this research. The fermentation media with different parameters (pH, incubation time and incubation temperature) was filtrated using wattman filter paper with vacuum filter. Sodium hydroxide was then added to the filtrate before calcium chloride to hinder the formation of hydrochloric acid. Addition of sodium hydroxide to the filtrate raised the pH between 8 and 9 forming trisodium citrate. As shown in the chemical reaction below to recover citric acid, calcium chloride solution formed by

dissolving 28.5 g calcium chloride in 70 mL of distilled water, the solution was added gradually while continuously stirred at 70 °C by hot plate magnetic stirrer.

$$2C_6H_5O_7Na_3.4H_2O + 3CaCl_2 \rightarrow (C_6H_5O_7)_2Ca_34H_2O + 6NaCl \quad (3\text{-}1)$$

When the medium's pH reached 7, an exothermic reaction led to the formation of a white tricalcium citrate precipitate. This precipitate was collected by centrifuging the suspension at 3000 revolutions per minute for 10 minutes and then filtered using Whatman No. 1 filter paper. After several washes with sterilized distilled water, the washed precipitate underwent conversion to soluble citric acid and insoluble calcium sulfate by the addition of diluted sulfuric acid (98%), following the stoichiometric relationship in the equation. The amount of sulfuric acid utilized depended on the recovered calcium citrate, with less sulfuric acid than required being used to ensure complete consumption. Separating sulfuric acid from citric acid is challenging, whereas any remaining calcium citrate can be easily filtered out. Subsequent stirring with a hot plate magnetic stirrer for 90 minutes at 90°C facilitated the precipitation of calcium sulfate from the mixture of insoluble calcium citrate and sulfuric acid [8].

$$(C_6H_5O_7)2Ca3.4H_2O + 3H_2SO_4 \rightarrow 2C_6H_8O_7 + 3CaSO_4.2H_2O + 2H_2O \quad (3\text{-}2)$$

The resulting suspension from the aforementioned reaction underwent centrifugation at 3000 revolutions per minute for 10 minutes, followed by filtration of the calcium sulfate using Whatman No. 42 filter paper. Subsequently, the filtrate underwent treatment with activated carbon and vacuum filtration to remove any colorants. Finally, the supernatant, presumed to contain citric acid in solution, was filtered through micro-membrane filter paper to eliminate any fungal residues.

3.10 Citric Acid Determination

The citric acid concentration in the solution was determined titrimetrically by employing 0.1M NaOH and phenolphthalein as indicator. A set volume of the analyte (presumed to be a citric acid solution) was placed in a titration flask and the burette filled with 0.1 M NaOH standard solution until the zero-mark using a funnel. Before the titration was began, three drops of phenolphthalein were poured into the citric acid solution. Gradually the standard solution (NaOH) was added, drop by drop with continuous magnetic stirrer to until the phenolphthalein indicator exhibited an intermediate color (weak pink) between that of acid and the color of the base.

Titration concludes at the endpoint, where the solution is neither acidic nor basic, indicating a stoichiometric balance between the acid and the base. At this point, the volume of NaOH in the burette is noted, marking the completion of the titration process.

For calculation purposes, it's established that 1 mL of 0.1 M NaOH is equivalent to 0.064 g of citric acid [72]. This conversion factor (0.064) is then utilized to express the amount of citric acid in grams per liter using the formula

$$.\frac{g}{L} citric\ acid = \frac{titre(volume\ of\ NaOH)X0.064\ X100X10}{X(ml\ anayte)\ X\ 1000ml} \quad (3\text{-}9)$$

3.11 Crystallization of Citric Acid

Freeze drying is widely considered the optimal method for removing water to obtain a final product of premium quality. This is due to the high content of liquid in the solution and the low temperature required for the process operation, which effectively prevents deterioration reactions and microbiological activities. As a result, the final product is of excellent quality. The mother liquor containing citric acid was ultimately concentrated in a freeze dryer, thereby forming citric acid as the final product.

3.12 Characterization of Citric acid

3.12.1 Determination of Purity of the Citric acid

The purity of the obtained product was determined using a melting-point apparatus, comparing the melting temperature of the product crystals with that of pure, anhydrous citric acid crystals.

Furthermore, determination of citric acid content was performed gravimetrically following the protocol outlined by Merrier and Boulet in 1958, which involved the use of pyridine-acetic anhydride. In brief, four grams of the sample were precisely weighed and dissolved in 40 mL of distilled water. Following this, 1.3 mL of pyridine was added for each 1 mL of sample, and the solution was thoroughly mixed. Subsequently, 5.7 mL of acetic anhydride was added, and the mixture was incubated at 32°C for 30 minutes in a water bath. Optical density values of the sample were measured at 405 nm using a UV-visible spectrophotometer. The concentration of citric acid in the sample was determined by

comparing it with a reference run conducted concurrently, after preparing a citric acid standard from citric acid monohydrate by dehydrating it at 90°C for 72 hours until a constant weight was achieved.

3.12.2 Determination of the Organic Impurities

The Sulphated Ash test, also known as the ash content test, is a common analytical technique used to determine the inorganic residue left behind after a sample is completely burned or ashed at a high temperature. This test is particularly relevant for organic compounds like citric acid to assess the purity and presence of inorganic impurities or contaminants.

Sulphated Ash test is typically conducted:

I. **Sample Preparation:** Take a known quantity of the citric acid sample (usually specified in the test method) and place it in a crucible. The crucible should be clean and heat-resistant. Silica crucible was dried over a burner for 10 minutes, it was cooled to room temperature in a silica gel containing desiccator.
II. **Drying:** Heat the crucible containing the sample to a moderate temperature (usually around 105-110°C) to remove any moisture. This step ensures that the weight loss during ashing is primarily due to the organic content.
III. **Ashing:** Five gram of the sample was weighed accurately in a tared crucible. It was ignited in a muffle furnace at 600 ± 50 ºC until the material was thoroughly charred.
IV. **Cooling:** The residue was cooled, moistened with 1 mL of sulphuric acid and ignited till the carbon source was completely oxidized.
V. **Calculation:** The crucible was cooled in a desiccator and weighted. Percentage of sulfated ash was calculated as follows:

$$\text{Sulfated ash, percent by mass} = \frac{M_1}{M_2} * 100 \qquad (3\text{-}9)$$

Where

M_1 = Mass, in g, of the residue

M_2 =Mass, in g, sample used

3.12.3 Determination of heavy metals

The sample preparation procedure adhered to the Wet-ashing technique developed by Maria in 2002. One gram of dried water hyacinth was carefully weighed and placed into an acid-washed glass round-bottom flask. To facilitate digestion, 20 mL of aqua regia (a mixture of HCl and HNO3 in a 3:1 ratio) was added to the flask. A condenser was then attached to the round-bottom flask, which underwent reflux for two hours at 90°C. Following digestion, the round-bottom flask was rinsed with distilled water, and the sample was filtered through Whatman filter paper No. 1 to separate the insoluble solid from the supernatant liquid. Distilled water was added to adjusted the volume to 100 mL with , and all samples and blanks were stored in plastic containers.

After digestion, the sample was analyzed using AAS to determine the concentration of metal ions present. Arsenic (As), Iron (Fe), and Lead (Pb) were analyzed using AAS equipped with a deuterium arc background corrector and a standard air-acetylene flame system. External calibration curves were used for analysis after optimizing parameters such as burner and lamp alignment, slit width, and wavelength adjustment to minimize signal intensity. Each sample underwent three separate determinations. Hollow cathode lamps were utilized under the manufacturer's recommended conditions at their respective primary source lines.

Calibrate the instrument using standard solutions (1, 5, 10, 20, 30, 40, 50) ppm concentrations to convert the instrument's response (e.g., absorbance) to metal concentrations. The absorbance of each of the standard solutions using the AAS instrument was measured. The calibration curve was plotted the concentration of the element (x-axis) and the corresponding absorbance values (y-axis) for the calibration standards. Linear regression analysis was performed on the calibration curve to determine the equation of the best fit line.

$$A = mx + b \tag{3-10}$$

Where:

A is the absorbance

m is the slope

x is the concentration,

b is the y-intercept.

The concentration of metal ions within the samples was ascertained by measuring their absorbance via AAS and subsequently comparing it to the corresponding standard calibration

curve. Finally, the absorbance of the prepared sample was measured using AAS then calculating using equation (3-10).

CHAPTER FOUR

4 RESULT AND DISCUSSION

4.1 Biochemical Analysis of water hyacinth

In this section, the results of the analysis carried out on substrate such as proximate analysis such as, moisture content ash content volatile matter, fixed carbon content (Table 4-1) and lignocellulosic content of water hyacinth (Figure 4-1).

4.1.1 Moisture content

The mean moisture content of water hyacinth utilized for this study was determined to be 90.86% through three trail runs. Samples reached a constant weight after 1 hours. Since water hyacinth is a floating aquatic plant, the higher moisture content was anticipated. The moisture content of oven dried water hyacinth was measured to be 5.5%

4.1.2 Ash

The ash content was determined using the equation as described in ASTM E17755-01 (2020) using equation 3-2. It was found to be 12.8%., the result on Table 4-1.

4.1.3 Volatile matter content

The values of volatile matter content were 65.5% result in Table 4-1 which was high and it indicates that water hyacinth has high organic loading.

4.1.4 Organic matter content

The assessment of organic matter, encompassing the nutritional components of crude lipids, proteins, and carbohydrates, reveals their respective percentages as 2.5%, 7.01%, and 52.25%. These findings strongly indicate the presence of abundant nutrients that can serve as a favorable substrate for microorganisms engaged in the biosynthesis of citric acid.

Table 4-1: Proximate analysis of water hyacinth

No.	Parameter analysis	Content	[74]	[75]	[76]
1	Moisture (%)	90.8			
2	Ash (% dry basis)	12.8	21.1	15.35	
3	Volatile matter content (% dry basis)	65.5	64.4	70.35	
4	Crude fiber (% dry basis)	20.44			
5	Crude lipid (% dry basis)	2.5			
6	Carbohydrate (% dry basis)	57.25			
7	Protein (% dry basis)	7.01			10.23±0.3

4.1.5 Determination of water hyacinth composition

The TGA analysis of water hyacinth revealed several distinct weight loss stages, as depicted in Figure 4-1. The discussion primarily centered on three critical stages.

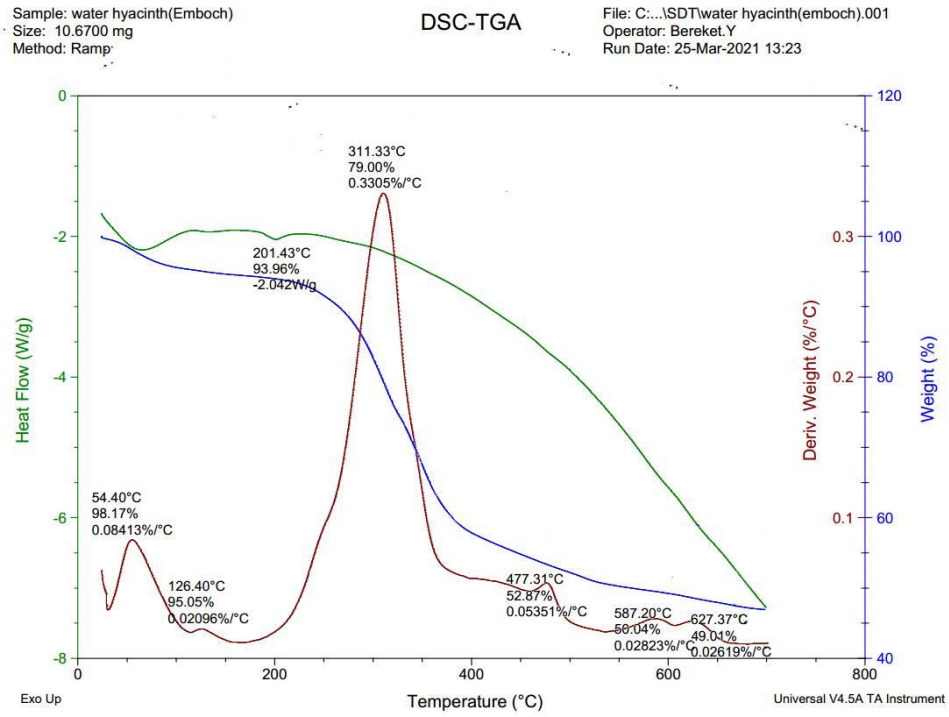

Figure 4- 1: Result of a TGA of water hyacinth

The first stage, occurring between 24 and 200°C, was due to the evaporation of residual water and volatiles. During this stage, the sample experienced a weight loss of approximately 12.29%. The second stage, spanning from 200 to 400°C, was associated with the degradation of cellulose and hemicellulose. According to the graphs, cellulose and hemicellulose

constituted 39% of the water hyacinth. In the final stage (400–500°C), the analysis determined that the sample contained 10% lignin.

4.2 Statistical Analysis

In this investigation, the BBD was used to execute of 17 experimental runs. For three-level three-factor experiments, BBD is the most widely utilized experimental design model. It is designed to fit a quadratic model always has three levels for each factor.

Table 4- 2: Box-Behnken Design for Citric Acid Production

Factors							**Response**
Run	**Coded value**			**Actual Value**			**Citric Acid g/L**
	X1	**X2**	**X3**	**X1**	**X2**	**X3**	
1	-1	+1	0	3	35	4	12.8079
2	+1	0	0	9	30	6	19.9106
3	-1	0	0	3	30	6	12.6082
4	+1	+1	0	9	35	4	19.3056
5	0	-1	-1	6	25	2	14.8649
6	+1	-1	0	9	25	4	18.0834
7	0	0	0	6	30	4	21.3867
8	-1	0	-1	3	30	2	13.3947
9	0	0	0	6	30	4	20.8725
10	0	+1	+1	6	35	6	17.4301
11	0	0	0	6	30	4	21.2718
12	0	0	0	6	30	4	21.3383
13	0	0	0	6	30	4	21.5100
14	0	+1	-1	6	35	2	18.6461
15	-1	-1	0	3	25	4	10.7085
16	0	-1	+1	6	25	6	15.8147
17	+1	0	-1	9	30	2	19.2880

The BBD does not include runs at the extreme combinations of all factors but makes up for it by providing higher prediction precision in the central region of the factor space. The least squares regression ANOVA was conducted using Design-Expert Software 13 in this study. The software was utilized to generate the model equation, examine the interaction effects of

the independent variables, and create surface plots based on the fitted equation obtained from the regression analysis while keeping one of the independent variables constant.

4.3 Effect of the Operating Conditions

The impact of individual operating conditions which were incubation temperature, fermentation time and pH on the citric acid yield were examined.

4.3.1 Effect of Incubation Time on Citric Acid Yield

The incubation time is a critical variable in citric acid production, and its impact on the process was investigated in this study. The experiment involved monitoring citric acid production over different incubation periods while keeping temperature and pH levels constant. Figure 4-2 illustrates the citric acid accumulation profile over time.

Citric acid accumulation was observed at three distinct time points: three days, six days, and nine days of incubation. Notably, there was a noticeable increase in citric acid production from the third day to the sixth day, with the concentration rising from 14.67 g/L to 21.51 g/L. However, a slight decline was observed after the sixth day, resulting in a citric acid concentration of 21.44 g/L. Based on the findings of this study, it was evident that the maximum citric acid yield was achieved at the sixth day of incubation. This duration of six days for the fermentation period aligns with the results of previous studies [77], [78].

The early stages of incubation were marked by robust citric acid production, attributed to the abundant availability of carbon sources. However, the decrease in citric acid production after the sixth day can be attributed to several factors. First, there was a reduction in the sugar content available for fermentation. Second, the fungal growth phase reached its peak, resulting in a diminished capacity for citric acid synthesis. Additionally, the declining availability of nitrogen in the medium and the presence of inhibitory compounds produced by the fungi themselves contributed to the decrease in citric acid production.

Furthermore, a decline in pH values was observed towards the end of the fermentation process, primarily due to the accumulation of citric acid. It is worth noting that maintaining

an appropriate pH value at the start of fermentation was crucial for achieving specific biomass formation.

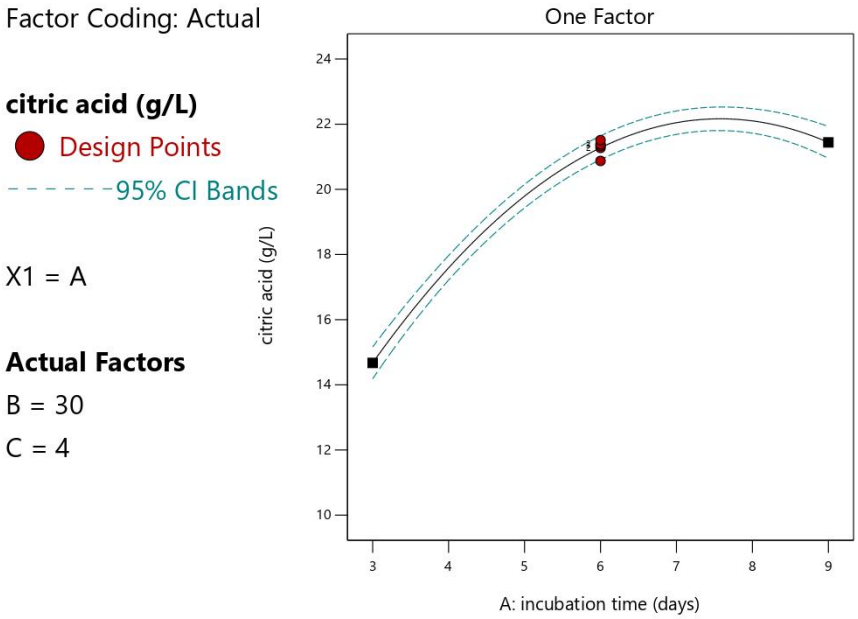

Figure 4- 2: Effect of Incubation Time on Citric Acid Yield

4.3.2 Effects of temperatures on citric acid yield

The temperature of the fermentation medium stands out as a critical factor with a profound influence on citric acid production. This study examined the impact of temperature while maintaining constant pH and fermentation time levels, as depicted in Figure 4-3.

The results clearly demonstrate the significant influence of temperature on citric acid synthesis. As the temperature increased from 25℃ to the central level, there was a remarkable rise in citric acid yield, elevating it from 17.35 g/L to 21.51 g/L. However, a slight decline was observed as the temperature exceeded 30℃, with a drop in citric acid yield to 19.53 g/L at 35℃.

This observed pattern can be attributed to the complex relationship between temperature and enzyme activity in citric acid production. At lower temperatures, enzyme activity tends to be subdued, resulting in a limited impact on citric acid production. Conversely, at higher temperatures, the risk of enzyme denaturation and inhibition rises, alongside the potential for excess moisture loss, all of which can hinder citric acid formation.

These findings align with the research conducted by Satheeshkumar Subramaniyan [79], as well as other studies that have identified 30°C as the optimal temperature for citric acid fermentation process [80].

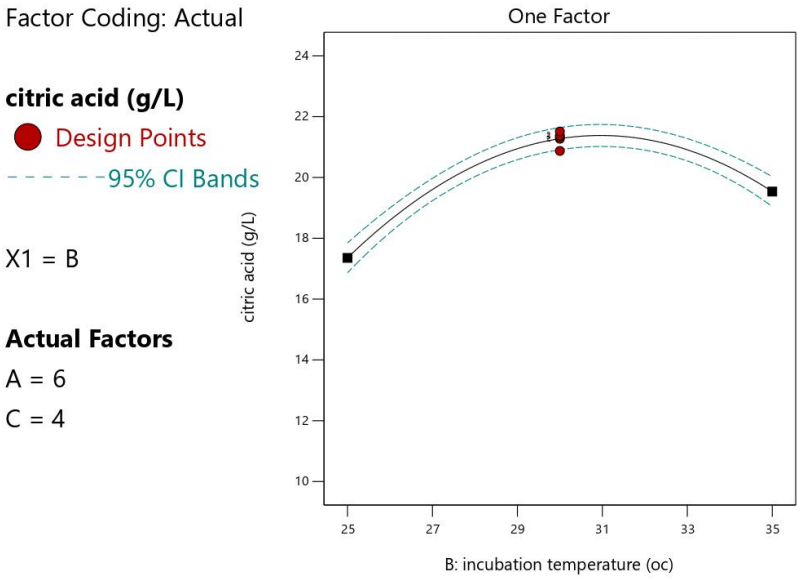

Figure 4- 3: Effect of Temperature on Citric Acid fermentation process

4.3.3 Effects of pH on citric acid yield

The pH of the fermentation medium emerges as another pivotal factor influencing citric acid yield. The study investigated the effect of pH while maintaining constant levels of other variables, and the results are presented in Figure 4-4.

The data unequivocally reveals the significant influence of pH on citric acid production. As the pH level increased from 2 to 4, there was a notable increase in citric acid yield, rising from 19.57 g/L to 21.51 g/L. Nonetheless, a decline in citric acid yield became evident as the pH surpassed 6 within the fermentation broth.

This observed trend can be ascribed to the inhibitory effect of low pH on the growth of A. niger, the microorganism responsible for citric acid production. This finding aligns with previous research, where an initial pH of 3.85 was identified as optimal for achieving maximum citric acid synthesis [81].

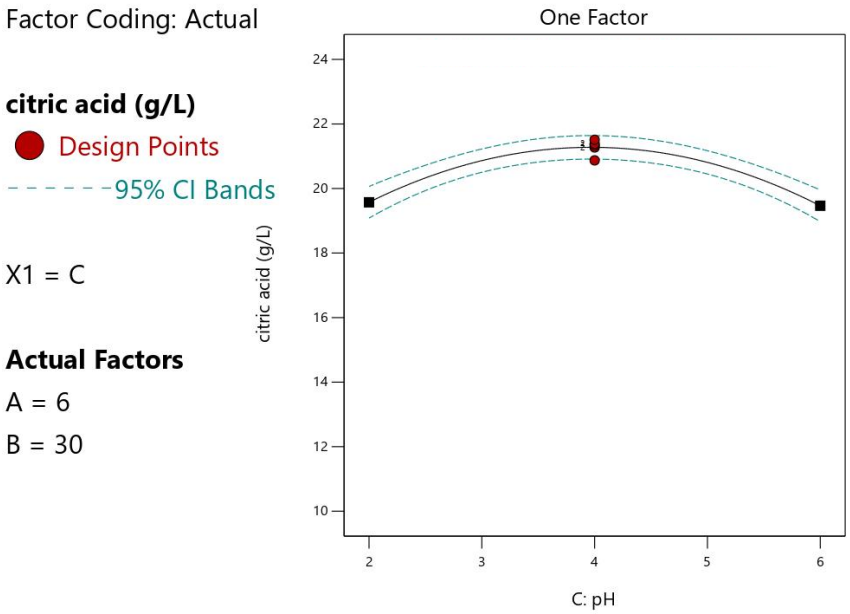

Figure 4- 4: Effect of pH on citric acid fermentation process

4.4 Experimental Design Analysis

In this study, the experimental design chosen for analysis of variance (ANOVA) was Response Surface Methodology (RSM), with a particular focus on Box-Behnken Design (BBD). The primary response variable under investigation was citric acid yield, as presented in Table 4-3. The observed citric acid yield values ranged from 10.7 g/L to 21.51 g/L, reflecting a considerable variation in the response.

To assess the need for data transformation, the ratio of the maximum to minimum citric acid yield was calculated and found to be 2.01. Ratios greater than 10 often indicate the necessity of a transformation. Conversely, when the ratio falls below 3, the impact of a power transformation tends to be minimal. In this context, the ratio of 2.01 signaled the importance of exploring potential transformations to enhance the analysis.

The primary objective of the model fit summary was to maximize the adjusted R-Squared and predicted R-Squared values, indicative of the model's explanatory power. The significance of the model and its individual factors, both linear and quadratic, was rigorously assessed. The linear model factors included Incubation Time (A), Incubation Temperature (B), and pH (C). Additionally, the quadratic model factors encompassed pure quadratic terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC), all evaluated based on their respective F and P values.

Table 4- 3: Analysis of variance (ANOVA) of the response surface model for citric acid yield of water hyacinth

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	203.15	9	22.57	190.22	< 0.0001	significant
A-incubation time	91.59	1	91.59	771.84	< 0.0001	
B-incubation temperature	9.50	1	9.50	80.07	< 0.0001	
C-pH	0.0231	1	0.0231	0.1949	0.6722	
AB	0.1924	1	0.1924	1.62	0.2436	
AC	0.4964	1	0.4964	4.18	0.0801	
BC	1.17	1	1.17	9.88	0.0163	
A^2	43.63	1	43.63	367.70	< 0.0001	
B^2	33.73	1	33.73	284.29	< 0.0001	
C^2	12.99	1	12.99	109.47	< 0.0001	
Residual	0.8306	7	0.1187			
Lack of Fit	0.5969	3	0.1990	3.40	0.1337	not significant
Pure Error	0.2337	4	0.0584			
Cor Total	552.19	16				

The Model F-value, which stands at an impressive 190.22, underscores the significance of the model employed in this study. To put it in perspective, there is merely a 0.01% probability that an F-value of this magnitude could be attributed to random variability or noise. The P-values, which indicate the significance of model terms, further affirm the model's validity. Specifically, model terms with P-values less than 0.0500 are considered significant. In this analysis, it was determined that the terms A, B, AC, BC, A^2, B^2, and C^2 all qualified as significant model terms, thus contributing significantly to the model's explanatory power.

Conversely, model terms with P-values exceeding 0.1000 are regarded as non-significant. In the context of this analysis, non-significant terms, except those necessary to maintain model hierarchy, may be candidates for model reduction to enhance its overall efficiency.

Additionally, the Lack of Fit F-value, which registers at 3.40, indicates that the Lack of Fit component is not statistically significant relative to the pure error. This means that there is a 13.37% probability that a Lack of Fit F-value of this magnitude could arise due to random

variation or noise. In essence, a non-significant Lack of Fit is a favorable outcome, as it implies that the model effectively captures the data without the need for further adjustments.

4.5 Model fit Summary

Tables 4-4 displayed he sequential model fittings which suggests that quadratic models were recommended for the experimental responses by design expert. Lack of fit tests indicates that the second-order quadratic model was suitable (prob>Fis 0.0001). Insignificant lack of fit is necessary to ensure a model to fits the experimental data adequately.

In summary, the proposed quadratic model can be efectively utilized to navigate the design space with high accuracy.

Table 4- 4: Sequential model fitting for citric acid yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	5267.39	1	5267.39			
Linear vs Mean	101.11	3	33.70	4.26	0.0266	
2FI vs Linear	1.86	3	0.6205	0.0614	0.9790	
Quadratic vs 2FI	**100.17**	**3**	**33.39**	**281.40**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	0.5969	3	0.1990	3.40	0.1337	Aliased
Residual	0.2337	4	0.0584			
Total	5471.37	17	321.85			

The coefficient of determination (R^2), shown in Table 4-5, for the regression model of citric acid yields was **0.9959**. Which indicates that the models accounted for **99.59** % variability within the experimental range. Whereas, the "predR^2" values of **0.9514** are in reasonable agreement with the "AdjR^2" of **0.9907**. The model equations can also predict the citric acid yield with predR^2 of **95.14**% variability while the study factors are out of the experimental range. In general, for a good model, the values of R^2 and prediction R^2 should be close to 1. The Predicted R^2 of 0.9514 is in agreement with the Adjusted R^2 of 0.9907; i.e., the difference is less than 0.2.

Table 4- 5: Model summary statistical for citric acid

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	2.81	0.4957	0.3793	0.2644	150.03	
2FI	3.18	0.5048	0.2077	-0.2135	247.52	
Quadratic	**0.3445**	**0.9959**	**0.9907**	**0.9514**	**9.92**	**Suggested**
Cubic	0.2417	0.9989	0.9954		*	Aliased

Table 4- 6: Model adequacy measures for citric acid yield

Std. Dev.	0.3445	**R^2**	0.9959
Mean	17.60	**Adjusted R^2**	0.9907
C.V. %	1.96	**Predicted R^2**	0.9514
		Adeq Precision	40.6597

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 40.6597 indicates an adequate signal. This model can be used to navigate the design space.

4.6 Development of model equation

The utilization of Response Surface Methodology (RSM) provides an empirical relationship between the response and the independent variables. Through software like Design Expert, mathematical relationships between the response and the independent variables, such as fermentation time (A), temperature (B), and pH (C), can be established in terms of both coded and actual factors. The model equation correlating the response (Y) to these variables are written below in equation (4-1):

Equation in Terms of Coded Factors

$$\text{Citric acid (g/L)} = 21.28 + 3.38A + 1.089B - 0.0538C - 0.2193AB + 0.3523AC - 0.5415BC - 3.22A^2 - 2.83049B^2 - 1.756465C^2 \quad (4\text{-}1)$$

Where, A = Incubation time

B = Incubation temperature

C = pH

The equation, expressed in terms of coded factors, this is used to prediction the response at specified levels of each factor. High levels of factors are coded positive (+1), while low levels are coded as negative (-1). The coding scheme enables the comparison of factor coefficients, aiding in the assessment of their relative impact.

According to the regression model equation developed, the yield response was influenced by linear terms including incubation period (A), temperature (B), and pH (C), as well as quadratic terms comprising pure quadratics (A^2, B^2, C^2) and interaction quadratics (AB, AC, BC). Examination of the coefficients in the equations revealed that the yield response increased with the incubation time (A) and temperature (B), both exhibiting positive linear effects on yield. However, the incubation time had a more significant linear impact on yield compared to incubation temperature. Pure quadratic terms (A^2, B^2, C^2) exhibited a negative influence on the response yield.

Additionally, the interaction between incubation time and temperature (AB) and the interaction between incubation time and pH (AC) displayed positive quadratic effects on yield. Conversely, the interaction between temperature and pH (BC) exhibited a negative quadratic effect on yield.

Equation in Terms of Actual Factors

citric acid (g/L) =	-121.44230	
	+5.62370	incubation time
	+7.31544	incubation temperature
	+4.75821	pH
	-0.014621	incubation time * incubation temperature
	+0.058710	incubation time * pH
	-0.054147	incubation temperature * pH
	-0.357672	incubation time²
	-0.113220	incubation temperature²
	-0.439116	pH²

4.7 Model Adequacy Check

The model's adequacy was assessed through analysis of variance to determine whether the equations provided satisfactory approximations to the actual values. Figures 4-5 depict the normal probability plot of residuals for the responses, indicating a normal distribution as the data closely align with the straight line and show no variance deviation. Internally studentized residuals plots were created to visualize the satisfactory fit of the developed model. Figure 4-6 also illustrates the graph of predicted values obtained from the correlation versus actual values. These results demonstrate that the regression model equation accurately describes the experimental data, as all points closely align with the line of perfect fit. This suggests that the model can be utilized to predict the optimal conditions for the three variables in citric acid production. Additional diagnostic plots (Residuals vs. Predicted, Residuals vs. Run) and influence plots (Cook's Distance, Box-Cox Plot for Power Transforms, DFFITS, and DFBETAS) can be found in the Appendix.

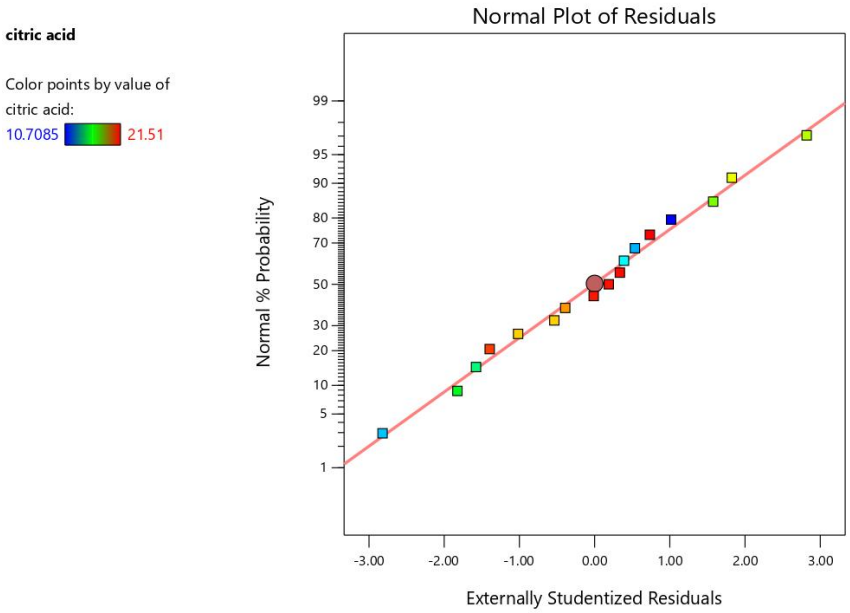

Figure 4- 5: Residual vs run plot for citric acid yield

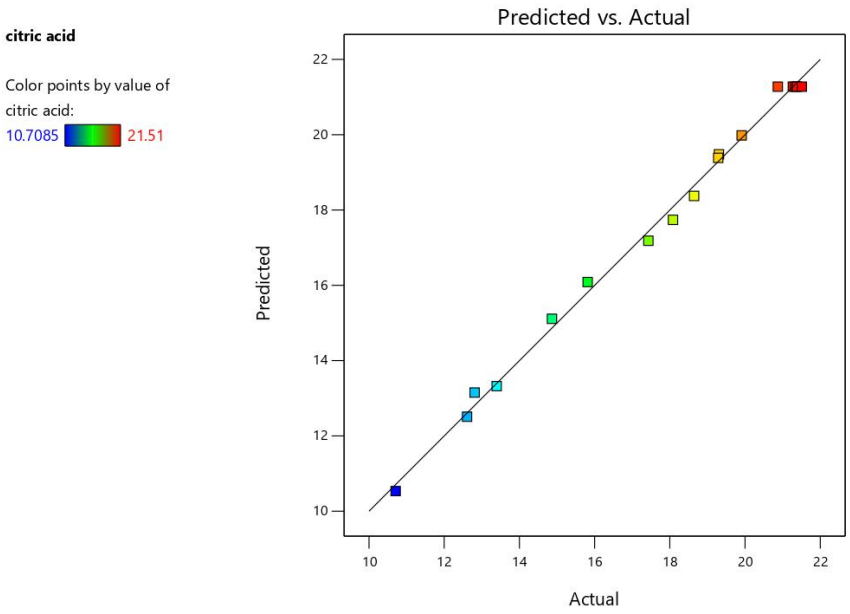

Figure 4- 6: Predicted Vs Actual Experimental Value for Citric Acid Yield

4.8 Effect of interactive parameters between process variables

The yield response was notably influenced by the interaction of two variables while holding a third variable constant. To observe the interaction between these factors, three-dimensional response surface curves were plotted. These curves were analyzed to comprehend how the variables interacted with each other and to determine the optimal levels of each variable for maximizing yield. Contour curves were also examined to understand the effect of the two chosen variables on the yield while maintaining the third variable at its center point. The interaction between the variables to analyze are:

1. Incubation temperature and pH at fixed fermentation time
2. Incubation temperature and incubation time at fixed pH
3. pH and incubation time at fixed temperature.

The significance of interaction between corresponding variables was denoted by the saddle nature of the contour plots. An interaction is observed when the response varies depending on the settings of two factors. The plots provide a straightforward interpretation of two-factor interactions, as they manifest with non-parallel lines, indicating that the effect of one factor depends on the level of the other. If the plotted points fall outside the range, it suggests that

the differences are unlikely to be solely due to error and can be attributed to the effects of the factors. If the “I beam“ overlap there is no significant difference (95% confidence is default) between the two points. We can then choose the most economical or convenient level for that factor. As it can be seen from Equation 4-1 the interaction factor effects on citric acid yield can be understood easily by the coefficients of interaction factors. There were three interaction factors analyzed by the model equation. These were:

- AB – Incubation time and temperature
- AC – Incubation fermentation time and pH
- BC – Incubation temperature and pH

The sign of the coefficient of the interaction factor indicates the effect of interaction factor on citric acid yield. Therefore, the interaction factors with positive sign have positive effect on citric acid yield. Whereas, interaction factors with negative signs have a negative effect on citric acid synthesis. The 3D response surface and contour plots of the effect of the interaction of incubation period, temperature and pH with the response of citric acid production are discussed below.

4.8.1 Effects of Incubation time and temperature on citric acid yield

Incubation time emerges as a pivotal variable in the fermentation process, significantly influencing citric acid production. The interactive effects of incubation time and fermentation temperature were meticulously examined, and their relationship was elucidated through 3D plots and surface contour plots, as presented in Figure 4-7 (a) and (b).

The graphical representation in Figure 4-8 vividly illustrates the dynamic interplay between incubation time and fermentation temperature on citric acid yield. Notably, as the fermentation period and temperature increased while maintaining a constant pH, there was a corresponding increase in citric acid yield. However, this upward trajectory was not indefinite; it was observed that citric acid yield began to decline as both the fermentation period and temperature continued to rise beyond certain thresholds.

The zenith of citric acid yield was attained at a moderate fermentation period and temperature. This intriguing observation can be attributed to the complex relationship between temperature,

enzyme activity, and citric acid production. At lower temperatures, enzyme activity tends to be subdued, resulting in a limited impact on citric acid production. Conversely, excessively high temperatures can lead to enzyme denaturation and inhibition, accompanied by issues such as excess moisture loss, halted growth, reduced sugar content, diminished nitrogen availability, and the presence of inhibitory compounds generated by the fungi within the fermentation medium [2].

In essence, the study underscores the critical importance of optimizing both incubation time and fermentation temperature to achieve maximum citric acid yield. The results highlight the delicate balance required for successful citric acid fermentation, where moderate conditions prove to be the most conductive for optimal production.

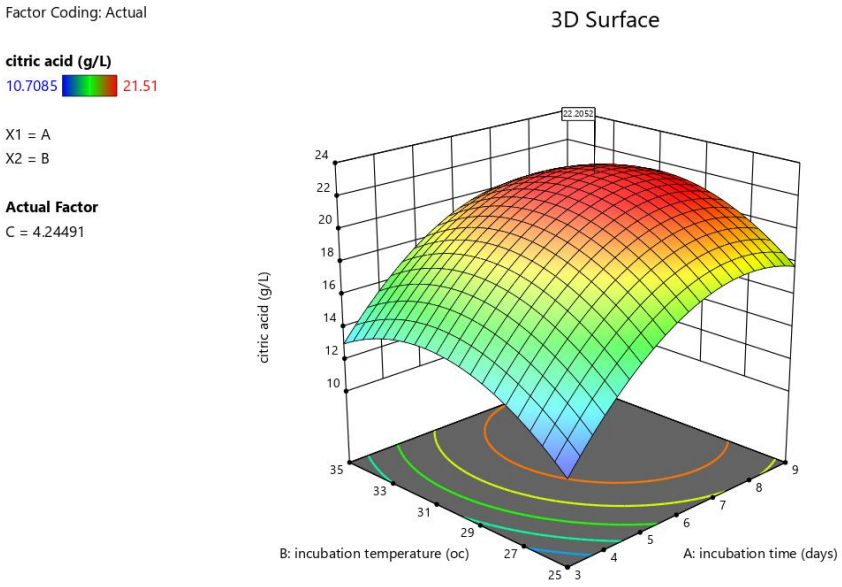

Figure 4- 7: 3D surface plot of interaction effect between fermentation time versus temperature at 4 pH.

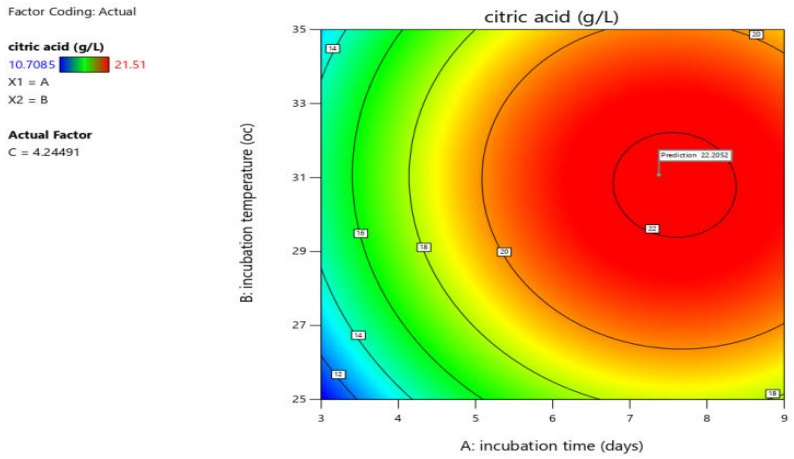

Figure 4- 8: 2D contour plot Interaction effect of fermentation time versus temperature at 4 pH.

4.8.2 Effects of fermentation period and pH on citric acid yield

The study also delved into the intriguing interplay between fermentation time and pH, focusing on their impact on citric acid yield while maintaining a fixed temperature. To visually represent this relationship, 3D plots and surface contour plots were employed, as depicted in Figure 4-8 (a) and (b).

The graphical depictions in Figure 4-9 offer valuable insights into the effects of fermentation time and pH on citric acid production. It becomes evident that as fermentation time and pH levels ascend towards the center point, there is a notable increase in citric acid yield. However, this upward trend does not persist indefinitely; once these variables surpass the center point, a decline in citric acid yield becomes apparent.

This intricate pattern can be attributed to the complex dynamics at play. As fermentation time extends towards the center point, a greater quantity of citric acid is synthesized, leading to a decline in the pH of the fermentation broth, shifting it towards acidity. This acidic environment can have inhibitory effects on the fungal growth, potentially impairing its productivity. One possible explanation for this decrease in productivity is the potential deterioration of the enzyme systems responsible for citric acid biosynthesis.

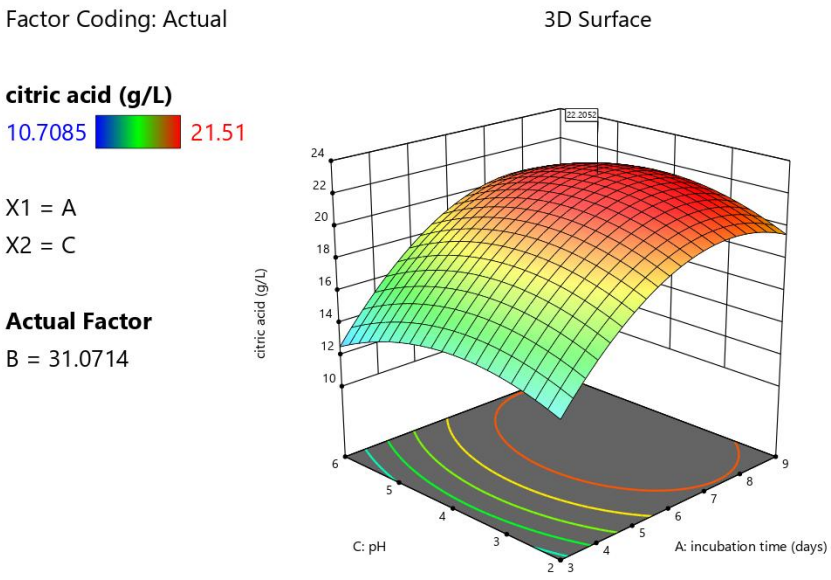

Figure 4-9: 3D surface plot of interaction effect between fermentation time versus pH at 30ºC

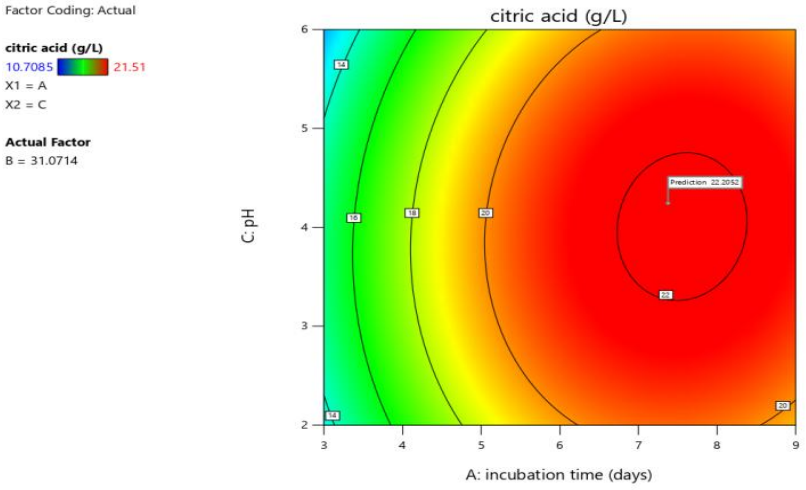

Figure 4- 10: 2D contour plot Interaction effect of fermentation time versus pH at 30ºC

4.8.3 Effects of incubation temperature and pH on citric acid yield

A comprehensive examination of the data, as presented in Figure 4-9, reveals that temperature and pH are the pivotal fermentation conditions that lead to the highest citric acid yield. It is evident that as temperature and pH ascend from lower to moderate levels, there is a corresponding increase in citric acid yield. However, this upward trajectory in yield is not without its limits; as temperature and pH continue to rise to higher levels, a noticeable decline in citric acid yield becomes apparent.

This intricate relationship between temperature, pH, and citric acid production can be attributed to a series of complex physiological and biochemical interactions. As temperature escalates beyond a certain threshold, it can induce the denaturation of crucial enzymes and hinder the germination of fungal spores. This, in turn, leads to a slowdown in metabolic activity within the fermentation medium, contributing to enzyme denaturation and a decrease in overall cell viability.

The consequences of these physiological changes are directly reflected in the citric acid yield, with higher temperatures and pH levels associated with reduced yields, as illustrated in Figure 4-9. It becomes evident that maintaining an optimal balance in temperature and pH is paramount for maximizing citric acid production.

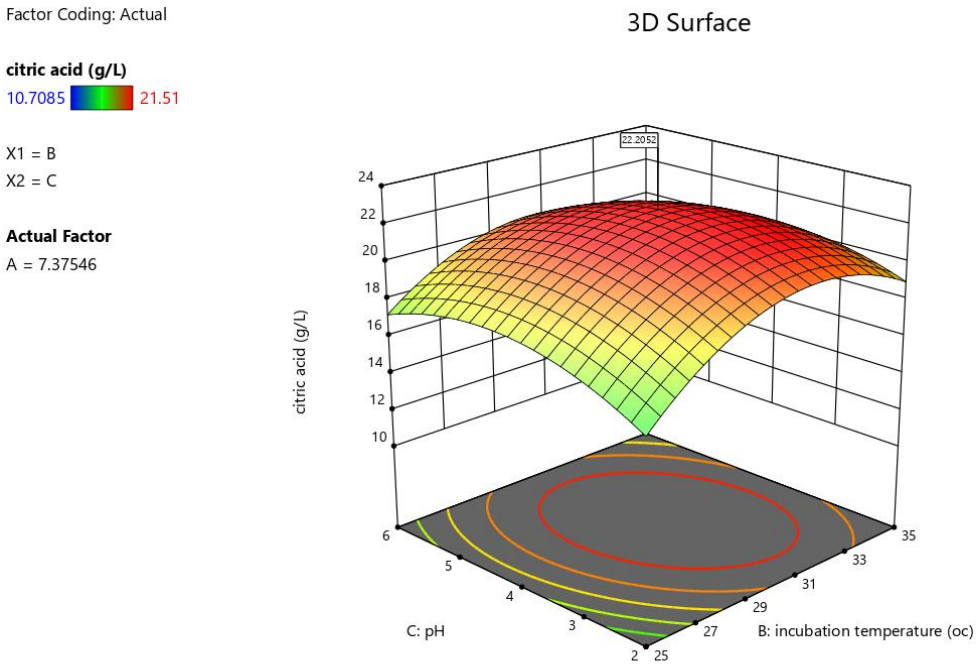

Figure 4-11: 3D surface plot of interaction effect between fermentation temperature versus pH at day 6.

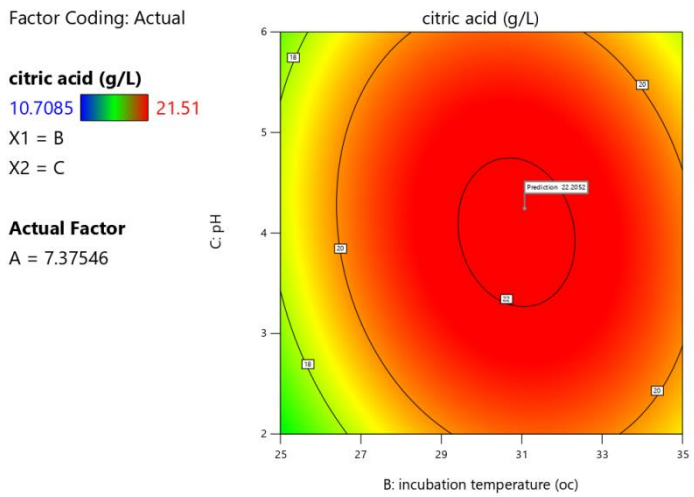

Figure 4- 12: 2D contour plot Interaction effect of fermentation temperature versus pH at day 6.

4.9 Experimental optimization

The primary objective of the present study was to determine the optimal process parameters that would yield the highest citric acid production while maintaining efficiency in the overall process. To achieve this objective, key process variables, including temperature, fermentation time, and pH, were carefully optimized.

In optimizing the fermentation process, temperature, fermentation time and pH are a set of process parameters held to be “in range” while the citric acid yield was the response that need to be “maximized”. Table 4-7 which provides a concise summary of the factors, responses, and objectives that were meticulously defined to attain the highest possible outcomes in the process conditions.

In essence, this research sought to strike a delicate balance between process efficiency and citric acid yield, with a keen focus on identifying the ideal combination of parameters that would yield the most favorable results.

Table 4-7: Constraints applies for optimization

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: incubation time	is in range	3	9	1	1	3
B: incubation temperature	is in range	25	35	1	1	3
C: pH	is in range	2	6	1	1	3
citric acid	maximize	20.7	37.55	1	1	3

The process of numerical optimization, guided by expert design, generated a diverse set of 100 alternative solutions in appendix aimed at optimizing the fermentation conditions. These alternatives were meticulously scrutinized, taking into account multiple factors, including yield, economic considerations, and energy efficiency. The objective was to identify the most favorable solution while carefully balancing these critical parameters.

Within the framework of these constraints, the optimal combination emerged, comprising a temperature of 31.071℃, a pH level of 4.245, and a fermentation duration of 7.375 days. This specific configuration yielded a remarkable citric acid production of 22.205 g/L, surpassing other optimizing alternatives provided by the expert design software.

To facilitate the selection of this optimal batch, a desirability function was employed. This function played a pivotal role in identifying the optimum levels of factors that would yield the most desirable responses. The culmination of this meticulous process resulted in the selection of the optimized batch, characterized by a maximum combined desirability value of 1.00.

The graphical representation of this optimized solution for the fermentation conditions is depicted in Figure 4-13, providing a visual representation of the culmination of the research's efforts in achieving the most favorable outcome.

Therefore, the numerical optimization can be taken as optimal value because the predicted value is close enough with actual value. The histogram shows how well each variables satisfied the criteria and values near one are good.

The ramp plot of the optimization solution for the fermentation conditions was shown in Figure 4-10. The ramp display combines the individual graphs for easier interpretation and the dot on each ramp reflects the factor setting or response prediction for that solution.

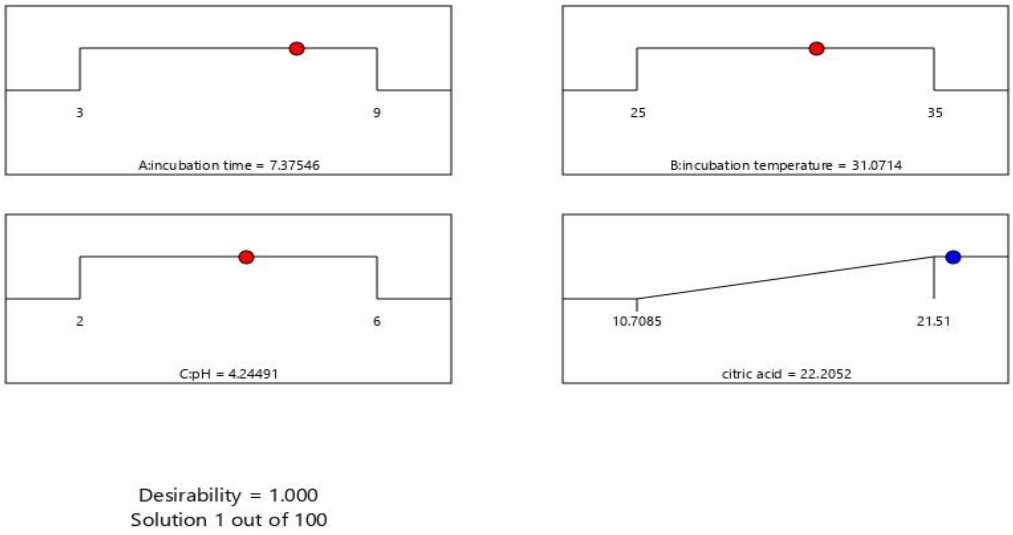

Figure 4- 13: Ramp plot of optimization solution for the responses

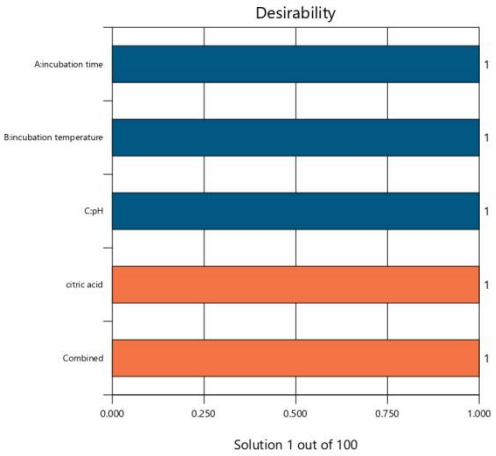

Figure 4- 14: Desirability plot of optimization solution for the responses

4.10 Validation of experimental model

To validate the optimum condition predicted by the model, triplicate experiments were conducted at the optimized process conditions. Accordingly, the citric acid yield of were obtained.

This result is in close agreement with the predicted data obtained from the optimization analysis. The small percentages of error 10.11%, 4.35% and 3.04%, between the optimization and the validation results shown in Table 4-8, further confirm that the models are suitable and sufficient to predict the responses.

Table 4-8: Validation of experimental

Run	A: Incubation time(days)	B: Fermentation temperature	C: pH	Citric Acid Yield(g/L)		Error (%)
				Predicted	Experimental	
1	7.375	31.071	4.245	22.205	19.960	10.11
2	7.375	31.071	4.245	22.205	21.239	4.35
3	7.375	31.071	4.245	22.205	21.411	3.58

4.11 Characterization and comparing of the product quality with Standard

After optimization of the fermentation conditions, citric acid was extracted from at optimized fermentation conditions as per the method presented in section three. The obtained results are described in Table 4-11 and compared with the standard as follows.

4.11.1 Sulphated Ash

The sulfated ash test follows a procedure to measure the amount of residue substance not volatilized from a sample when the sample is ignited in the presence of sulphuric acid. The test is usually used for determining the content of inorganic impurities in an organic substance. Total sulfated ash content of the product was calculated by direct substitution in the relationship expressed in equation 3-3. The result of a current study in terms of sulfated ash content was satisfactory as compared to the standard IS 13186 and Citrique Belge as mentioned in the Table 4-11. The sulfated ash content of the extracted citric acid in this study was lower than the standard which was 0.02 ppm <0.05 ppm. The amount of residue obtained did not exceed the limit specified in the standard. This implies that the product has good quality when compared to the standard value.

4.11.2 Arsenic

Arsenic, a toxic element with trivalent and pentavalent forms, is a heavy metal that is both odorless and tasteless. In its inorganic form, arsenic is a well-known carcinogen, associated with the development of skin, lung, liver, and bladder cancers. Even at lower levels of exposure, it can lead to adverse health effects such as nausea, vomiting, reduced red and white blood cell production, irregular heart rhythms, damage to blood vessels, and a sensation of tingling or numbness in the extremities.

To address the concerns associated with arsenic intake, the quality of the product under study was rigorously evaluated using Atomic Absorption Spectroscopy (AAS) with the advanced Buck Scientific VGP AAS instrument from the USA. As indicated in Table 4-11, the results of the elemental analysis in this research demonstrated that the concentration of arsenic in the sample was "BDL" (Below Detection Limit), with a value of less than 0.06. This outcome indicates that the arsenic concentration in the sample was well below the maximum allowable value specified in the relevant standard (1 ppm maximum).

4.11.3 Iron

Iron is a mineral in our body that comes from foods like red meat and fortified cereals or from supplements we take; it is an essential element that is required in trace amount for various physiological processes especially for human being at recommended dose, but intake of this element at higher concentration, above the standard, may tend to be toxic and derange various physiological processes leading thereby to disease. Therefore, it is important to determine the metal concentration in the extracted citric acid; this is because the product may use as food grade and added to various food items in the industries and consumed directly affect the human health.

In this study, as can be shown in the Table 4-11, results of the iron content of the extracted citric acid were 3 ppm as assessed by Atomic Absorption Spectroscopy (Buck of Scientific VGP AAS, USA), gave lower iron content than IS 13186 standard (5 ppm max) but higher than Citrique Belge (1 ppm max) which is one the greatest citric acid producer in the world.

4.11.4 Lead

Lead is one of the limited classes of elements that can be described as purely toxic. Lead is a highly toxic metal and a very strong poison. Lead poisoning is a serious and sometimes fatal condition. It occurs when lead builds up in the body. Most other elements thought toxic and high concentration is actually required nutrient at a lower level. There is no exposure level below which lead appears to be safe. High level of lead is particularly of great concern especially due to the fact that citric acid was consumed by a human who is uniquely susceptible to the effect of lead. As it can be illustrated in Table 4-9, the result showed the maximum amount of lead (BDL<0.05) in the sample was significantly lower than that of the standard (1 ppm max).

Table 4-9: Physical and chemical properties of citric acid produced at optimized fermentation condition

Parameter	Present result	IS 13186, 2010 standard	Citrique Belge
Appearance (%)	White powder	Colorless crystals or white powder	Colorless crystals or white powder
Odor	Odorless	Odorless	Odorless
Assay (%)	99.1	99.5	99.8
Melting point (^{0}C)	150	153	153
Lead (ppm)	BDL(<0.05)	3ppm max	0.5ppm max
Iron (ppm)	2.6	5ppm max	1ppm max
Arsenic (ppm)	BDL(<0.06)	1ppm max	1ppm max
Sulphated ash (%)	0.03	0.05ppm	

The research paper revealed that the citric acid test results closely matched those outlined in both the IS 13186, 2010 standard and Citrique Belge specifications. These findings suggest that the citric acid produced could meet food-grade standards. Considering the increasing demand for citric acid and the importance of environmental sustainability, utilizing water hyacinth as a feed-stock for citric acid synthesis appears prudent. This approach not only addresses environmental hazards associated with water hyacinth proliferation but also offers a sustainable solution by re-purposing this invasive species into a valuable product.

CHAPTER FIVE

5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Citric acid production from water hyacinth by solid state fermentation using A. niger was investigated in this research. A three-variable, three-level BBD was used to study the simultaneous effect of pH, incubation time and fermentation temperature. A statistically significant model ($p<0.0001$) was developed to describe the relationship between citric acid concentration and the chosen independent variables. The fermentation conditions were optimized using RSM.

The statistical model showed a good fit with the experimental data ($R^2=0.9959$) with a low standard deviation. Based on the experimental result obtained, it was found that the process variables revealed a significant interaction effect on citric acid yield was fitted into a quadratic model equation.

According to the model regression equation developed, the yield was positively affected by linear effects except pH and negatively affected by all quadratic effects. In this study based on the analysis of experimental results, it is realized that the individual factors and their interaction effects are significant model terms on citric acid yield. This shows that the capability of the design of the experimental analysis is successfully capturing these effects.

Moderate level of incubation time, fermentation temperature and pH were favorable for citric acid production. Optimal values of synthesis conditions which gave maximum citric acid yield were selected using numerical optimization of design of expert. The maximum citric acid yield obtained after conducting the experiment at selected optimum condition was 22.205 g/L at 31.071 ºC, 7.375 days and pH of 4.245 with a high value of combined desirability. These values were selected from 100 alternative optimal solutions set by design expert, based on considerations and compromise of yield, energy, and economy during extraction.

The observed quantitative difference in the quantity of the citric acid produce was due to, incubation time fermentation temperature and pH variability. Thus, determination of optimum parameters needs to have the consideration to get the maximum amount of citric acid. Based on the study conducted, it was found that the properties of citric acid produced at the optimum condition (31.92 g of substrate loading, 5.82 days incubation time and 31.55ºC fermentation temperature) were assessed by using different methods and the findings are fulfill the standard requirement as listed in the Table 4-11. It can be concluded from these findings that comparable good quality citric acid can be produced from water hyacinth by using solid state fermentation which is the most environmentally friendly type of fermentation process.

Finally, it can be concluded that utilization of invasive aquatic weed for citric acid production is not only solve the problem of infestation the lakes but also save valuable foreign exchange by reducing the citric acid imported from abroad.

5.2 Recommendation

Given the demonstrated possibility of utilizing water hyacinth for citric acid production therefore; further works are still necessary. Future researches that would be complementary to the present study aiming at investigation of the better citric acid yield and concerning the environment, therefore, strongly recommended to focus on the following points:

- It is recommended to conduct researches on the effects and measure optimum amount sugar concentration, moisture content, substrate loading, particle size, nutrient media type and concentration on citric acid yield.
- Feasibility studies need to be carried out if water hyacinth can produce a commercially acceptable yield.
- Further studies of disposal or recycle system for the substrate.
- Water hyacinth being a high moisture content lignocellulose material further studies on the cost-effective evaporation technique.
- Further studies are needed to determine the concentration of citric acid by using Fourier Transformation Infrared (FTIR) and High-Performance Liquid Chromatography (HPLC).

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Appendix

Table A-1: Determination of moisture content

Run	1	2	3
Weight of Hw before drying (g)	25	25	25
Weight of Hw after drying (g)	2.125	2.472	2.270
Moisture content (%)	91.50	90.11	90.92
Average moisture content (%)		90.86	

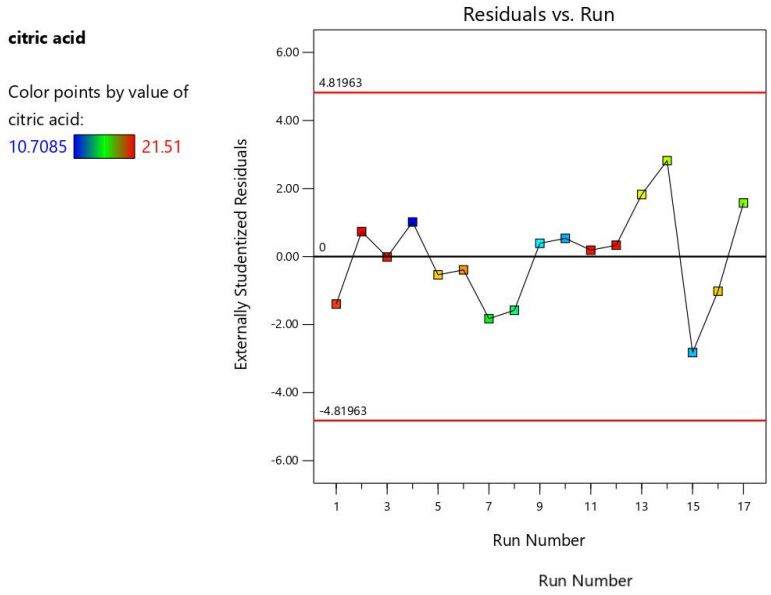

Figure A-1: Residual vs. Run

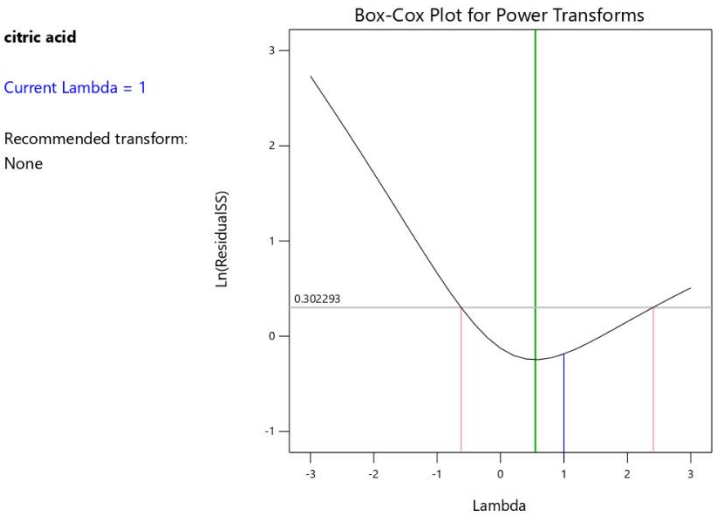

Figure A-2: Box-Cox plot for power transformation

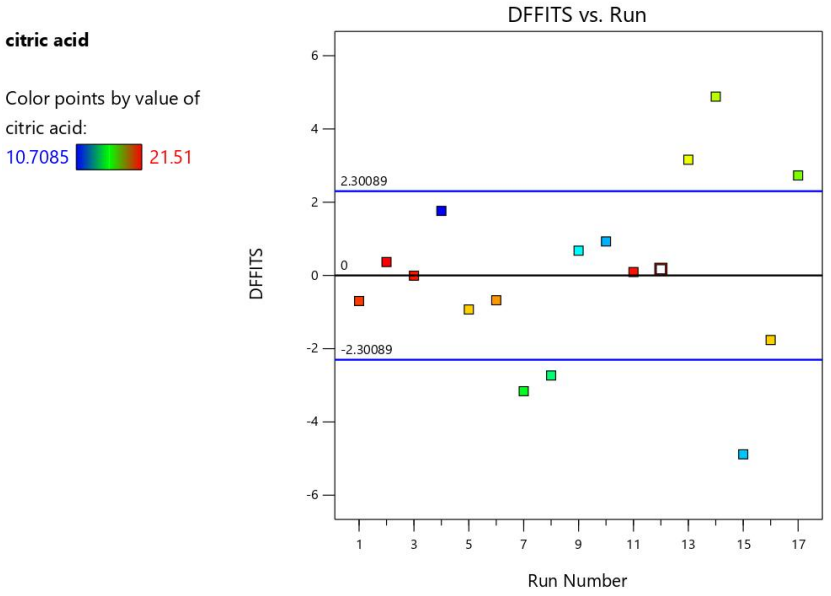

Figure A-3: DFFITS vs. Run

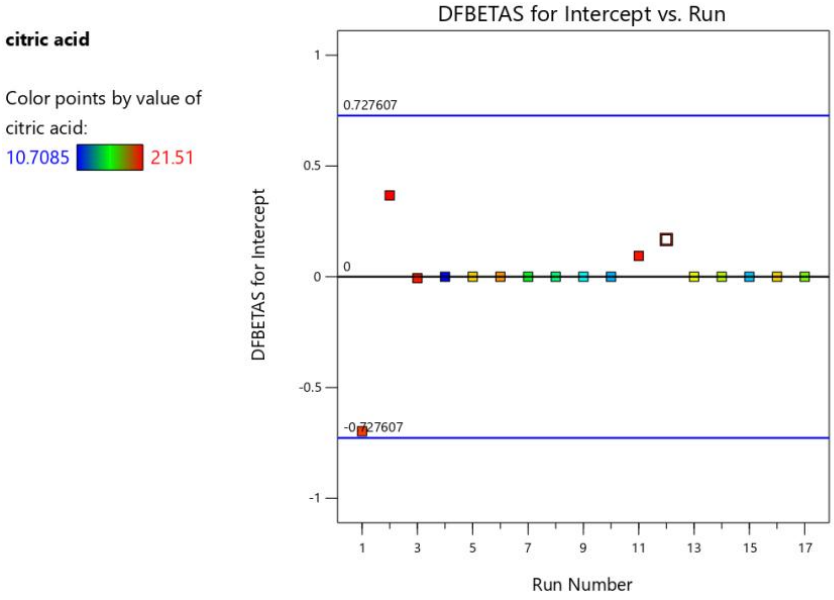

Figure A-4: DFBETAS for vs. Run

Table A-2: Sequential Model Sum of Squares

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	7946.65	1	7946.65			
Linear vs Mean	160.76	3	53.59	1.23	0.3386	
2FI vs Linear	13.95	3	4.65	0.0841	0.9672	
Quadratic vs 2FI	**538.39**	**3**	**179.46**	**88.40**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	5.15	3	1.72	0.7577	0.5732	Aliased
Residual	9.06	4	2.27			
Total	8673.95	17	510.23			

Table A-3 Different alternative optimization solutions

Number	incubation time	incubation temperature	pH	citric acid	Desirability	
1	**7.375**	**31.071**	**4.245**	**22.205**	**1.000**	**Selected**
2	8.109	29.872	3.583	21.918	1.000	
3	6.667	31.111	3.733	21.942	1.000	
4	7.886	30.966	3.188	21.894	1.000	
5	7.802	29.099	3.621	21.771	1.000	
6	8.281	31.593	3.664	21.938	1.000	
7	7.333	30.444	3.333	21.996	1.000	
8	7.380	29.226	4.648	21.808	1.000	
9	8.104	30.802	4.375	22.101	1.000	
10	7.548	32.222	4.731	21.764	1.000	
11	7.548	32.222	3.269	21.846	1.000	
12	6.600	30.444	4.889	21.533	1.000	
13	7.133	31.333	2.756	21.522	1.000	
14	6.917	30.928	3.530	22.016	1.000	
15	6.638	32.121	3.931	21.788	1.000	
16	7.792	31.749	2.808	21.533	1.000	

17	7.243	31.785	4.511	21.981	1.000
18	7.571	29.869	5.271	21.520	1.000
19	6.667	30.205	3.488	21.792	1.000
20	7.252	31.890	3.553	22.037	1.000
21	6.220	30.607	3.533	21.521	1.000
22	6.476	32.220	3.808	21.649	1.000
23	8.581	31.242	3.377	21.647	1.000
24	8.459	30.492	3.803	21.914	1.000
25	8.420	30.089	4.326	21.915	1.000
26	7.264	29.588	3.781	21.992	1.000
27	8.206	29.412	4.885	21.650	1.000
28	7.451	31.592	4.642	21.987	1.000
29	6.724	31.938	3.373	21.765	1.000
30	7.363	32.566	3.339	21.776	1.000
31	6.528	30.148	3.638	21.744	1.000
32	7.240	31.109	3.737	22.180	1.000
33	6.784	31.596	4.211	21.948	1.000
34	7.296	29.682	3.069	21.620	1.000
35	8.134	32.041	4.514	21.841	1.000
36	7.396	29.799	5.223	21.533	1.000
37	7.354	30.038	3.389	21.960	1.000
38	6.705	30.432	4.791	21.680	1.000
39	8.352	30.316	4.998	21.653	1.000
40	8.147	31.244	3.836	22.089	1.000
41	7.904	29.453	4.826	21.784	1.000
42	7.837	29.027	4.320	21.846	1.000
43	7.988	32.809	3.359	21.601	1.000
44	7.436	33.110	3.385	21.577	1.000
45	6.646	29.745	3.699	21.747	1.000

46	7.742	31.531	3.519	22.087	1.000
47	7.499	30.867	4.505	22.143	1.000
48	7.406	31.752	4.713	21.902	1.000
49	7.028	30.731	5.128	21.580	1.000
50	6.675	31.702	3.247	21.711	1.000
51	8.332	32.233	4.253	21.776	1.000
52	7.973	30.929	3.073	21.774	1.000
53	6.536	30.982	3.631	21.833	1.000
54	8.261	32.088	4.605	21.724	1.000
55	7.018	32.263	3.368	21.814	1.000
56	7.166	31.376	5.047	21.651	1.000
57	7.384	30.733	5.039	21.777	1.000
58	8.177	31.051	3.411	21.928	1.000
59	7.750	32.187	4.218	22.003	1.000
60	7.300	31.215	2.862	21.662	1.000
61	8.340	30.857	5.100	21.570	1.000
62	7.871	28.853	3.609	21.642	1.000
63	7.005	30.462	4.422	22.043	1.000
64	8.227	31.062	3.435	21.917	1.000
65	7.203	30.262	3.077	21.759	1.000
66	7.524	31.392	4.998	21.767	1.000
67	7.241	31.288	2.979	21.760	1.000
68	6.853	31.959	4.293	21.884	1.000
69	6.355	31.185	3.737	21.712	1.000
70	7.750	29.942	4.418	22.099	1.000
71	6.370	32.316	3.714	21.531	1.000
72	7.817	31.450	2.997	21.740	1.000
73	7.284	30.554	3.812	22.191	1.000
74	7.619	31.230	5.105	21.697	1.000

75	8.593	30.225	4.250	21.831	1.000	
76	7.742	28.606	3.813	21.624	1.000	
77	7.988	30.329	3.524	22.021	1.000	
78	8.152	30.086	3.651	21.975	1.000	
79	7.449	28.875	4.378	21.777	1.000	
80	6.785	31.783	4.680	21.693	1.000	
81	6.879	30.952	4.897	21.707	1.000	
82	8.163	31.806	3.150	21.690	1.000	
83	8.671	30.062	4.658	21.641	1.000	
84	7.578	30.218	5.284	21.547	1.000	
85	6.931	29.076	4.717	21.560	1.000	
86	8.390	32.195	3.493	21.674	1.000	
87	7.861	32.338	4.577	21.792	1.000	
88	6.538	31.334	3.487	21.777	1.000	
89	7.917	31.906	4.917	21.689	1.000	
90	8.096	30.515	2.987	21.615	1.000	
91	7.849	31.031	2.869	21.624	1.000	
92	8.661	30.074	3.622	21.647	1.000	
93	8.187	30.646	4.591	21.990	1.000	
94	7.152	30.261	3.688	22.094	1.000	
95	6.505	31.190	3.784	21.837	1.000	
96	8.136	30.378	2.930	21.521	1.000	
97	7.719	30.211	3.088	21.771	1.000	
98	7.310	31.404	3.369	22.037	1.000	
99	7.989	31.728	3.232	21.836	1.000	
100	7.259	29.045	4.547	21.758	1.000	

Appendix B

Diagrammatic illustration of the experiment

(A) (B)

Figure A-5 (A) Drying oven (B) Incubator

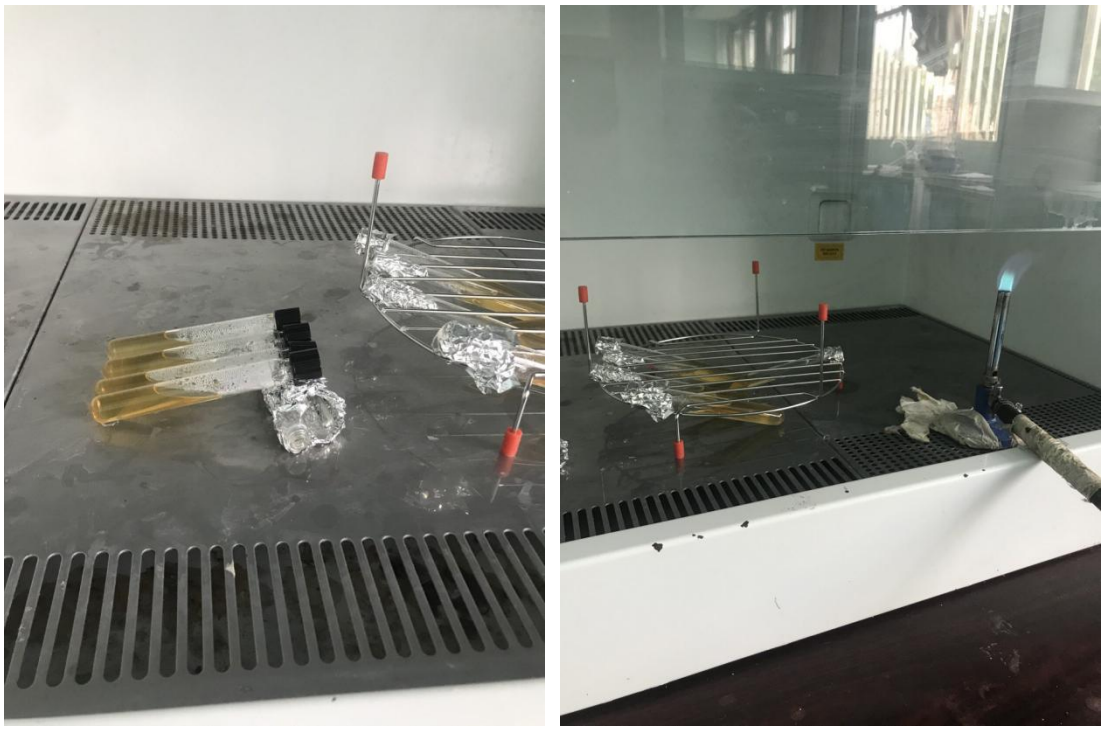

Figure A-6 PDA for innoculation

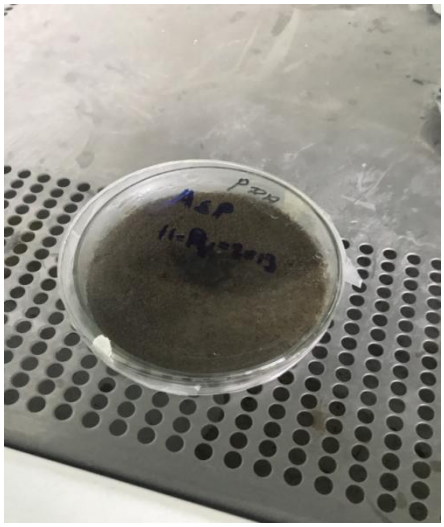

Figure A-7 Aspergillus niger After 5 days

(A) (B)

Figure A-8 (A)Shaker incubator with fermentation media (B) After Fermentation

(A) (B)

Figure A-9 (A) Micro-membrane filtration (B) Fermentation media after filtration

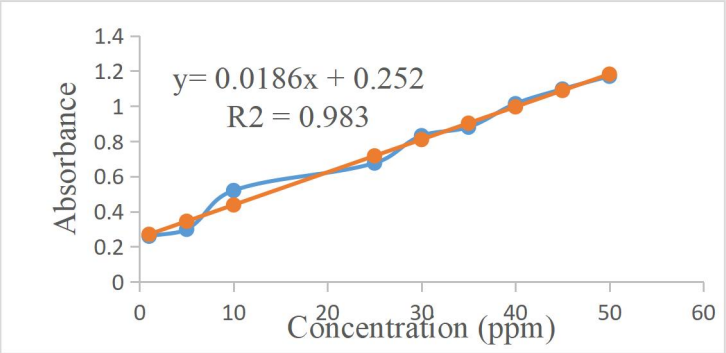

Figure A-10 Standard curve for iron